# NanoLC Ultra® System

## for TripleTOF™ 5600, QTRAP® 5500 and 4000 QTRAP® Systems

***System Integration Test***


eksigent®
part of AB SCIEX

**Document Number: D5030175 A**
**Release Date: March 2012**

**For research use only. Not for use in diagnostic procedures.**



Eksigent
5875 Arnold Road, Dublin, CA 94568.
AB Sciex LP is ISO 9001 registered.

Printed in Canada.

# Contents

# Foreword

This foreword contains general safety-related information, describes the symbols and conventions used in the documentation, and provides regulatory compliance information. It also describes potential hazards and associated warnings for the system, and the precautions that should be taken to minimize the hazards. In addition to this foreword, refer to the *Site Planning Guide* for site requirements.

## General Safety Information

Before operating any instrument, become familiar with its operation and with the potential hazards. To prevent personal injury or instrument damage, read, understand, and obey all safety precautions. Warnings in this document and labels on the device are shown with international symbols. Failure to heed these warnings could result in serious injury.

This safety information is intended to supplement federal, state or provincial, and local environmental health and safety (EHS) regulations. The information provided covers instrument-related safety with regard to the operation of the device. It does not cover every safety procedure that should be practised. Ultimately, you and your organization are responsible for compliance with federal, state or provincial, and local EHS regulations and for maintaining a safe laboratory environment.

For more information, refer to the appropriate laboratory reference material and standard operating procedures.

## Symbols and Conventions

The following conventions may be used throughout the guide.

**DANGER! Danger signifies an action which leads to severe injury or death.**

**WARNING! Personal Injury Hazard: A warning indicates an operation that could cause personal injury if precautions are not followed.**

**WARNING! Electric Shock Hazard: This symbol indicates a warning of electrical shock hazard. Read the warning and follow all precautions before performing any operation described in the guide. Failure to do so can result in serious injury.**

**WARNING! Burn Hazard: This symbol indicates a warning of potential burns from hot surfaces. Read the warning and follow all precautions before performing any operation described in the guide. Failure to do so can result in serious injury.**

**WARNING! Biohazard: This symbol indicates a warning of biohazardous materials. Read the warning and follow all precautions before performing any operation described in the guide. Failure to do so can result in serious injury.**

**Caution: A caution indicates an operation that could cause damage to the instrument or loss of data if precautions are not followed.**

**Tip!** Provides useful information that helps apply the techniques and procedures in the text for a specific need, and provides shortcuts, but is *not essential* to the completion of a procedure.

**Note:** A note emphasizes significant information in a procedure or description.

# Safety Instructions

The following safety instructions apply to the NanoLC Ultra system:

**WARNING! Potential Operator Injury: Use of this equipment in a manner not approved by the manufacturer may inhibit its safety protection.**

**Caution: Changes or modifications to this unit not expressly approved by the manufacturer could void the instrument warranty and render the system inoperable.**

**WARNING! Electrical Shock Hazard: Only use fuses of the type and current rating specified. Do not use repaired fuses or by-pass the fuse holder.**

**WARNING! Electrical Shock Hazard: The supplied power cord must be used with a power outlet containing a protective ground contact.**

**WARNING! Biohazard: When replacing tubing or fittings on the ekspert microLC 200 system, exposure to solvents may occur. It is therefore recommended that appropriate safety procedures be followed and personal protective equipment be used, according to the applicable Material Safety Data Sheets supplied by the solvent vendor.**

**WARNING! Electrical Shock Hazard: Do not change the external or internal grounding connections. Tampering with or disabling these connections could create a safety hazard and/or damage the system. The instrument, as shipped, is properly grounded in accordance with normal safety regulations.**

**WARNING! Potential System Damage: Do not turn the system on if you suspect that it has incurred any kind of electrical damage. Instead, disconnect the power cord and evaluate the system.**

**WARNING! Potential System Damage: Electrical damage may have occurred if any part of the system shows visible signs of damage, exposure to liquids or of having been transported under severe stress.**

**WARNING! Electrical Shock Hazard: Continue to exercise caution as capacitors inside the system may still be charged even after the system has been turned off.**

**WARNING! Electrical Shock Hazard: Disconnect power cords from the power supply before attempting any type of maintenance.**

**WARNING! Electrical Shock Hazard: The combination of the pump and autosampler with a LC/MS system may require additional safety measures as described by AB SCIEX. See the mass spectrometer *Safety Guide* for instructions for the safe grounding on the LC/MS system.**

**WARNING! Electrical Shock Hazard: Use a grounding cable connected between the injection valve's sample loop and an appropriate grounding point at the LC/MS source. This supplementary grounding will reinforce the safety configuration specified by AB SCIEX.**

**WARNING! Potential System Damage: Damage can result if the system is stored for prolonged periods under extreme conditions (for example, subjected to heat, water, etc.).**

**WARNING! Environmental Hazard: Do not allow flammable and/or toxic solvents to accumulate. Follow a regulated, approved waste disposal program. Never dispose of flammable and/or toxic solvents into a municipal sewage system.**

**WARNING! Potential System Damage: To avoid damaging electrical parts, do not disconnect an electrical assembly while power is applied to the system. Once the power is turned off, wait approximately 30 seconds before disconnecting an assembly.**

**WARNING! Potential System Damage: The system contains a number of sensitive electronic components that may be damaged if exposed to excessive line voltage fluctuations and/or power surges.**

**WARNING! Potential Operator Injury: To avoid injury during operation, keep hands and loose objects away from the autosampler arm and syringe assembly.**

**WARNING! Potential Operator Injury: Use caution when working with any polymeric tubing under pressure:**
**– Always wear proper eye protection when near pressurized polymer tubing.**
**– Do not use polymer tubing that has been severely stressed or kinked.**
**– Do not use polymer tubing, in particular PEEK or DuPont Tefzel tubing, with tetrahydrofuran (THF), dimethylsulfoxide (DMSO), chlorinated organic solvents, concentrated mineral acids, such as nitric, phosphoric or sulfuric acids, or any related compounds.**

**WARNING! Puncture Hazard: Do not operate the autosampler without the safety shield properly installed.**

**Caution: Potential System Damage: An on-board lithium battery maintains the autosampler firmware when the instrument is turned off. It should only be replaced by a factory-authorized service engineer.**

**Caution: Potential Data Corruption: When you use the HTC-*xt* PAL autosampler for chromatographic analyses and observe a change in the retention of a particular compound, the resolution between two compounds or peak shapes, immediately determine the reason for the changes. Do not rely on the analytical results until the cause of the change is determined.**

# Qualified Personnel

After installing the system, the FSE (Field Service Employee) uses the *Customer Familiarization Checklist* to train the customer on system operation, cleaning, and basic maintenance. Only AB SCIEX trained personnel shall operate and maintain the equipment. Equipment installation and service shall only be conducted by AB SCIEX Field Service Employees. Contact an AB SCIEX FSE for more information.

# Equipment Use and Modification

Use the system indoors in a laboratory that complies with the environmental conditions recommended in the system *Site Planning Guide*. If the system is used in an environment or in a manner not prescribed by AB SCIEX, the protection provided by the equipment can be impaired.

Unauthorized modification or operation of the system may cause personal injury and equipment damage, and may void the warranty. Contact an AB SCIEX representative for more information on servicing the system.

# Mains Supply

**WARNING! Electrical Shock Hazard: Use only qualified personnel for the installation of all electrical supplies and fixtures, and make sure that all installations adhere to local regulations.**

For information on system electrical specifications, refer to the *Site Planning Guide*.

## Protective Conductor

The mains supply should include a correctly installed protective earth conductor that must be installed or checked by a qualified electrician before connecting the instrument.

Do not intentionally interrupt the protective conductor. Any interruption of the protective conductor is likely to make the installation dangerous.

# Environmental Conditions

Use qualified personnel for the installation of electrical mains, heating, ventilation, and plumbing supplies and fixtures. Make sure that all installations follow local bylaws and biohazard regulations. For more information about the required environmental conditions for the system, refer to the *Site Planning Guide* for the instrument.

**WARNING! Explosion Hazard: The instrument is not designed for operation in an explosive environment. Do not operate the instrument in an environment containing explosive gases.**

**WARNING! Asphyxiation Hazard: The use of instruments without adequate ventilation to outside air may constitute a health hazard. In addition, certain procedures required during the operation of the instrument may cause gases to be discharged into the exhaust stream; under these conditions, inadequate ventilation may result in serious injury. Take extreme care to vent exhaust gases properly.**

**WARNING! Toxic Chemical Hazard: Make sure that the source exhaust system is properly connected, particularly if samples containing toxic or highly volatile chemicals or solvents are being analyzed. A minimum 20% positive air flow into the laboratory is required.**

**WARNING! Biohazard: This instrument or any part is not intended to act as a biological containment safety cabinet. For biohazardous material use, always apply local regulations for hazard assessment, control, and handling.**

## Instrument Disposal (Waste Electrical and Electronic Equipment)

Do not dispose of system components or subassemblies, including computer parts, as unsorted municipal waste. Follow local municipal waste ordinances for proper disposal provisions to reduce the environmental impact of WEEE (waste, electrical, and electronic equipment). To make sure that you safely dispose of this equipment, contact an FSE for instructions.

European Union customers: Contact a local AB SCIEX Customer Service office for complimentary equipment pick-up and recycling.

# Regulatory Compliance

This system complies with the standards and regulations listed in this section. Applicable labels have been affixed to the system.

### Canada

- **Safety**—CSA 61010-1

### Europe

- Low Voltage Directive 2006 / 95 / EC
- **Electromagnetic Compatibility**—61326-1 EN 55011 Class A, EMC Directive 2004 / 108 / EC
- **Safety**—EN 61010-1

For more information on EU compliance, see the Declaration of Conformance included with the system.

### International

- **Electromagnetic Compatibility**—CISPR 11 Class A, IEC 61326-1
- **Safety**—IEC 61010-1

### United States

- **Safety**—UL 61010-1

# Additional Documentation

- *NanoLC Ultra® System Operator's Manual*—Printed and electronic copies are included with the system
- *Eksigent® Control Software User Guide*—installed with the Eksigent control software
- *Analyst® Software Getting Started Guide*—installed with the Analyst software

# Technical Support

AB SCIEX and its representatives maintain a staff of fully-trained service and technical specialists located throughout the world. They can answer questions about the instrument or any technical issues that may arise. For more information, visit the web site at www.absciex.com.


eksigent®
part of AB SCIEX

# LC/MS System Configuration Test— AB SCIEX TripleTOF™ 5600 System

# 1

This chapter describes the steps for preparing and performing LC/MS system configuration tests for the NanoLC Ultra® system configured with the cHiPLC® Nanoflex system (or external ChromXP column) and the AB SCIEX TripleTOF™ 5600 instrument.

**Note:** The tests in this chapter are written for Gradient 2 as the low-flow channel. If this is not true for your system (for example, if you have a 1D or 1D+ system), then make appropriate changes throughout the tests.

The tests in this chapter are divided as follows:

- Fast test to condition the column and determine the functional status of the system. See Create the Methods and Batch for the System Functional Test for details.

  Perform these tests after completing the NanoSpray® ion source infusion tests in order to first confirm the spray performance of the tip. Refer to the *NanoSpray® Ion Source Operator Guide* for more information.

- Longer test to determine the performance level of the instrument for proteomics applications such as protein identification and quantification. See Create the Methods and Batch for the System Performance Test for details.

  These tests can be used as a measure of the NanoLC Ultra system performance in isolation of performance of the other components. Results from these tests can become the baseline performance for the system and can be performed regularly and used as a system quality control test in the future.

Approximate time required:

1. Create the methods and batch: 45 minutes
2. Prepare the system for testing: 3-4 hours
3. Perform the test.
   i. System functional test: 90 minutes
   ii. System performance test: 180 minutes

Recommended solvents can be ordered from VWR:

- Burdick and Jackson acetonitrile with 0.1% formic acid, P/N BJLC441-1.0
- Burdick and Jackson water with 0.1% formic acid, P/N BJLC452-1.0

Required materials for a Nanoflex system installation:

- Reverse phase cHiPLC column (75 µm x 15 cm ChromXP C18-CL 3 µm 120 Å)
- cHiPLC trap (200 µm x 0.5 mm ChromXP C18-CL 3 µm 120 Å)
- LC/MS Peptide/Protein Mass Standards Kit (P/N 4368624)

**Note:** Make sure that the Nanoflex system is in the Load position before beginning these tests.

Required materials for an external column installation:

- Reversed phase ChromXP nanoLC column (75 µm ID x 15 cm, ChromXP C18 3 µm 120 Å, P/N 805-00120)
- ChromXP nanoLC Trap column (350 µm ID x 0.5 mm, ChromXP C18 3 µm 120 Å, P/N 5016752)
- LC/MS Peptide/Protein Mass Standards Kit (P/N 4368624)

**Note:** After successfully completing the tests, create a backup of the EKSettings.reg file. See Create a Backup of the EKSettings.reg File for more information.

# Create the Methods and Batch for the System Functional Test

This section describes a test for the NanoLC Ultra system to condition the column and determine the functional status.

Perform these tests when the mass spectrometer is known to be operating well and meeting performance specifications. If the NanoLC system has been idle for two weeks or more, then calibrate the system. Refer to the appendix, System Calibration, for more information.

**Note:** The steps in this section do not constitute a NanoLC Ultra system performance test. See Create the Methods and Batch for the System Performance Test.

The expected test duration is 30 minutes using the NanoSpray® ion source. Repeat the test until you have consistent peak shape and intensity (approximately 90 minutes).

## Create the Acquisition Methods and Batch—Functional Test

**Note:** Use Gradient 2 for the autosampler method. Gradient 2 is the nanoflow module for LC configuration.

1. Plumb the autosampler valve with a 10 µL sample loop.
2. In the **AS1/AS2 Autosampler** status window, click **Method Editor**.
3. Create the autosampler method for a trap-elute configuration, as shown in Figure 1-1.

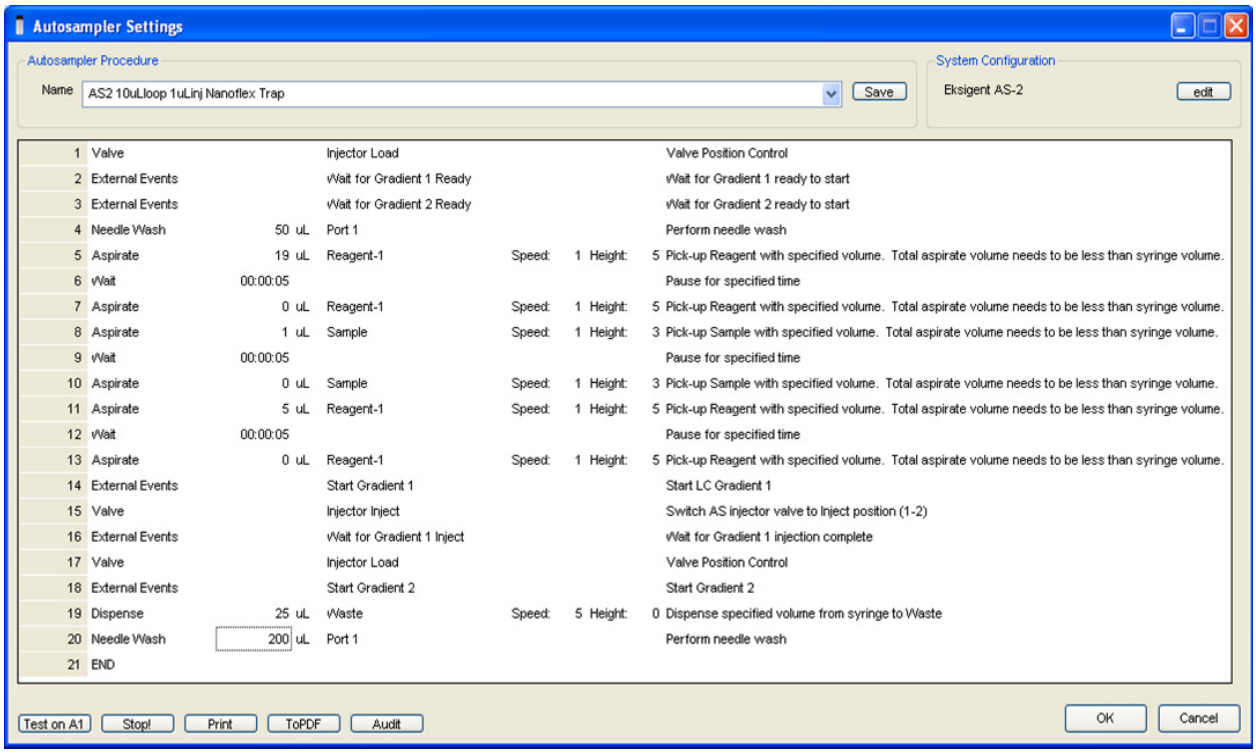


**Figure 1-1 Autosampler Settings dialog—trap-elute configuration (Nanoflex system)**

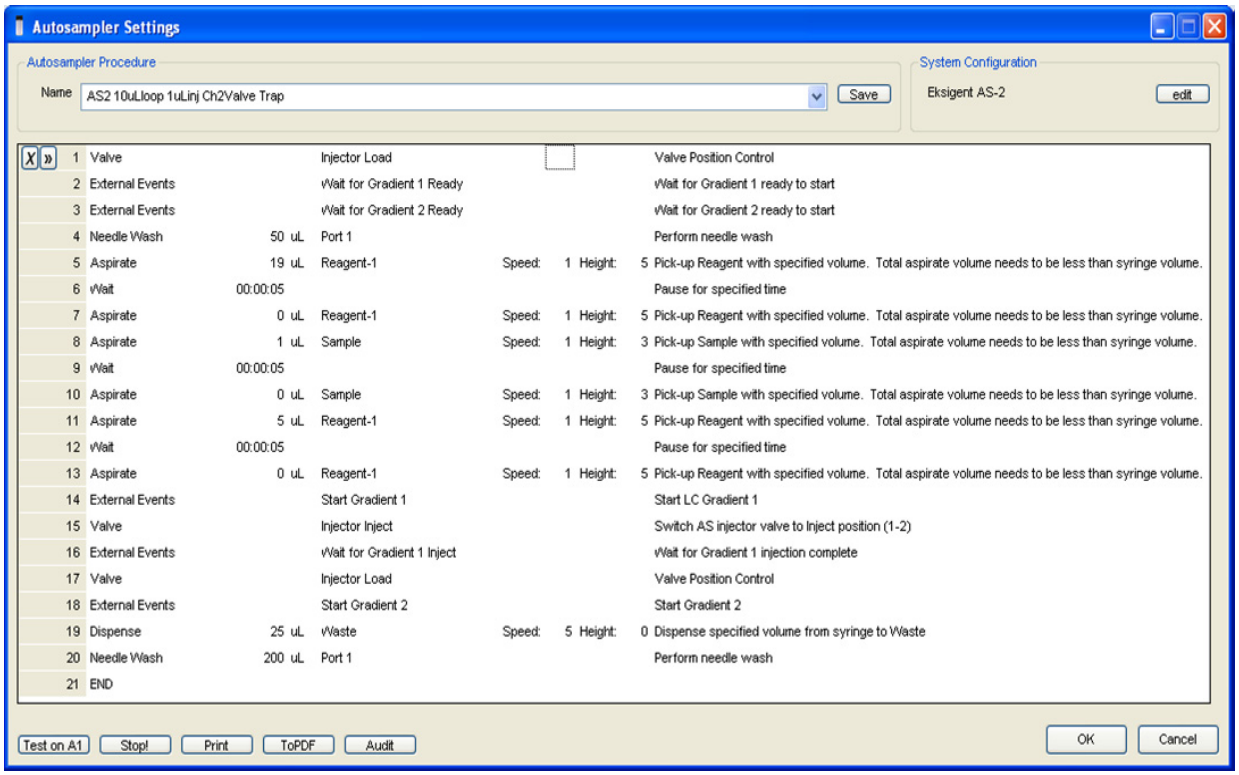


**Figure 1-2 Autosampler Settings dialog—trap-elute configuration (column)**

4. In the **Name** field, specify the name of this method as AS2 10uLloop 1uLinj Nanoflex trap elute or AS2 10uLloop 1uLinj Ch2Valve Trap, depending on the installation.
5. Click **Save**.

# Create the LC Methods

The aqueous channel for each pump (Channel A) will be filled with Buffer A. The organic channel (Channel B) will be filled with Buffer B. For the Loading Pump, Buffer A is always used. Typical buffer mixtures are shown in Table 1-1.

**Table 1-1 Typical Buffer Mixtures**

| Buffer | Mixture | Channel |
|---|---|---|
| Buffer A | 100% water:0.1% formic acid | Channel A |
| Buffer B | 100% acetonitrile:0.1% formic acid | Channel B |

In the method below, the loading pump will be the pump with the microflow module.

## Create the Pump Method in the Eksigent® Control Software

1. Make sure that **Loading Pump** is selected as the channel (top, right corner of window).
2. Click **LC Methods**.
3. In the **Name** field, type **Load Pump 2 min Trap Wash**, and then click **Save**.
4. On the **Gradient Table** tab, revise the method for the loading pump (the loading pump will be the pump with the microflow module), as shown in Figure 1-3.

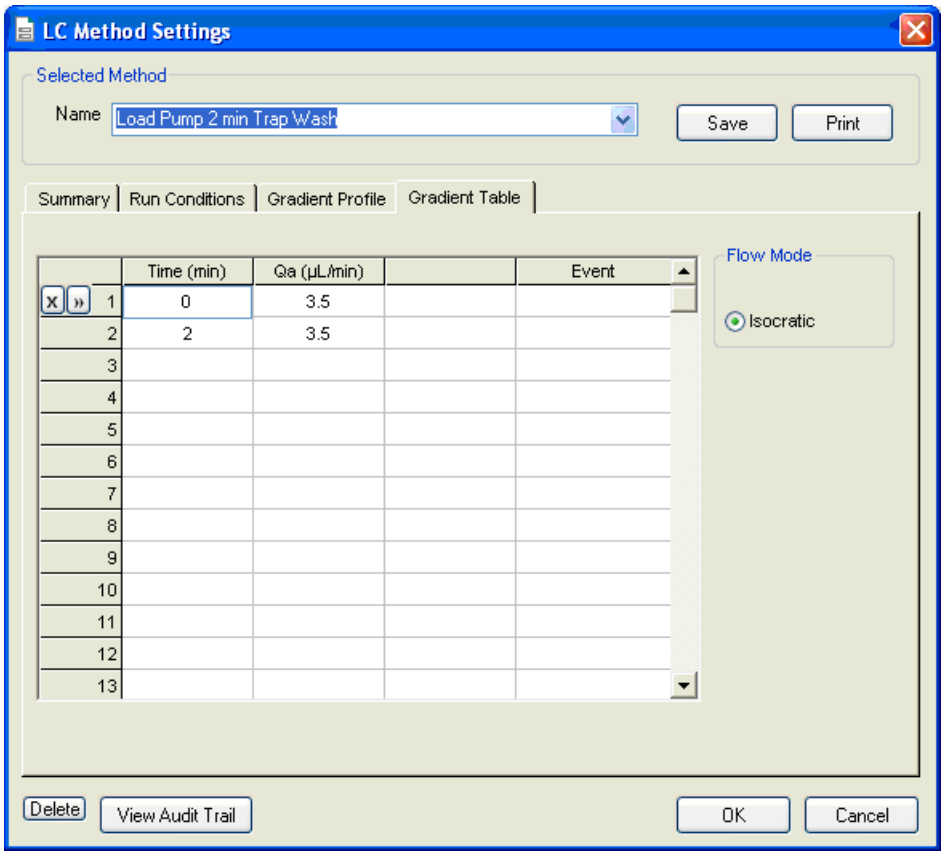


**Figure 1-3 LC Method Settings dialog—Gradient Table tab**

5. On the **Run Conditions** tab, specify conditions as shown in Figure 1-4.

**Note:** For a trap-elute configuration, the Sample Injection method should be Standard.

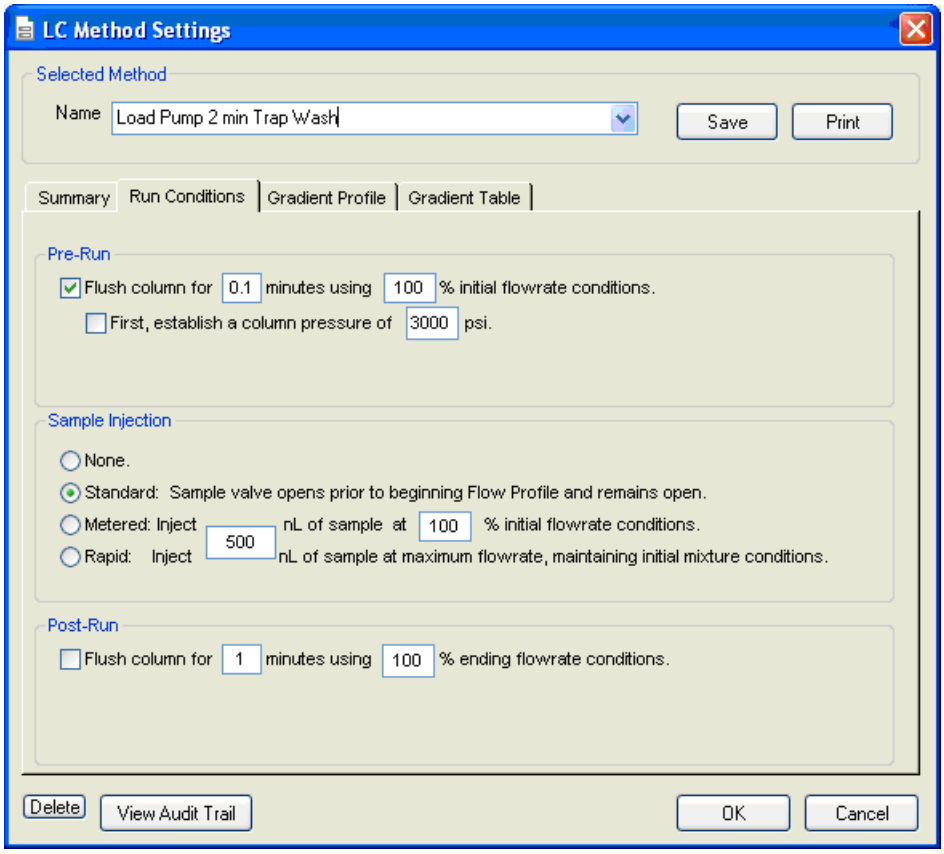


**Figure 1-4 LC Method Settings dialog—Run Conditions tab**

6. Click **Save**.

## Create the Gradient Method in the Eksigent Control Software

For the analytical gradient (typically, the Gradient 2 pump with the nanoflow module), create the gradient method.

1. Make sure that **Gradient 2** is selected as the channel (top, right corner of window).
2. Click **LC Methods**.
3. If the installation includes a Nanoflex system, then in the **Name** field, type **CH2 15min 400nLmin nanoflex trap**, and click **Save**.

   Or

   If the installation includes an external column, then in the **Name** field, type **CH2 15min 400nLmin trap**, and click **Save**.
4. On the **Gradient Table** tab, specify the method as shown in Figure 1-5 or Figure 1-7, depending on the installation.

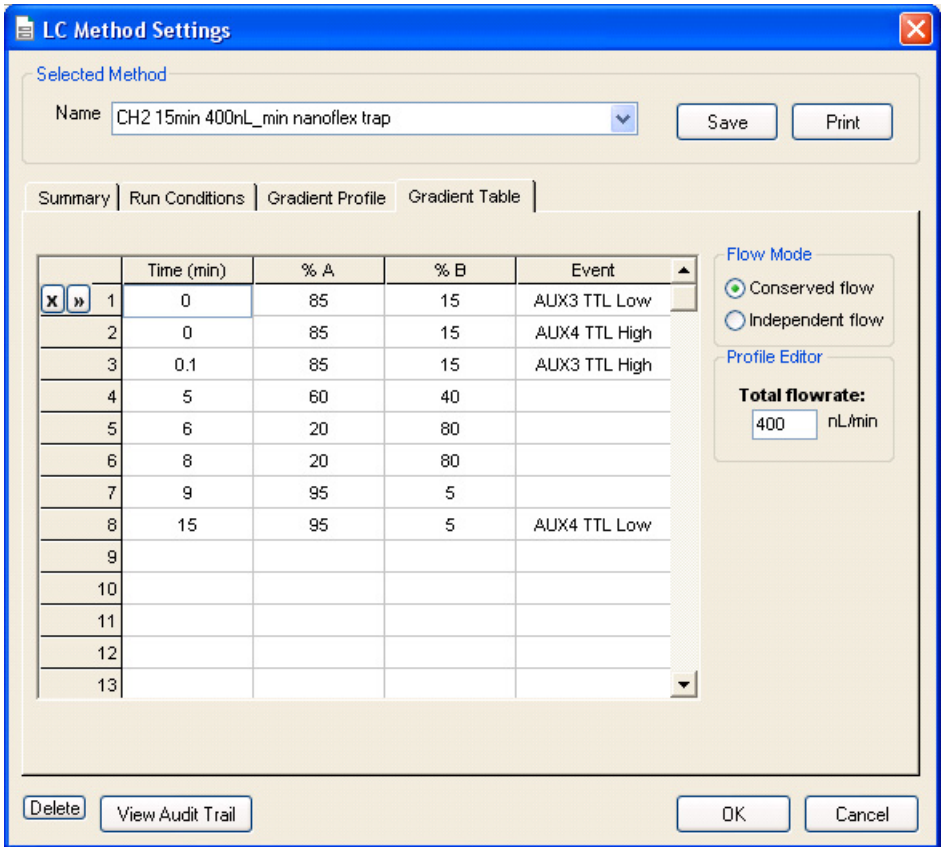


**Figure 1-5 LC Method Settings dialog—Gradient Table tab (Nanoflex system)**

**Note:** The events shown verify the correct switching of the Nanoflex valve. The signals at Time 0 will move the Nanoflex valve to the Inject position and the signals at Time 15 will move the Nanoflex valve back to the Load position.

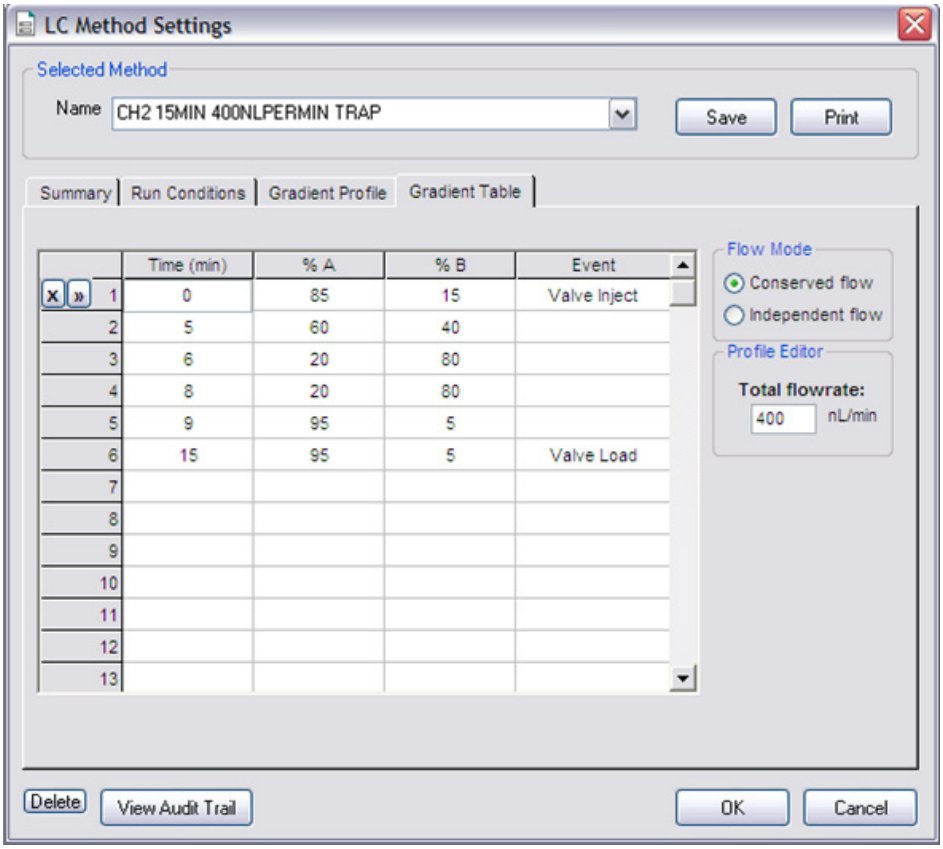


**Figure 1-6 LC Method Settings dialog—Gradient Table tab (column)**

5. On the **Run Conditions** tab, specify the method as shown in Figure 1-7.

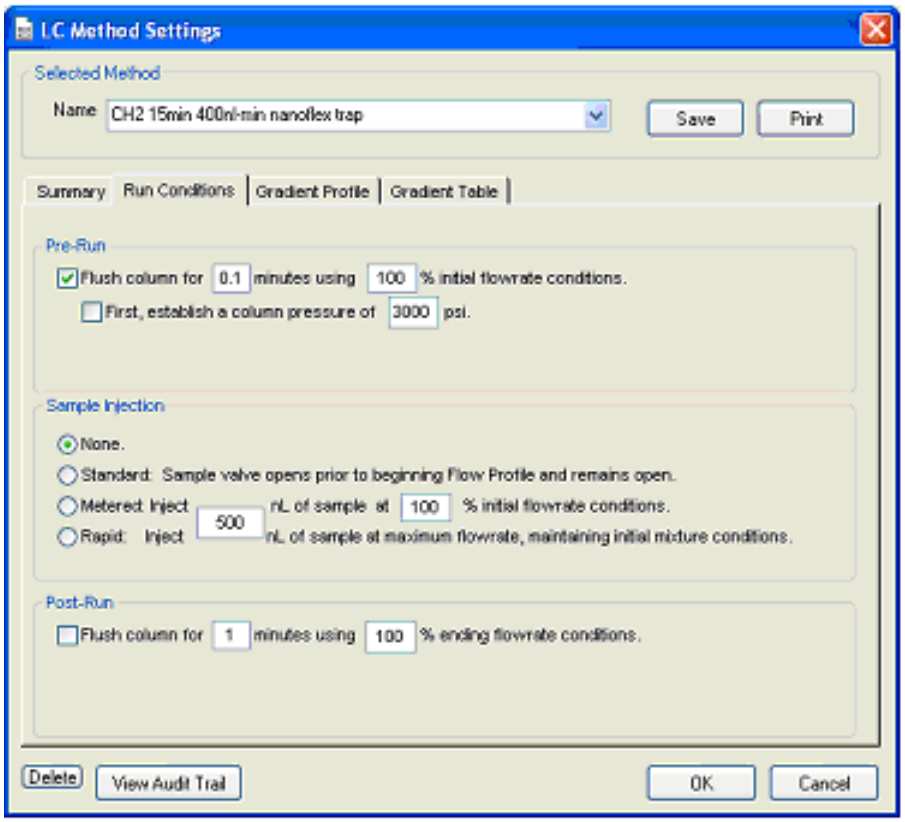


**Figure 1-7 LC Method Settings dialog—Run Conditions tab (Nanoflex system)**

6. Click **Save**.

## Create the Acquisition Method in the Analyst® TF Software

1. In the **Acquire** section of the **Navigation** toolbar, click the **Method Wizard** button to open the wizard.
2. On the **Choose Methods** tab of the **Method Wizard** window, specify the MS method.
   - In the **Choose MS Method** list, select **ToF MS + Hi Sensitivity Product Ion (+)** method.
3. If an autosampler and LC method has already been associated with a previous acquisition method, in the **Choose LC Method** list, select an existing .dam method.
4. If an LC method has not been used before, then select **No LC Method**.

**Tip!** A method can be defined later in this test. See Add LC Information to the Acquisition Method.

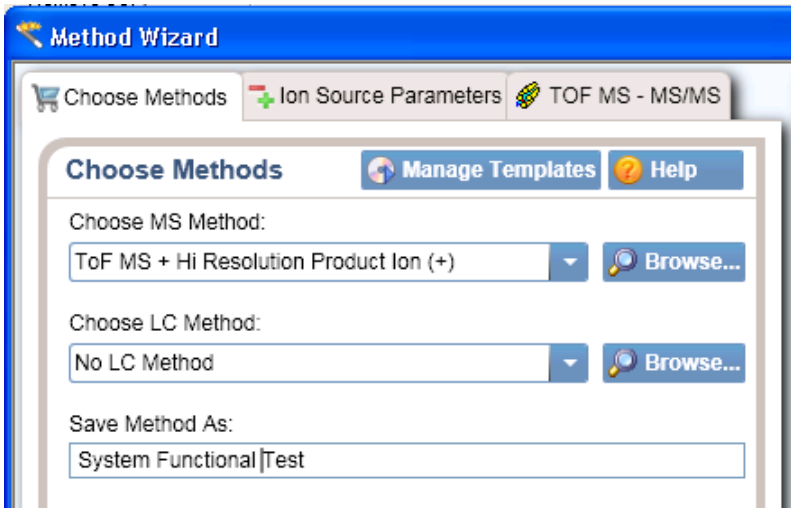


**Figure 1-8 Method Wizard window—Choose Methods tab**

5. In the **Save Method As** field, type **System Functional Test** and press **Enter**.
6. Click **Next**.
7. On **Ion Source Parameters** tab, specify the source conditions that were determined during the NanoSpray® ion source infusion test. Refer to the *NanoSpray® Ion Source Operator Guide* for more information.

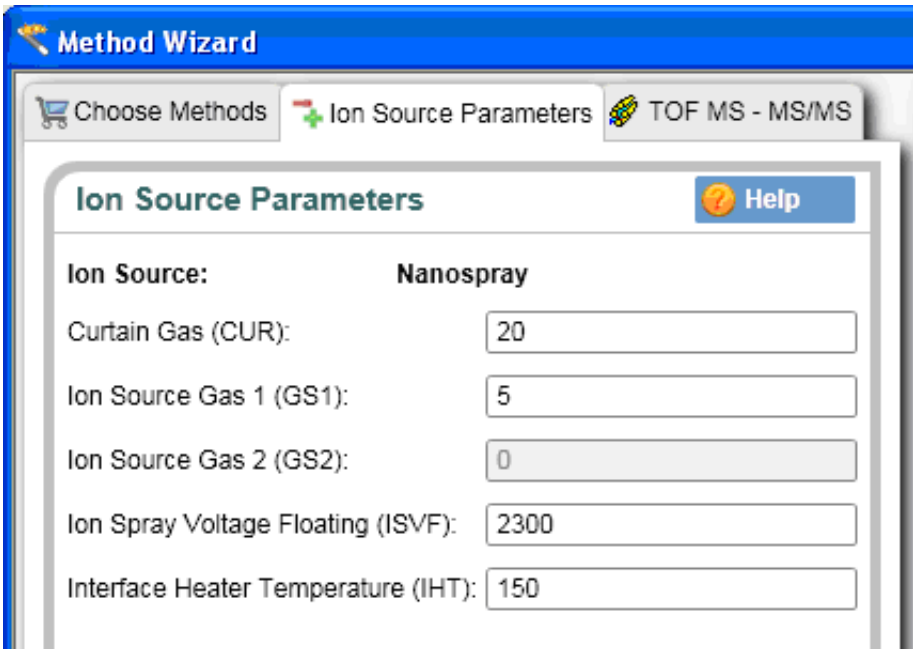


**Figure 1-9 Method Wizard window—Ion Source Parameters tab**

8. Click **Next**.
9. On the **TOF MS - MS/MS** tab, specify the acquisition parameters for the TripleTOF 5600 instrument.

i. Enter the scan information for the TOF MS experiment: In the **Start TOF Mass (Da)** field and the **Stop TOF Mass (Da)** field, specify the mass range to 400 and 1800, respectively. The **TOF Accumulation Time** is 0.250 seconds.

ii. Enter the scan information for the TOF MS/MS experiment: In the **MS2 Start Mass (Da)** and **MS2 Stop Mass (Da)** fields, specify the mass range to 100 and 1800, respectively. The **MS2 Accumulation Time** is 0.5 seconds.

iii. The total **Cycle Time** is automatically calculated from the times specified.

iv. In the **Mass Spec Acquisition Duration** field, specify the **Duration Time** for the entire LC/MS run.
This value depends on the length of time of the analytical gradient. In this example, it is 14 minutes.

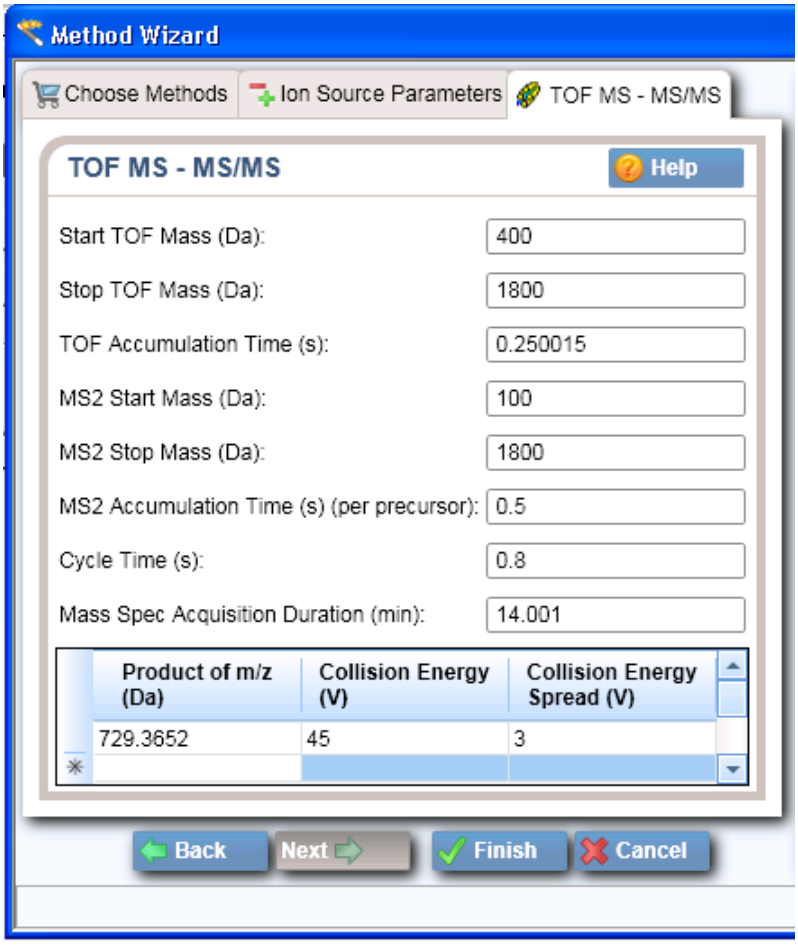


**Figure 1-10 Method Wizard window—TOF MS - MS/MS tab**

10. In the table at the bottom of the tab, define the precursor ion for the product ion scan.

**Note:** As this method is a looped MS and MS/MS method, the precursor ion must be defined.

- For the Beta-Galactosidase digest, a good peptide to fragment for testing is the 729.3652 ion.

- In the table, specify the m/z of this peptide along with the collision energy (45) and a CES of 3 V.
- The Declustering Potential is 70.

11. Click **Finish** to save the method.

## Add LC Information to the Acquisition Method

If the autosampler and pump method information was not specified above, add it now.

**Note:** The Q1 Isolation window is automatically set to Unit. This is the correct setting for this test.

1. Click **Acquisition Method** in the left pane, and then select **LC Sync** as the **Synchronization Mode**.

2. Click **Eksigent AS2** and then select the autosampler method, AS2 10uLloop 1uLinj nanoflex trap elute.ini or AS2 10uLloop 1uLinj trap elute.ini, depending on the installation.

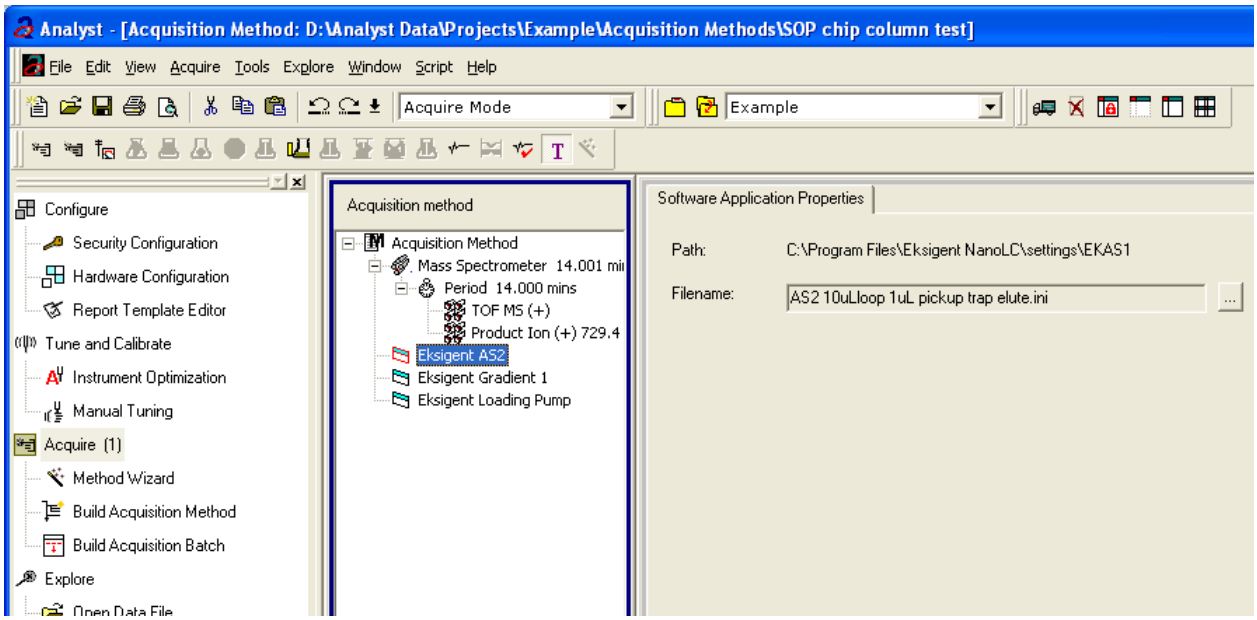


**Figure 1-11 Software Application Properties tab—autosampler filename**

3. Click **Eksigent Gradient 1**, and then select the gradient 2 pump method, CH2 15min 400nLmin nanoflex trap.ini or CH2 15min 400nLmin trap.ini, depending on the installation.

4. Click **Eksigent Loading Pump**, and then select the loading pump method, Load Pump 2 min Trap Wash.ini.

5. Save the method as “System Functional Test”.

**Note:** This method can also function as the LC auto calibration method when using Beta-Galactosidase as a calibrant.

# Create the Methods and Batch for the System Performance Test

This section provides tests for the NanoLC Ultra system that indicate of the performance level of the instrument for proteomics applications such as protein identification and quantification.

Perform these tests when the mass spectrometer is known to be operating well and meeting performance specifications. If the NanoLC system has been idle for two weeks or more, then calibrate the system. Refer to the appendix, System Calibration, for more information.

The expected test duration is 60 minutes using the NanoSpray ion source. Repeat the test until you have consistent peak shape and intensity (approximately 180 minutes).

## Create the Acquisition Methods and Batch— Performance Tests

1. Plumb the autosampler valve with a 10 µL sample loop.
2. In the **AS1/AS2 Autosampler** window, click **Method Editor**.
3. Create the autosampler method for a trap-elute configuration, as shown in Figure 1-12.

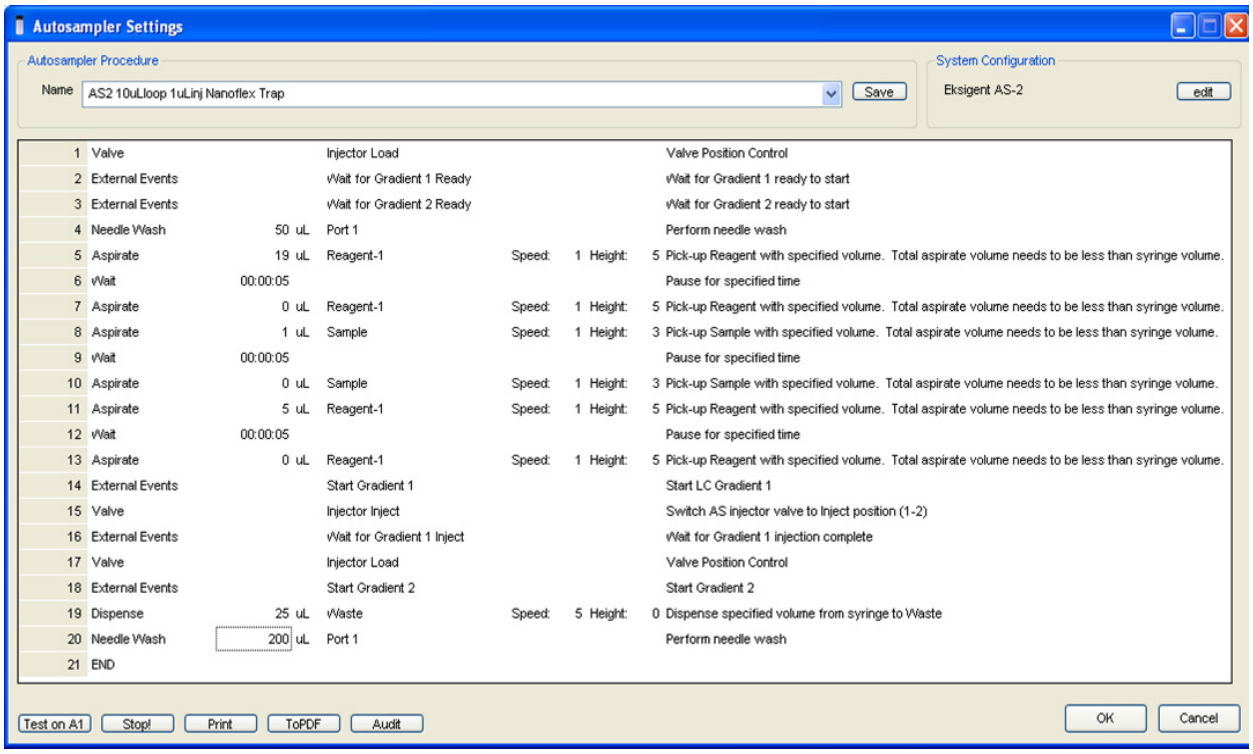


**Figure 1-12 Autosampler Settings dialog—trap-elute configuration (Nanoflex system or column installation)**

4. In the **Name** field, specify the name of this method as AS2 10uLloop 1uLinj trap elute or AS2 10uLloop 1uLinj Ch2Valve Trap, depending on the installation.
5. Click **Save**.

# Create the LC Method

The aqueous channel for each pump (Channel A) will be filled with Buffer A. The organic channel (Channel B) will be filled with Buffer B. For the Loading Pump, Buffer A is always used. Typical buffer mixtures are shown in Table 1-2.

**Table 1-2 Typical Buffer Mixtures**

| Buffer | Mixture | Channel |
|---|---|---|
| Buffer A | 100% water:0.1% formic acid | Channel A |
| Buffer B | 100% acetonitrile:0.1% formic acid | Channel B |

In the method below, the loading pump will be the pump with the microflow module.

## Create the Pump Method in the Eksigent Control Software

1. Make sure that **Loading Pump** is selected as the channel (top, right corner of window).
2. Click **LC Methods**.
3. In the **Name** field, type **Load Pump 10min Trap Wash**, and then click **Save**.

4. On the **Gradient Table** tab, revise the method for the loading pump (the loading pump will be the pump with the microflow module), as shown in Figure 1-13.

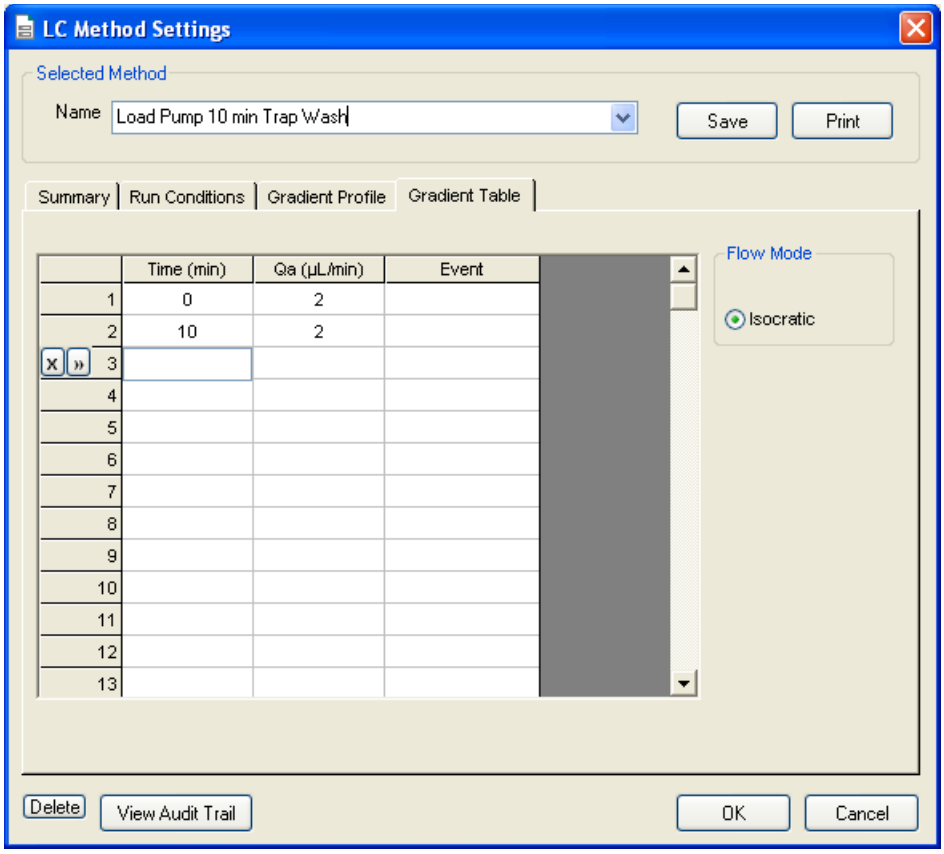


**Figure 1-13 LC Method Settings dialog—Gradient Table tab**

5. On the **Run Conditions** tab, specify conditions as shown in Figure 1-14.

**Note:** For a trap-elute configuration, the Sample Injection method should be Standard.

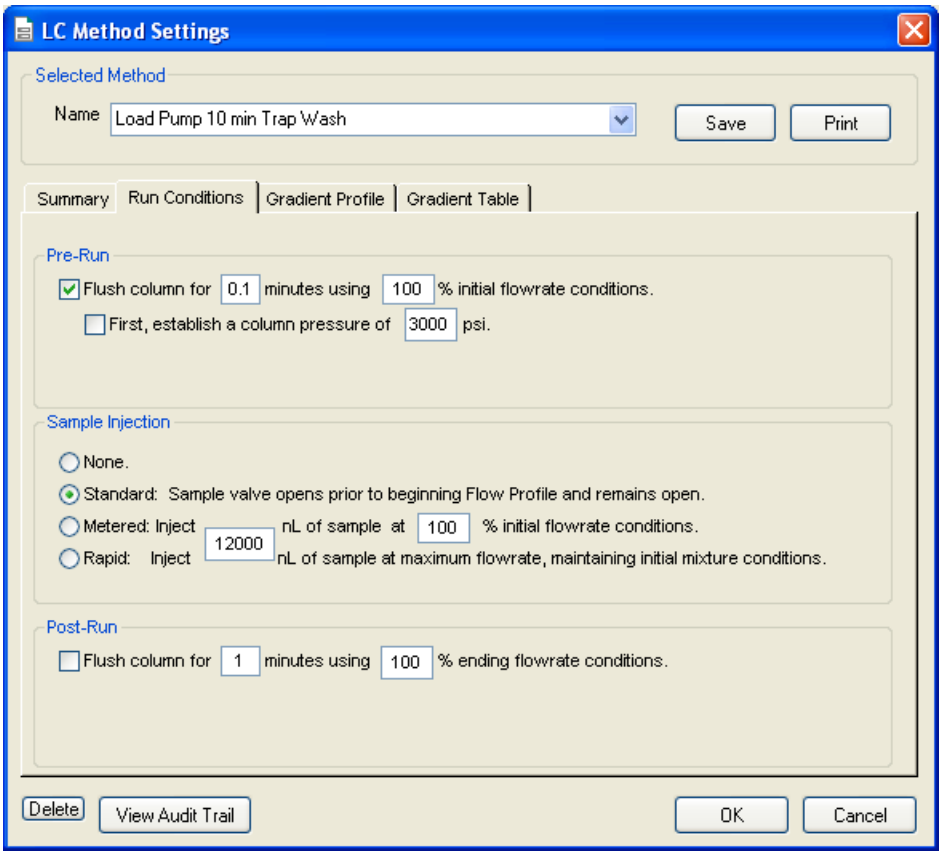


**Figure 1-14 LC Method Settings dialog—Run Conditions tab**

6. Click **Save**.

## Create the Gradient Method in the Eksigent Control Software

For the analytical gradient (typically on the Gradient 2 pump with the nanoflow module), create the gradient method.

1. Make sure that **Gradient 2** is selected as the channel (top, right corner of window).
2. Click **LC Methods**.
3. If this is a Nanoflex system installation, then in the **Name** field, type **CH2 45min 300nL/min Nanoflex trap**, and click **Save**.

   Or

   If this is a column installation, then in the **Name** field, type **CH2 45min 300nLmin column trap**, and click **Save**
4. On the **Gradient Table** tab, specify the method, as shown in Figure 1-15.

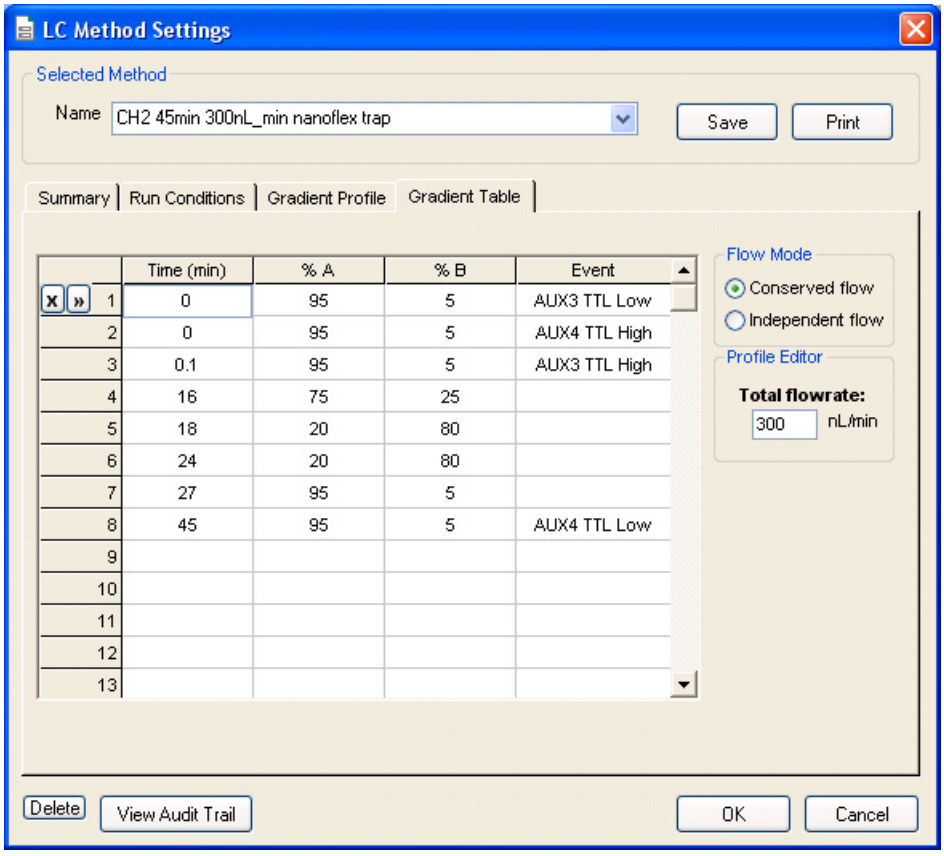


**Figure 1-15 LC Method Settings dialog—Gradient Table tab (Nanoflex system)**

**Note:** The events shown verify the correct switching of the Nanoflex valve. The signals at Time 0 will move the Nanoflex valve to the Inject position and the signals at Time 45 will move the Nanoflex valve back to the Load position.

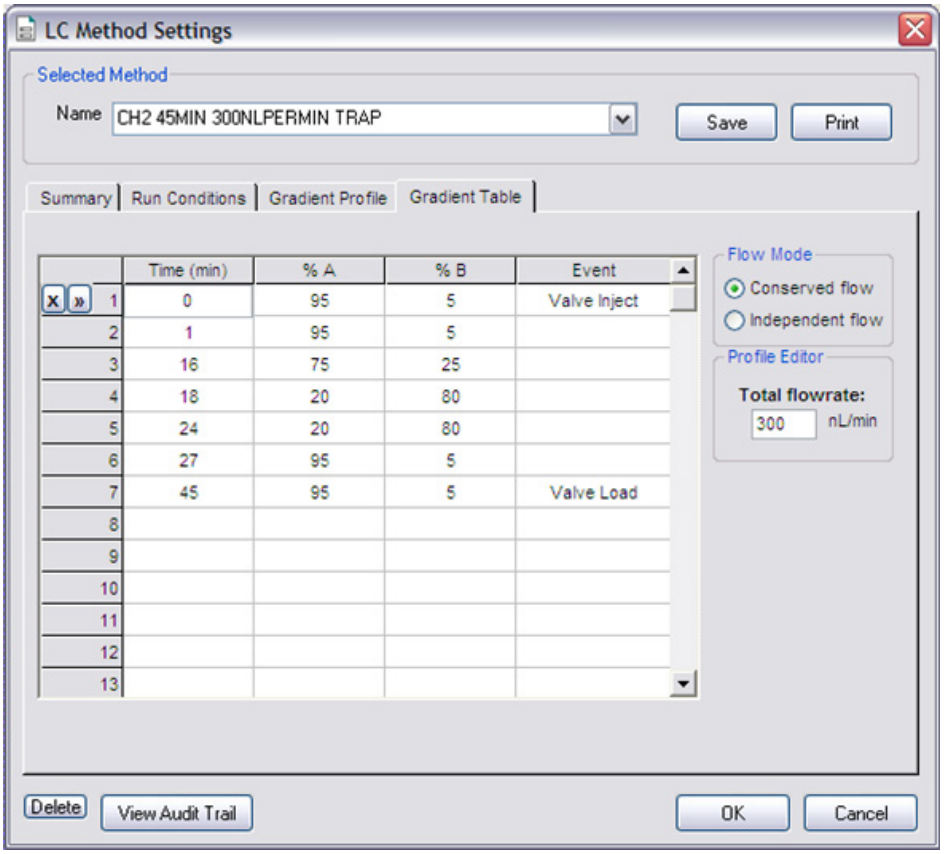


**Figure 1-16 LC Method Settings dialog—Gradient Table tab (column)**

5. On the **Run Conditions** tab, specify the method as shown in Figure 1-17.

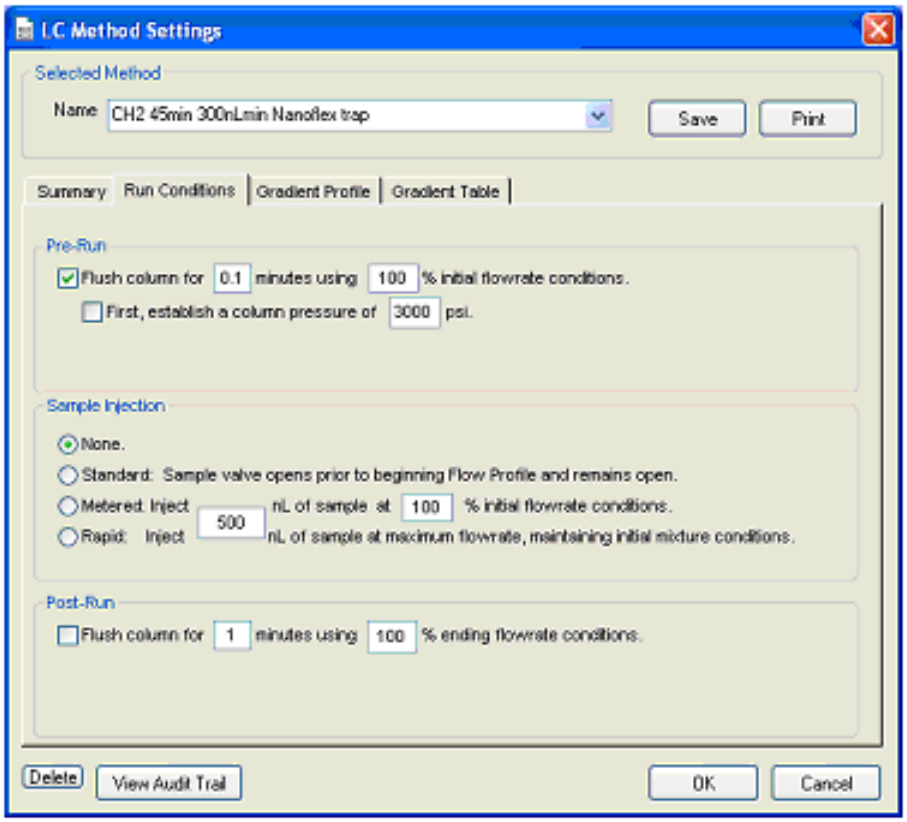


**Figure 1-17 LC Method Settings dialog—Run Conditions tab**

6. Click **Save**.

## Specify the Acquisition Method in the Analyst TF Software

1. In the **Acquire** section of the **Navigation** toolbar, click the **Method Wizard** button to open the wizard.
2. On the **Choose Methods** tab of the **Method Wizard** window, specify the MS method.
   - In the **Choose MS Method** list, select **ToF MS + Hi Sensitivity Product Ion (+)** method.
3. If an autosampler and LC method has already been associated with a previous acquisition method, in the **Choose LC Method** list, select the existing .dam method.
4. If an LC method has not been used before, then select **No LC Method**.

**Tip!** Any method can be selected as it can be edited after creation of this acquisition method.

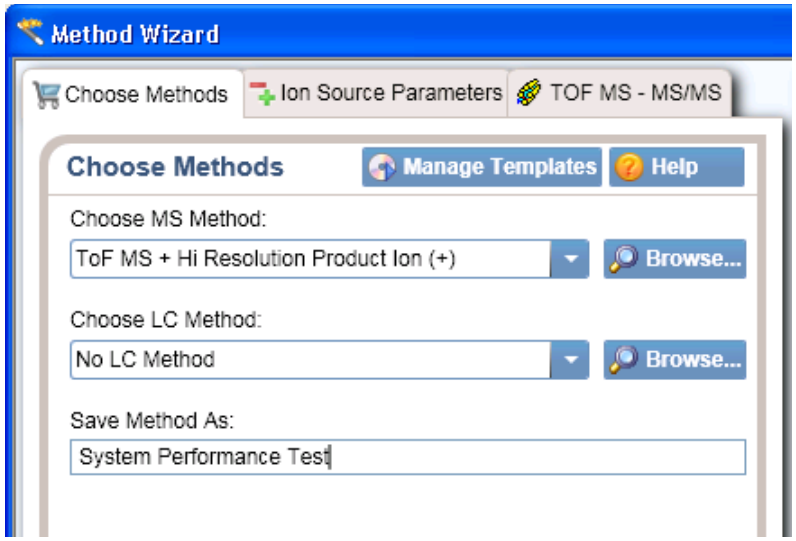


**Figure 1-18 Method Wizard window—Choose Methods tab**

5. In the **Save Method As** field, type **System Performance Test** for the acquisition method and press **Enter**.
6. Click **Next**.
7. On **Ion Source Parameters** tab, specify the source conditions that were determined during the NanoSpray ion source infusion test. Refer to the *NanoSpray® Ion Source Operator Guide* for more information.

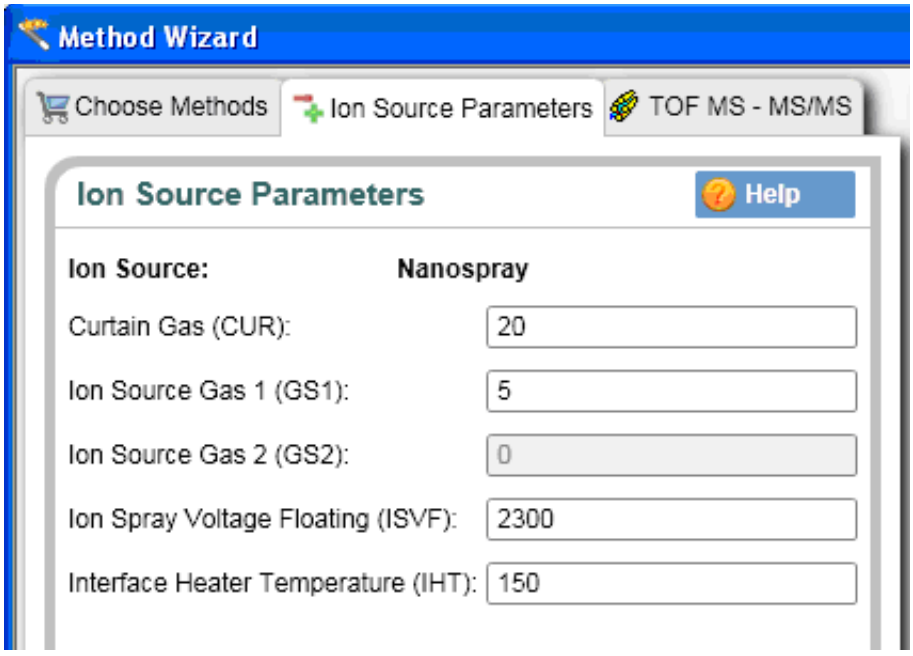


**Figure 1-19 Method Wizard window—Ion Source Parameters tab**

8. Click **Next**.
9. On the **TOF MS - MS/MS** tab, specify the acquisition parameters for the TripleTOF 5600 system.

i. Enter the scan information for the TOF MS experiment: In the **Start TOF Mass (Da)** and **Stop TOF Mass (Da)** fields, specify the mass range to 400 and 1800, respectively. The **TOF Accumulation Time** is 0.250 seconds.

ii. Enter the scan information for the TOF MS/MS experiment: In the **MS2 Start Mass (Da)** and **MS2 Stop Mass (Da)** fields, specify the mass range to 100 and 1800, respectively. The **MS2 Accumulation Time** is 0.5 seconds.

iii. The total **Cycle Time** is automatically calculated from the times specified.

iv. In the **Mass Spec Acquisition Duration** field, specify the **Duration Time** for the entire LC/MS run.
This value depends on the length of time of the analytical gradient. In this example, it is 44 minutes.

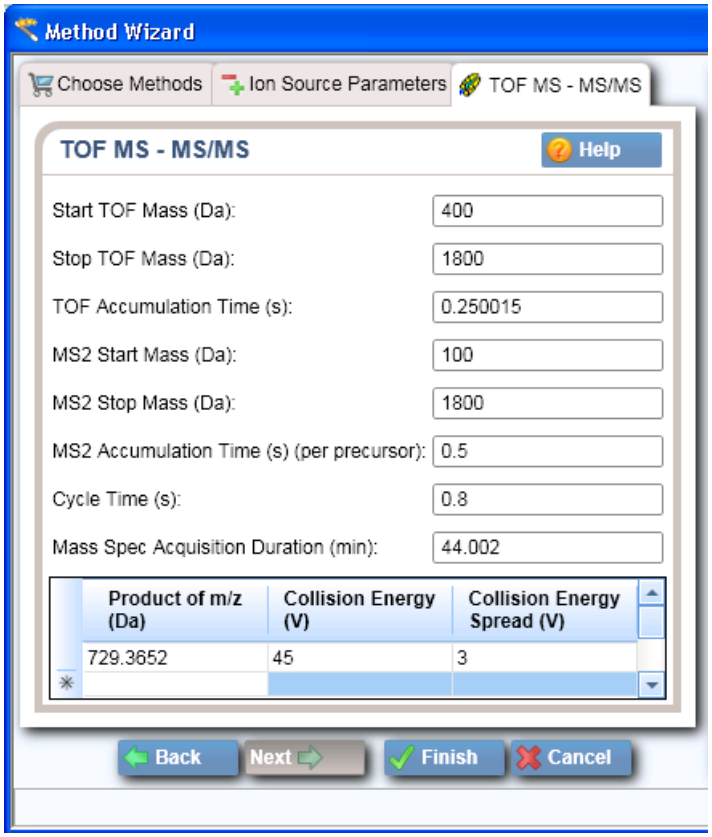


**Figure 1-20 Method Wizard window—TOF MS - MS/MS tab**

10. In the table at the bottom of the tab, define precursor ion as 729.3652. for the product ion scan.
11. Specify the m/z of the peptide with the collision energy (45) and a CES of 3 V.
12. Click **Finish** to save the method.

### Add LC Information to the Acquisition Method

If the autosampler and pump method information was not specified, add it now.

**Note:** The Q1 Isolation window is automatically set to Unit. This is the correct setting for this test.

1. Click **Acquisition Method** in the left pane, and then select **LC Sync** as the **Synchronization Mode**.
2. Click **Eksigent AS2** and then select the autosampler method, AS2 10µLinj nanoflex trap elute.ini or AS2 10µLinj trap.ini, depending on the installation.

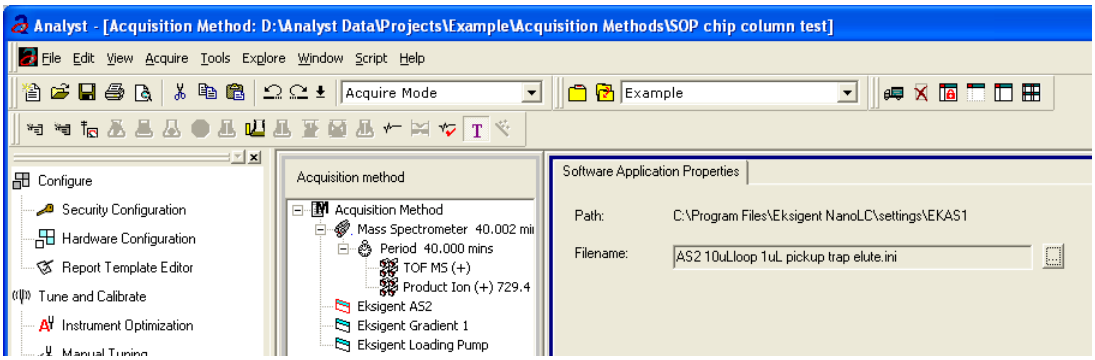


**Figure 1-21 Software Application Properties—Eksigent AS2**

3. Click **Eksigent Gradient 2** and then select the gradient pump method, CH2 45min 300nLmin Nanoflex trap.ini or CH2 45min 300nLmin trap.ini, depending on the installation.
4. Click **Eksigent Loading Pump** and then select the loading pump method, Load Pump 10 min Trap Wash.ini.
5. Save the method as “System Performance Test”.

**Note:** This method can also function as the LC auto calibration method when using Beta-Galactosidase as a calibrant.

# Prepare the System for Testing

Plumb the system in trap-elute configuration to perform the pre-column desalting workflow.

## Prepare the Solution and Dilution

Prepare the Beta-Galactosidase stock solution from the Beta-Galactosidase vial provided in the LC/MS Peptide/Protein Mass Standards Kit as described below. This will produce a stock solution of 1 pmol/µL.

1. Add 625.0 µL of Buffer A (100% water:0.1% formic acid) to the Beta-Galactosidase vial.
2. Vortex the vial for at least 30 seconds.

3. Using a centrifuge, spin the vial to bring the liquid down to the bottom of the vial before opening.
4. Repeat these steps to confirm dissolution.
5. Aliquot the stock solution (1 pmol/µL concentration) into 50 µL volumes and freeze for future use.

**Note:** Solutions can be stored at 4°C for up to 3 days after thawing.

### Prepare the Dilution for Functional Evaluation

1. Combine 40 µL of Buffer A (100% water:0.1% formic acid) with 10 µL of the Beta-Galactosidase protein digest stock solution in a clean vial. A 1 µL injection of a 200 fmol/µL solution will be performed.
2. Vortex the vial for at least 30 seconds to properly mix the solution. This is a 1/5 dilution and will give a final concentration of 200 fmol/µL.
3. Transfer the solution to the autosampler vial and make sure there is no bubble on the bottom of the vial.

### Prepare the Dilution for Performance Evaluation

1. Prepare the solution as described above. A 1 µL injection of a 25 fmol/µL solution will be performed.
2. Prepare 400 µL of the working solution of Beta-Galactosidase.
    - Combine 390 µL of Buffer A (100% water:0.1% formic acid) with 10 µL of the Beta-Galactosidase protein digest stock solution in a clean vial.
    - Vortex the solution for at least 30 seconds to properly mix the solution. This is a 1/40 dilution and will give a final concentration of 25 fmol/µL.
    - Transfer the solution to the autosampler vial and make sure there is no bubble on the bottom of the vial.

## Condition the System

A trap and column typically require 2 to 3 runs with 200 fmol of protein digest for conditioning.

- Verify that the trap and analytical column are well conditioned with protein digest injections before performing this test.

# Verify System Readiness

Make sure the NanoLC Ultra system is meeting performance specifications.

1. Connect effluent from the NanoLC Ultra system to the NanoSpray ion source and verify that the spray is stable by monitoring the background signal in the Analyst TF software.
2. Equilibrate the LC/MS system with the starting conditions of the method outlined above.
3. Make sure the spray is stable.
4. Double-click **Manual Tune** in the left **Navigation** bar.
5. Enter the key parameters in Table 1-3, and then click **Start** to begin acquisition.

**Table 1-3 Key Parameters**

| Parameter | Value |
|---|---|
| **MS** | |
| Scan type | TOF MS |
| Polarity | Positive |
| Start Mass | 400 |
| Stop Mass | 1000 |
| Run Time | 2 min |
| **Source/Gas**** | |
| Curtain Gas (CUR) | 20-25 |
| IonSpray Voltage (IS) | 2100-2400 V |
| Ion Source Gas 1 (GS1) | 2-15 |
| Interface Heater | 150°C |
| **Compound** | |
| Declustering Potential (DP) | 70 |

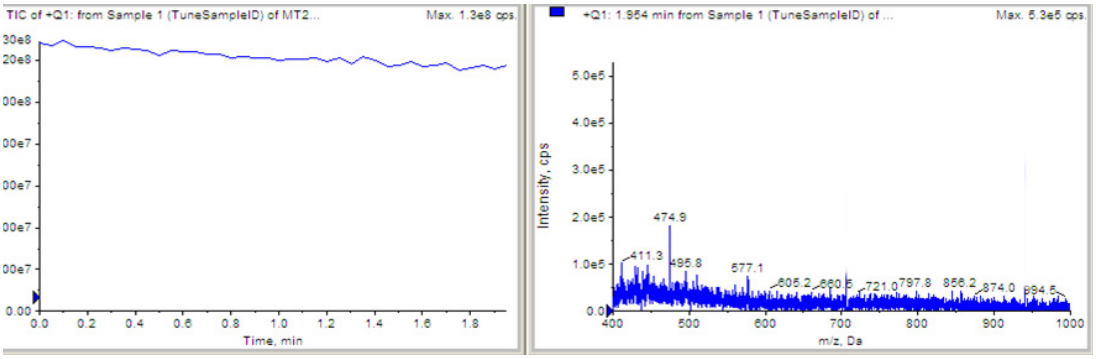


**Figure 1-22 Analyst TOF MS scan for background noise**

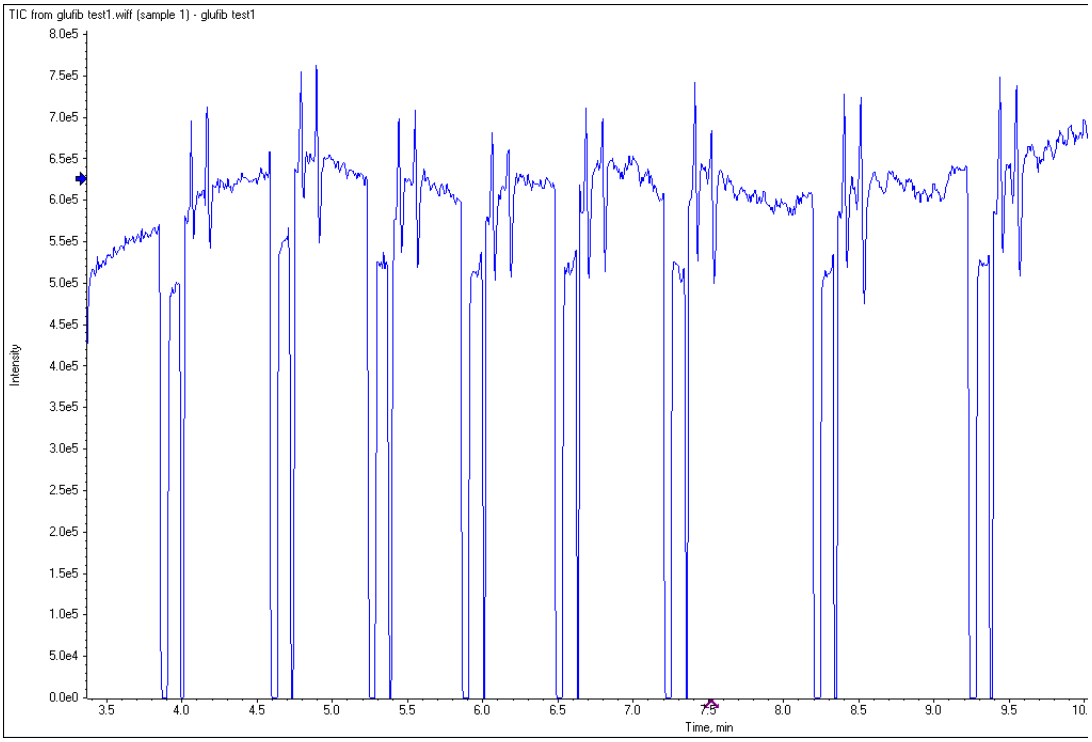


**Figure 1-23 Unstable spray induced by air bubbles**

6. Make sure that the spray is still stable by monitoring the background signal with a TOF MS scan.
   - Stable spray appears as shown in Figure 1-22.
   - Unstable spray appears as shown in Figure 1-23 (typical unstable spray induced by air bubbles).
   - If the spray is not stable, retune the NanoSpray ion source by infusion. Refer to the *NanoSpray® Ion Source Operator Guide* for more information.

# Perform the System Functional Test

Create the LC/MS acquisition batch, run the batch and then verify the results.

### Create the LC/MS Acquisition Batch in the Analyst TF Software

1. Double-click **Build Acquisition Batch** in the left **Navigation** bar.
2. Build the acquisition batch, as shown in Figure 1-24.
   i. In the **Acquisition** group, select the acquisition method from the list.
   ii. Click **Add Set**, and then click **Add Samples**.

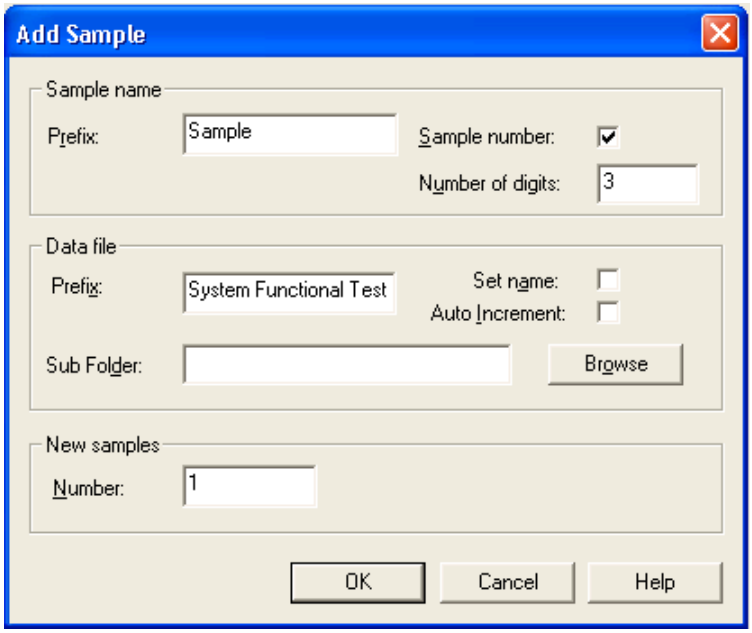


**Figure 1-24 Add Samples dialog—System Functional Test**

3. Click **OK**.
4. Save the data file as TT5600 system LC BGal functional status check <date>.
5. On the **Location** tab, specify the location of the Beta-Galactosidase sample in the autosampler.

## Run the Batch

1. On the **Submit** tab, click **Submit**.
2. In the **View** menu, click **Sample Queue**.
3. In the **Acquire** menu, click **Start Sample**.

## Verify the Results in the PeakView Software

1. Open the data file and double-click the magenta arrow to display the individual TICs.
2. Right-click the TIC of the TOF MS and select **Remove all Traces Except Active** to display the overlaid TOF MS TIC.
3. Extract the TOF MS peak XICs for the target peptides.
    i. On the **Show** menu, click **Extract Ions Using**.
    ii. Specify the masses and extraction width for the peptides as shown in Figure 1-25. Only the masses and widths are required.

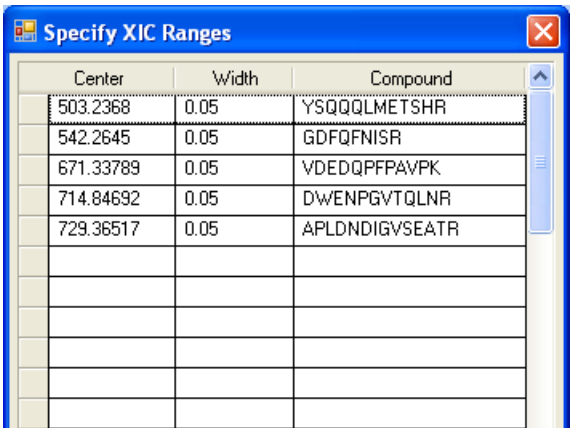


**Figure 1-25 Specify XIC Ranges dialog**

4. Click **OK**.
   The XICs for each peptide are generated.

**Note:** The XIC can be used to evaluate the NanoLC Ultra LC/MS peak retention times and shapes. Peak widths, retention times, and XIC width vary from LC to LC system depending on transfer line volumes, column type used, column age, and more.

5. In the **XIC** pane, click and drag across the range of the more intense peaks, as shown in Figure 1-26.

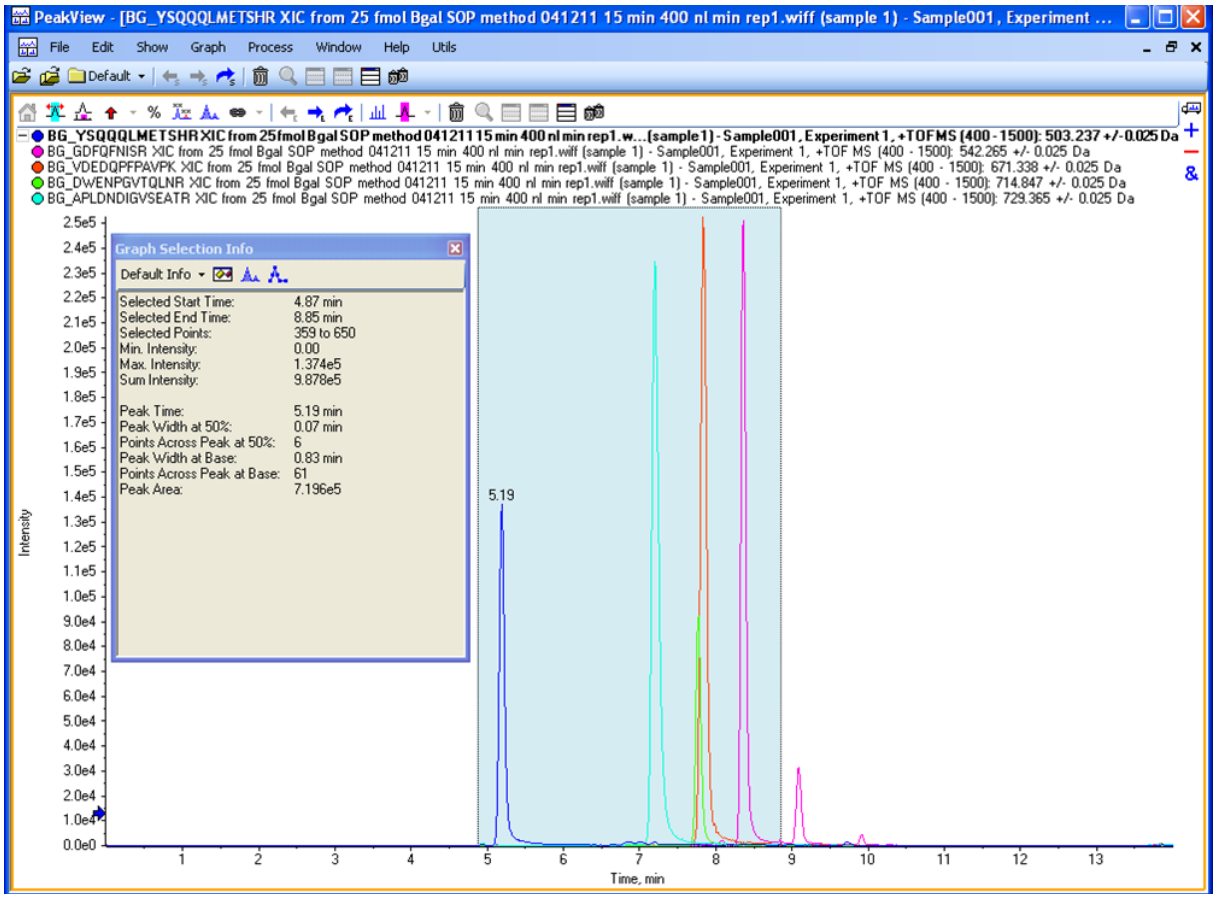


**Figure 1-26 TOF MS XICs of 5 peptides from Beta-Galactosidase NanoLC Ultra trap elute run (200 fmol on column)**

**Note:** This test is not a specification. Use this example to confirm injection and peak separation and shape only.

6. Make sure the peaks have good separation and shape.
   i. In the PeakView software, on the **Window** menu, click **Graph Selection Window**.
   ii. To collect the Peak Area for each XIC, click each (color-keyed) line in the upper left.

   The retention time appears on the top of the peak selected. The bottom line of the **Graph Selection Info** displays the Peak Area.

## Measure the Performance of the TOF MS/MS Scan

1. In the main TIC pane, extract individual TICs by double-clicking the magenta arrow.
2. Select the MS/MS scan, and then select **Remove all Traces Except Active** to display the pane shown at the bottom of Figure 1-27.

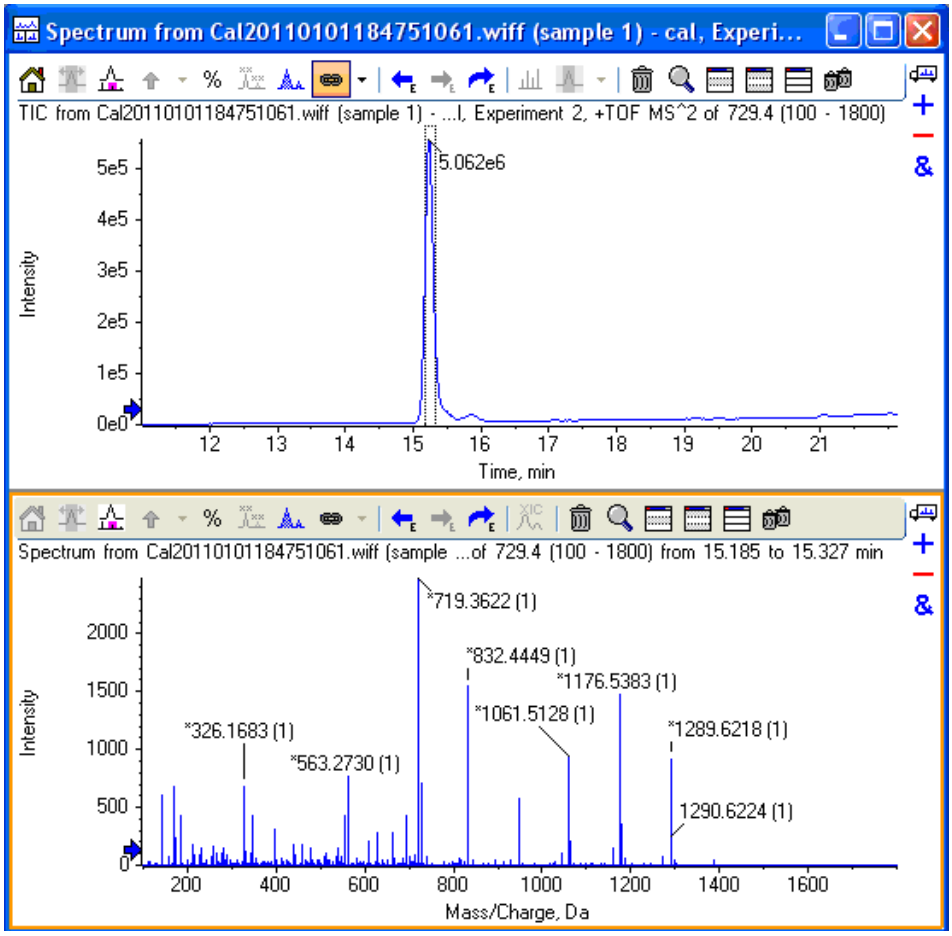


**Figure 1-27 TOF MS/MS XIC of m/z 729.3652 peak**

**Note:** This XIC is not a specification. Use this example to verify the TOF MS/MS scan.

3. Click and drag across the peak and double-click the peak to display the underlying MS/MS spectra.
4. Make sure the MS/MS spectra looks as shown in Figure 1-27 with fragment ions across the whole mass range.
5. Repeat the acquisition until you have consistent peak shape and peak intensity. If required, refer to Troubleshoot Peak Problems for more information.

# Perform the System Performance Test

Create the LC/MS acquisition batch, run the batch and then verify the results.

## Create the LC/MS Acquisition Batch in the Analyst TF Software

1. Double-click **Build Acquisition Batch** in the left **Navigation** bar.
2. Build the acquisition batch, as shown in Figure 1-28.
    i. In the **Acquisition** group, select the acquisition method from the list.
    ii. Click **Add Set**, and then click **Add Samples**.

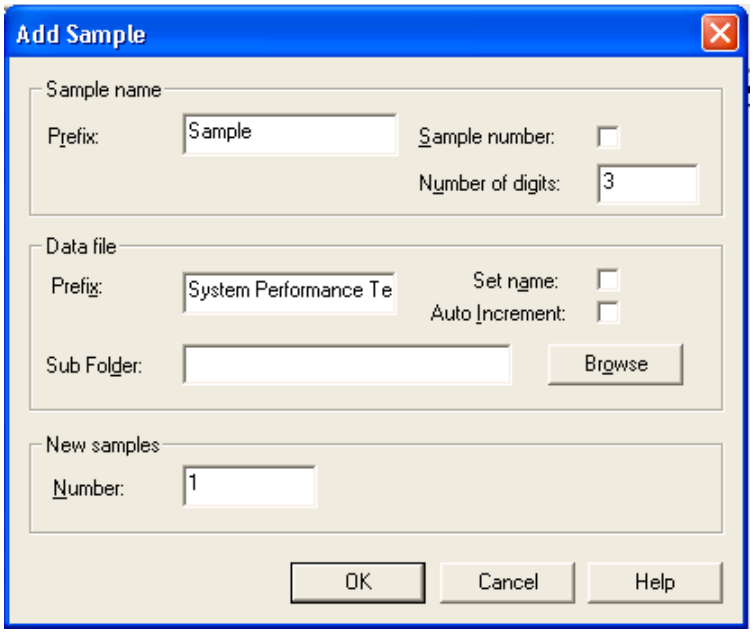


**Figure 1-28 Add Sample dialog—System Performance Test**

3. Click **OK**.
4. Save the data file as TT5600 system LC BGal Performance Test <date>.
5. On the **Location** tab, specify the location of the Beta-Galactosidase sample in the autosampler.

## Run the Batch

1. On the **Submit** tab, click **Submit**.
2. In the **View** menu, click **Sample Queue**.
3. In the **Acquire** menu, click **Start Sample**.

## Verify the Results in the PeakView software

1. Open the data file and double-click the magenta arrow to display the individual TICs.
2. Right-click the TIC of the TOF MS and select **Remove all Traces Except Active** to display the overlaid TOF MS TIC.
3. Extract the TOF MS peak XICs for the target peptides.

i. On the **Show** menu, click **Extract Ions Using**.

ii. Specify the masses and extraction width for the peptides as shown in Figure 1-25. Only the masses and widths are required.

4. Click **OK**.
The XICs for each peptide are generated. The peak areas of the extracted peaks can be used to evaluate the NanoLC Ultra LC/MS sensitivity and the peak retention times can shapes can be used to evaluate the chromatography.

**Note:** The XIC can be used to evaluate the NanoLC Ultra LC/MS peak retention times and shapes. Peak widths, retention times, and XIC width vary from LC to LC system depending on transfer line volumes, column type used, column age, and more.

5. In the **XIC** pane, click and drag across the range of the more intense peaks, as shown in Figure 1-29.
6. On the **Window** menu, click **Graph Selection Window**.
7. Collect the peak area value for each XIC.

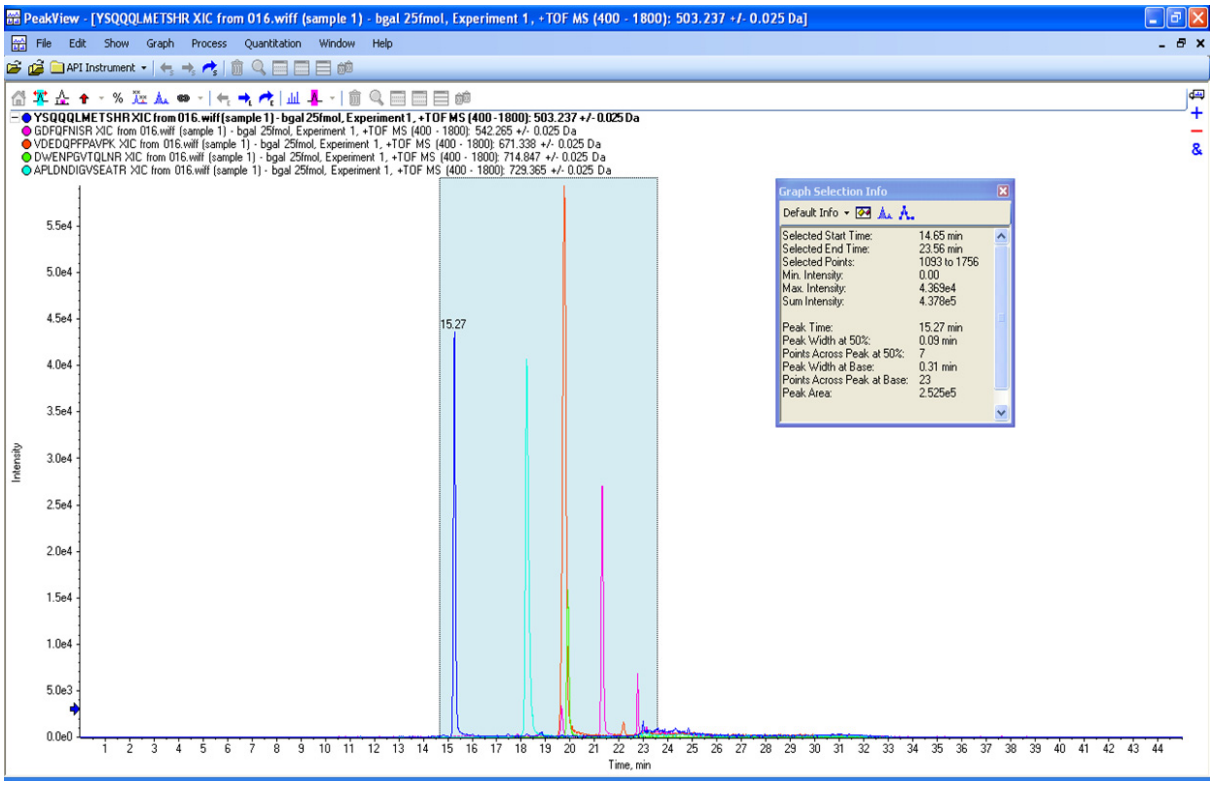


**Figure 1-29 TOF MS XICs of 5 peptides from Beta-Galactosidase NanoLC Ultra trap elute run (25 fmol on column)**

**Note:** This test is not a specification. Use this example to confirm injection and peak separation and shape only. Refer to the peak area specification in the Test Results—TripleTOF 5600 Instruments section of this chapter.

8. Make sure the peaks have good separation and shape using the PeakView software.
   i. In the PeakView software, on the **Window** menu, click **Graph Selection Window**.
   ii. Note the retention times of the chosen peaks.
   This will vary with each system. Time of elution of the first is about 14 to 16 minutes. The longer elution time indicates you need to minimize LC dead volume.

**Note:** Most of the XICs obtained should have peak widths of ~0.17 minute half height on average and have peak intensities similar to that shown in Figure 1-29. Some peaks will be narrower and some will be broader.

## Measure the Performance of the TOF MS/MS Scan

1. In the main TIC pane, extract individual TICs by double-clicking the magenta arrow.
2. Select the MS/MS scan, and then select **Remove all Traces Except Active** to display the pane shown in Figure 1-30.

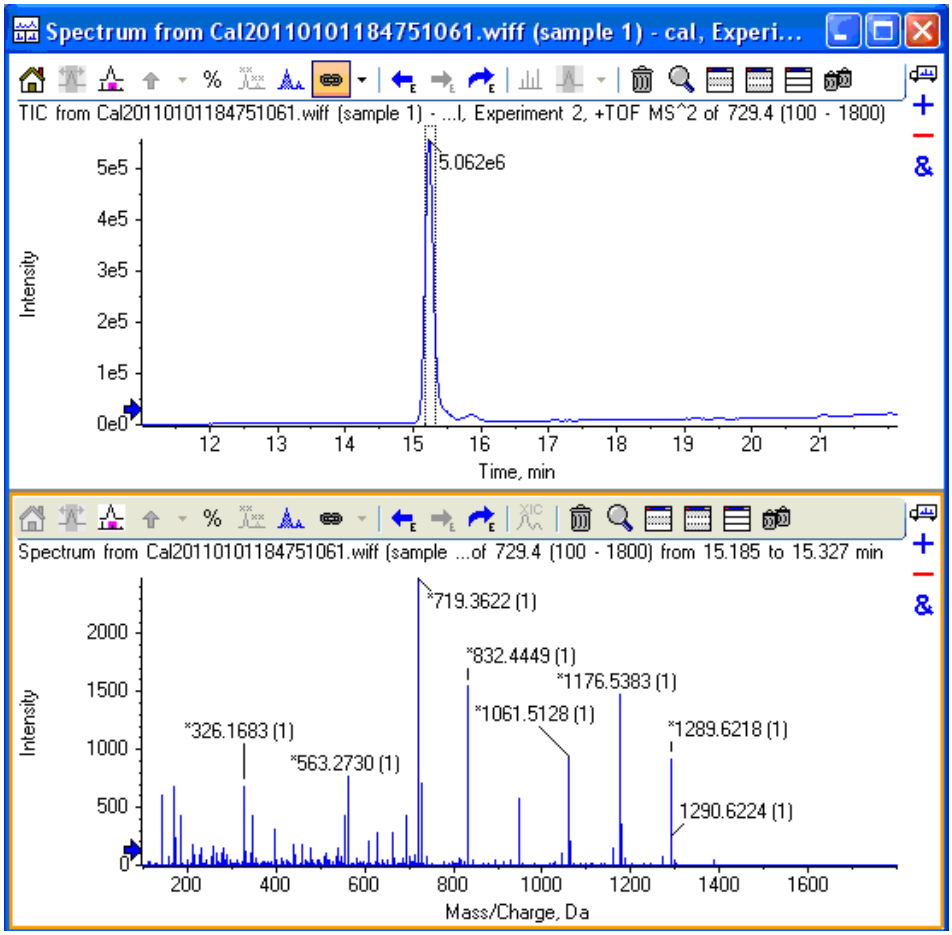


**Figure 1-30 TOF MS/MS XIC of m/z 729.3652 peak**

**Note:** This XIC is not a specification. Use this example to verify the TOF MS/MS scan.

3. Click and drag across the peak and double-click the peak to display the underlying MS/MS spectra.
4. Make sure the MS/MS spectra has fragment ion across the whole mass range. Refer to Figure 1-25.

**Note:** This test is not a specification. Use this example to confirm injection and peak shape only.

5. Repeat the acquisition until you have consistent peak shape, retention time, and peak intensity (a minimum of 3 times).

   For new columns, this may require that you repeat the acquisition 10 or more times in order to obtain consistent peak shape, retention time, and peak intensity. If required, refer to Troubleshoot Peak Problems for more information.
6. Record the results for each acquisition.
7. Record the average peak area of the acquisitions in the section, Test Results—TripleTOF 5600 Instruments on page 50.
8. Make sure that the average peak area meets the minimum requirements specified in the section, Test Results—TripleTOF 5600 Instruments on page 50.

# Troubleshoot Peak Problems

This section provides information for troubleshooting peak related problems such as broad or tailing peak widths, lack of separation between peaks, and low peak area.

## Peak widths are too broad or are tailing

- Inspect all connections in the flow path to verify that there are no dead volumes.
- Look at connections post-column and around trap column. A small increase in peak width is often seen when a trap column is used.

**Caution: Potential Instrument Damage: If using the Nanoflex system and problems persist, do not attempt to troubleshoot the fittings connected to the chip.**

## No separation between the peaks

- Make sure that both pumps are delivering the correct amount of solvent.
- Make sure that the pressure spike upon injection is not too severe in the high-flow channel (less than 300 psi change in pressure).
- Large pressure change upon injection suggests an air bubble has been introduced to the sample loop or is present in the trap column plumbing.

**Note:** The overall separation of the chromatography itself will often be less than direct injection. Components that elute comparably on the trap and analytical column will not re-resolve on the analytical column and, as a result, spread out or bunch together.

## Peak intensity or peak area is too low

- Verify the performance of the mass spectrometer and the ion source spray using the infusion tests in the *Nanospray® Ion Source Operator Guide*.
- Verify that the trap and analytical column are well conditioned with protein digest injections before performing this test. A trap typically requires 2 to 3 runs with 250 fmol to 500 fmol of protein digest on the column for conditioning.
- Verify that the correct amount of sample has been withdrawn from the autosampler vial.
- Perform a direct injection with a protein digest on the analytical column to determine if the problem is related to the trap.
- If the first LC peak does not elute for a long time, inspect the system for dead volume before the trap.
- If the early eluting peaks are not visible or are very low in intensity, this could mean that trapping efficiency is low. Replace the trap.

**Tip!** Minimize tubing length wherever possible and make sure all tubing for the nanoflow path has an inner diameter of approximately 25 µm i.d.

- If the late eluting peaks are not visible or are very low in intensity, this is usually a sign that the column is getting old. In rare cases, it could mean that the Beta-Galactosidase standard is degraded. See Figure 1-31 and Figure 1-32 for an example of a scan with 10 compounds and an older column.

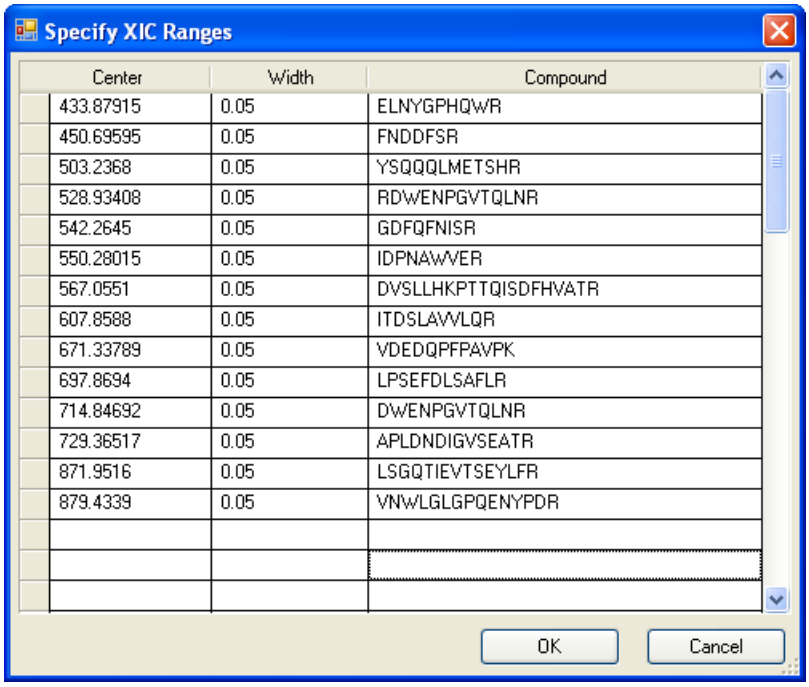


**Figure 1-31 Specify XIC Ranges dialog**

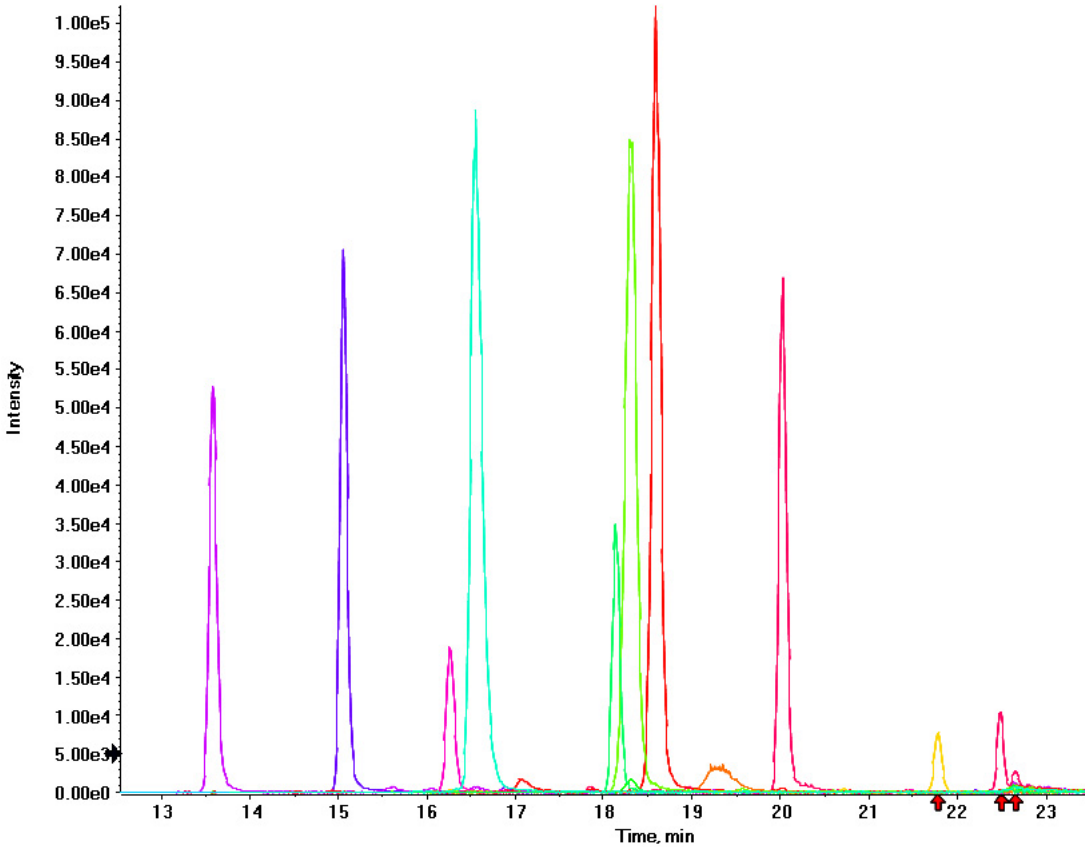


**Figure 1-32 Extraction of all peaks—Late eluting peaks not present**

- Always monitor the column and trap pressure over time; increasing pressure may indicate increasing blockage; probably at the Nanospray ion source tip. If, when the connection between the column and the ion source head is unfastened and the pressure changes quickly, then the tip is getting clogged and should be changed.
- For better long-term column lifetime, verify that there is at least a 30% drop in pressure observed during the high organic flush of the column. Increase the duration of the high organic flush until a good pressure change is observed. This time might increase for the trap column configuration relative to the direct injection configuration.
- Figure 1-33 shows a minimal pressure change upon injection and a 30% pressure decrease during the high organic flush.

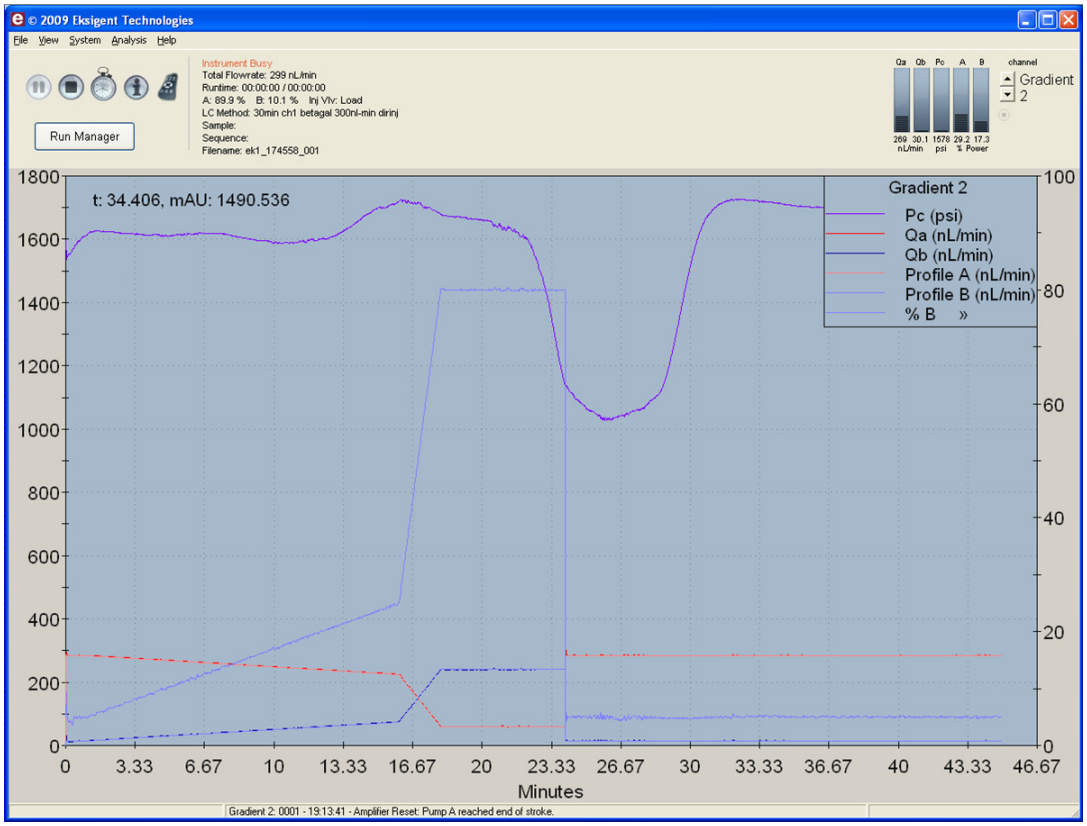


**Figure 1-33 Good pressure profile for a direct injection NanoLC Ultra system run**

# Create a Backup of the EKSettings.reg File

The EKSettings.reg file can be used to re-establish the system settings derived on installation if they are lost. Create a copy of the REG file upon completion of these tests.

1. In the Eksigent control software, on the **System** menu, click **Instrument Configuration**.
2. Click **Export Settings**.

   A backup of the REG file is created.
3. Navigate to the system settings folder (for example, C:\Program Files\Eksigent NanoLC\settings).
4. Copy the previous_settings.reg file to another location, separate from the host computer.

# Test Results—TripleTOF 5600 Instruments

For TripleTOF 5600 instruments, complete this table with the results from the ion from the Beta-Galactosidase digest solution stock. Make sure that the peak area is within specification.

Most of the XICs obtained should have peak widths of ~0.17 minute half height on average and have peak intensities similar to that shown in Figure 1-29. Some peaks will be narrower and some will be broader Additionally, verify that the peaks elute within 5 minutes of each other.

Beta-Gal Lot Number: _____

**Table 1-4 LC/MS Specification Test—TripleTOF 5600 Instruments**

| Q1 | Peptide ID | Spec. (peak area) | Actual |
|---|---|---|---|
| 503.2368 | YSQQQLMETSHR | 1.0E+05 | |
| 542.2645 | GDFQFNISR | 1.0E+05 | |
| 671.3379 | VDEDQPFPAVPK | 2.0E+05 | |
| 714.8469 | DWENPGVTQLNR | 5.0E+04 | |
| 729.3652 | APLDNDIGVSEATR | 2.0E+05 | |
| | | Specification Passed? | ❑ |

## Notes

| |
|---|
| |
| |
| |

# LC/MS System Configuration Test— AB SCIEX QTRAP® 5500 System

# 2

This chapter describes the steps for preparing and performing LC/MS system configuration tests for the NanoLC Ultra® system configured with the cHiPLC® Nanoflex system (or external ChromXP column) and the AB SCIEX QTRAP® 5500 instrument.

**Note:** The tests in this chapter are written for Gradient 2 as the low-flow channel. If this is not true for your system (for example, if you have a 1D or 1D+ system), then make the appropriate changes throughout the tests.

The tests in this chapter are divided as follows:

- Fast test to condition the column and determine the functional status of the system. See Create the Methods and Batch for the System Functional Test for details.

  Perform these tests after completing the NanoSpray® ion source infusion tests in order to first confirm the spray performance of the tip. Refer to the *NanoSpray® Ion Source Operator Guide* for more information.

- Longer test to determine the performance level of the instrument for proteomics applications such as protein identification and quantification. See Create the Methods and Batch for the System Performance Test for details.

  These tests can be used as a measure of the NanoLC Ultra system performance in isolation of performance of the other components. Results from these tests can become the baseline performance for the system and can be performed regularly and used as a system quality control test in the future.

Approximate time required:

1. Create the methods and batch: 45 minutes
2. Prepare the system for testing: 3-4 hours
3. Perform the test.
   i. System functional test: 90 minutes
   ii. System performance test: 180 minutes

Recommended solvents can be ordered from VWR:

- Burdick and Jackson acetonitrile with 0.1% formic acid, P/N BJLC441-1.0
- Burdick and Jackson water with 0.1% formic acid, P/N BJLC452-1.0

Required materials for a Nanoflex system installation:

- Reverse phase cHiPLC column (75 µm x 15 cm ChromXP C18-CL 3 µm 120 Å)
- cHiPLC trap (200 µm x 0.5 mm ChromXP C18-CL 3 µm 120 Å)
- LC/MS Peptide/Protein Mass Standards Kit (P/N 4368624)

**Note:** Ensure that the Nanoflex system is in the Load position before beginning these tests.

Required materials for an external column installation:

- Reversed phase ChromXP nanoLC column (75 µm ID x 15 cm, ChromXP C18 3 µm 120 Å, P/N 805-00120)
- ChromXP nanoLC Trap column (350 µm ID x 0.5 mm, ChromXP C18 3 µm 120 Å, P/N 5016752)
- LC/MS Peptide/Protein Mass Standards Kit (P/N 4368624)

**Note:** After successfully completing the tests, create a backup of the EKSettings.reg file. See Create a Backup of the EKSettings.reg File for more information.

# Create the Methods and Batch for the System Functional Test

This section describes a test for the NanoLC Ultra system to condition the column and determine the functional status.

Perform these tests when the mass spectrometer is known to be operating well and meeting performance specifications.

**Note:** The steps in this section do not constitute a NanoLC Ultra system performance test. See Create the Methods and Batch for the System Performance Test.

The expected test duration is 30 minutes using the NanoSpray® ion source. Repeat the test until you have consistent peak shape and intensity (approximately 90 minutes).

## Create the Acquisition Methods and Batch—Functional Test

**Note:** Use Gradient 2 for the autosampler method. Gradient 2 is the nanoflow module for LC configuration.

1. Plumb the autosampler valve with a 10 µL sample loop.
2. In the **AS1/AS2 Autosampler** status window, click **Method Editor**.
3. Create the autosampler method for a trap-elute configuration, as shown in Figure 2-1 or Figure 2-2.

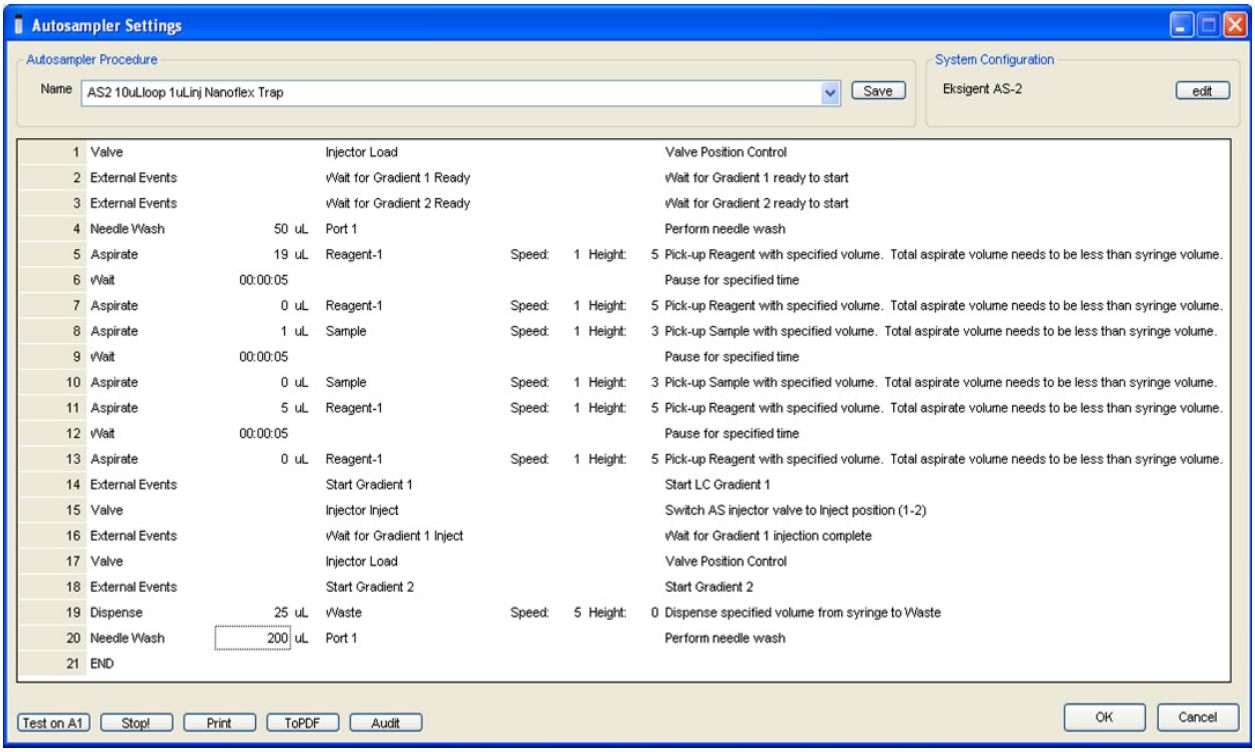


**Figure 2-1 Autosampler Settings dialog—trap-elute configuration (Nanoflex system)**

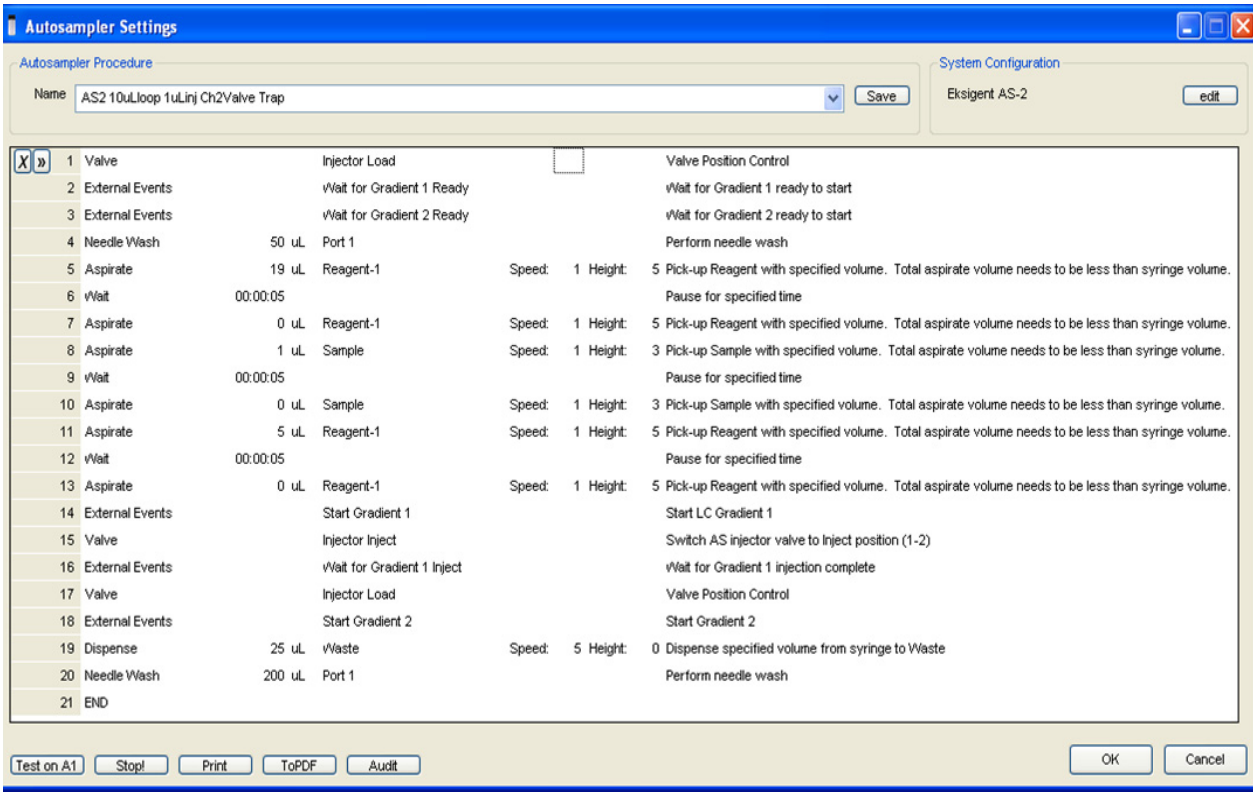


**Figure 2-2 Autosampler Settings dialog—trap-elute configuration (column)**

4. In the **Name** field, specify the name of this method as AS2 10uLloop 1uLinj Nanoflex Trap (Nanoflex system installation) or AS2 10uLloop 1uLinj Ch2Valve Trap (column installation), and then click **Save**.

# Create the LC Methods

The aqueous channel for each pump (Channel A) will be filled with Buffer A. The organic channel (Channel B) will be filled with Buffer B. For the Loading Pump, Buffer A is always used. Typical buffer mixtures are shown in Table 2-1.

**Table 2-1 Typical Buffer Mixtures**

| Buffer | Mixture | Channel |
|---|---|---|
| Buffer A | 100% water:0.1% formic acid | Channel A |
| Buffer B | 100% acetonitrile:0.1% formic acid | Channel B |

In the method below, the loading pump will be the pump with the microflow module.

## Create the Pump Method in the Eksigent® Control Software

1. Ensure that **Loading Pump** is selected as the channel (top, right corner of window).
2. Click **LC Methods**.
3. In the **Name** field, type **Load Pump 2 min Trap Wash**, and then click **Save**.

4. On the **Gradient Table** tab, revise the method for the loading pump (the loading pump will be the pump with the microflow module), as shown in Figure 2-3.

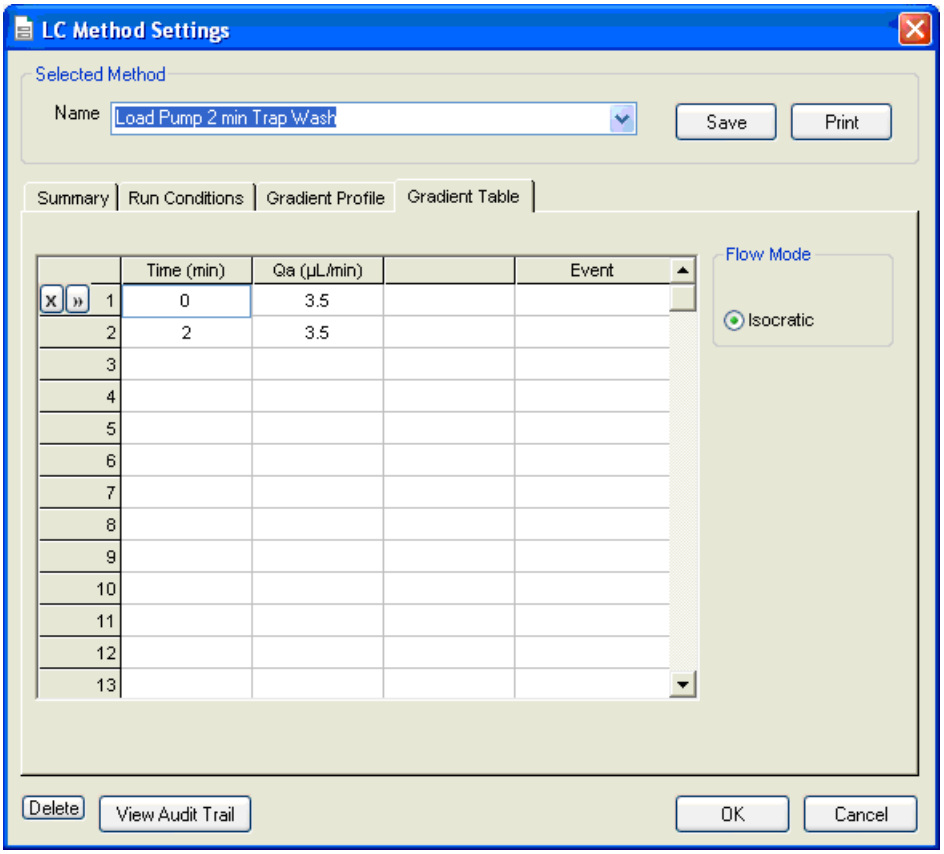


**Figure 2-3 LC Method Settings dialog—Gradient Table tab**

5. On the **Run Conditions** tab, specify conditions as shown in Figure 2-4.

**Note:** For a trap-elute configuration, the Sample Injection method should be Standard.

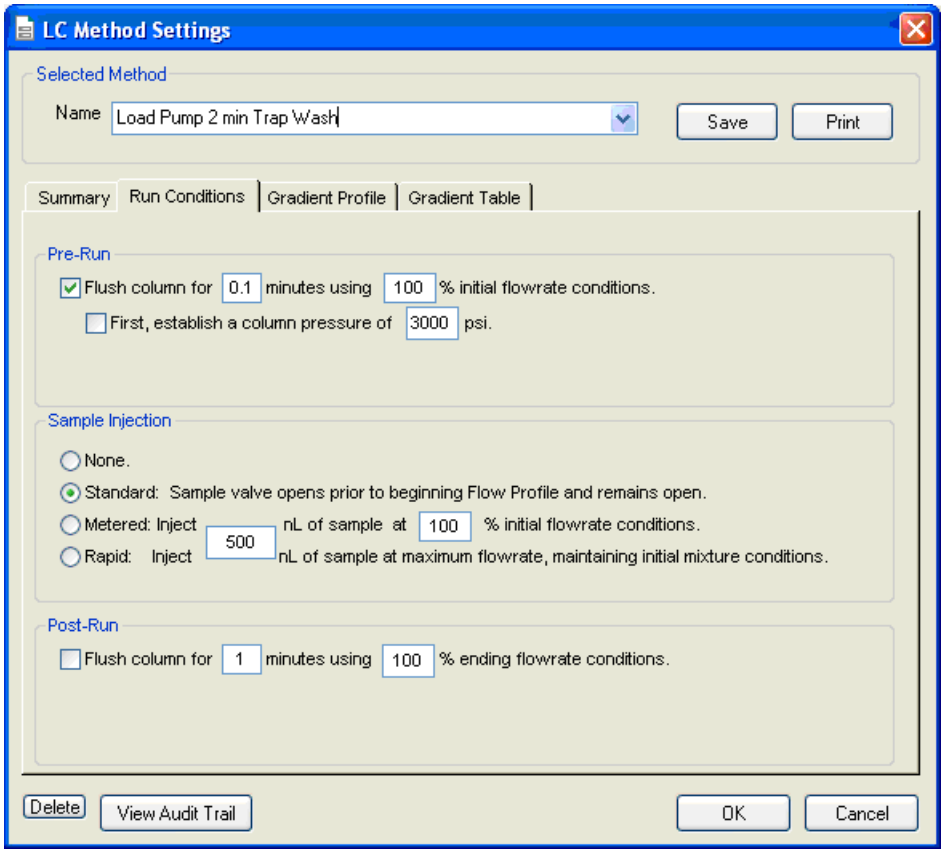


**Figure 2-4 LC Method Settings dialog—Run Conditions tab**

6. Click **Save**.

## Create the Gradient Method in the Eksigent Control Software

For the analytical gradient (typically on the Gradient 2 pump with the nanoflow module), create the gradient method.

1. Ensure that **Gradient 2** is selected as the channel (top, right corner of window).
2. Click **LC Methods**.
3. If the installation includes a Nanoflex system, then in the **Name** field, type **CH2 15min 400nLmin nanoflex trap**, and click **Save**.

   Or

   If the installation includes an external column, then in the **Name** field, type **CH2 15min 400nLmin trap**, and click **Save**.
4. On the **Gradient Table** tab, specify the method as shown in Figure 2-5 or Figure 2-6, depending on the installation.

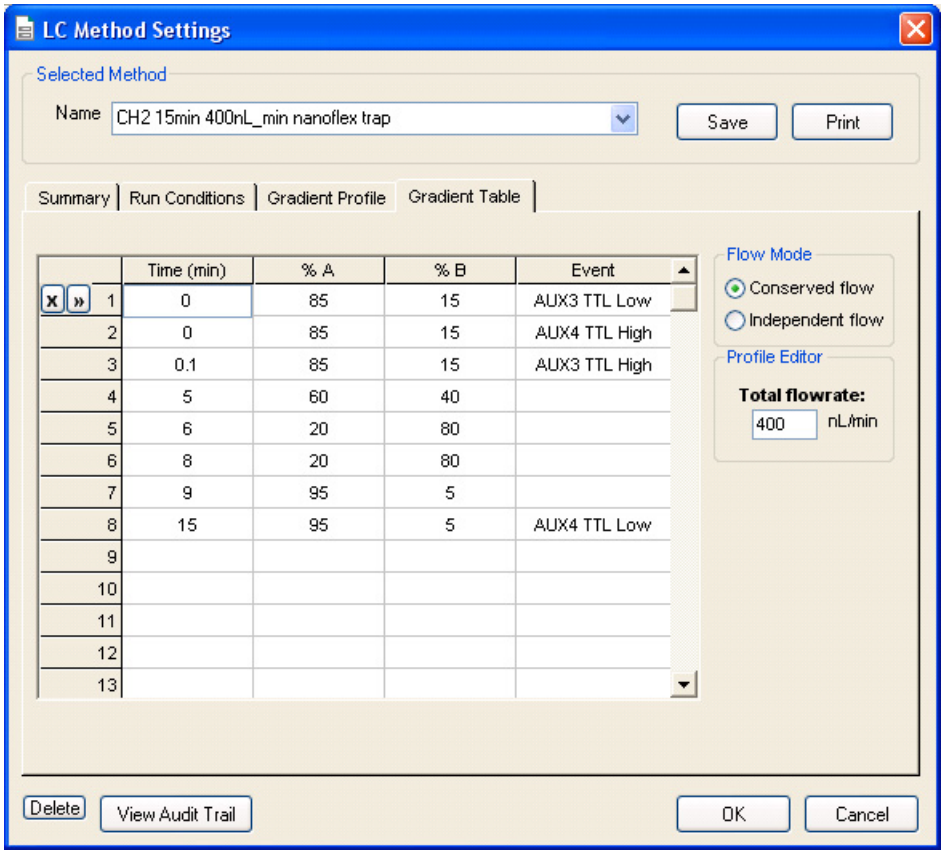


**Figure 2-5 LC Method Settings dialog—Gradient Table tab (Nanoflex system)**

**Note:** The events shown verify the correct switching of the Nanoflex valve. The signals at Time 0 will move the Nanoflex valve to the Inject position and the signals at Time 15 will move the Nanoflex valve back to the Load position.

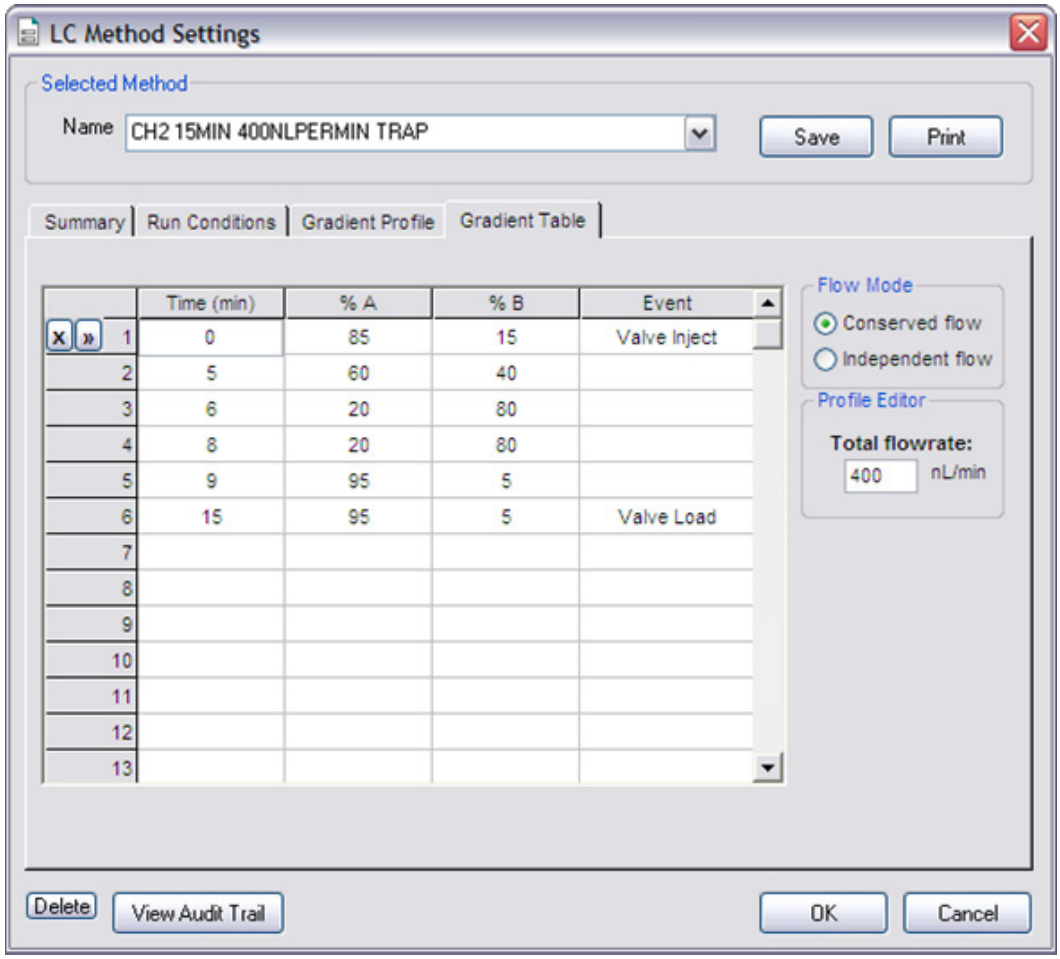


**Figure 2-6 LC Method Settings dialog—Gradient Table tab (column)**

5. On the **Run Conditions** tab, specify the method as shown in Figure 2-7.

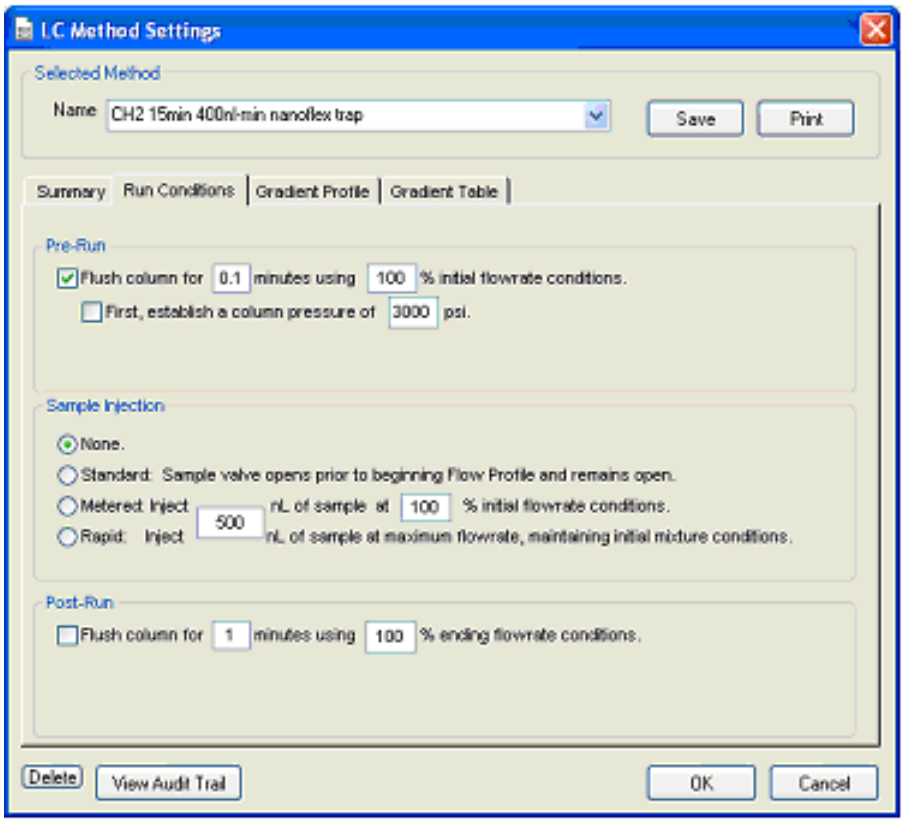


**Figure 2-7 LC Method Settings dialog—Run Conditions tab (Nanoflex system)**

6. Click **Save**.

# Create the Acquisition Method in the Analyst® Software

1. Double-click **Build Acquisition Method** on the left **Navigation** bar to create an acquisition method.
2. Specify the key parameters, as shown in Table 2-2.

**Note:** The acquisition time should be shorter than the LC run time.

**Table 2-2 Key Parameters**

| Parameter | Value |
|---|---|
| **MS** | |
| Scan Type | MRM Scan |
| Polarity | Positive |
| Q1/Q3 Masses and CE | See Table 2-3. |
| Acquisition time | 14 min |

**Table 2-2 Key Parameters (Continued)**

| Parameter | Value |
|---|---|
| **Advanced MS** | |
| Q1 Resolution | Unit |
| Q3 Resolution | Unit |
| Curtain Gas (CUR) | 20 |
| CAD Gas | HIGH |
| IonSpray Voltage (IS) | 2300V |
| Ion Source Gas 1 (GS1) | 2-15 |
| Interface Heater | 150°C |
| Declustering Potential (DP) | 70 |

** Source/Gas parameters may vary between systems and spray tip. Determine the best value for the system you are working with. Make sure the spray tip position is optimized before creating the acquisition method.

3. Enter the MRM transitions from Table 2-3.

**Note:** In the Analyst MRM transition table, verify that the additional CE (collision energy) column is added to the table view by right-clicking the table and selecting CE from the menu that appears.

**Table 2-3 MRM Transitions for Beta-Galactosidase**

| Q1 | Q3 | Dwell | ID | CE |
|---|---|---|---|---|
| 503.2 | 760.3 | 50 | BG_YSQQQLMETSHR | 27 |
| 542.3 | 636.4 | 50 | BG_GDFQFNISR | 26 |
| 671.3 | 755.5 | 50 | BG_VDEDQPFPAVPK | 33 |
| 714.9 | 884.5 | 50 | BG_DWENPGVTQLNR | 32 |
| 729.4 | 832.5 | 50 | BG_APLDNDIGVSEATR | 48 |

## Add LC Information to the Acquisition Method

1. Click **Acquisition Method** in the left pane, and then select **LC Sync** as the **Synchronization Mode**.

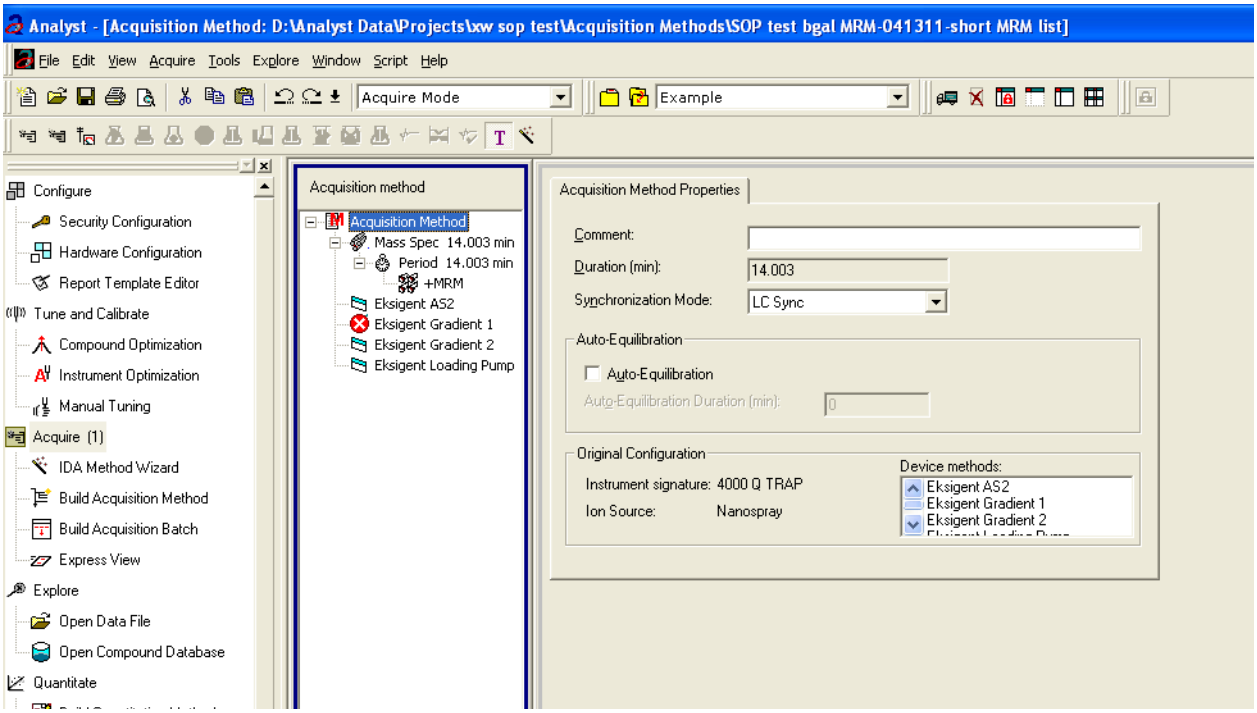


**Figure 2-8 Acquisition Method Properties tab—synchronization mode**

2. Click **Eksigent AS2** and then select the autosampler method, AS2 10uLloop 1uLinj Nanoflex Trap elute.ini or AS2 10uLloop 1uLinj Trap elute.ini, depending on the installation.

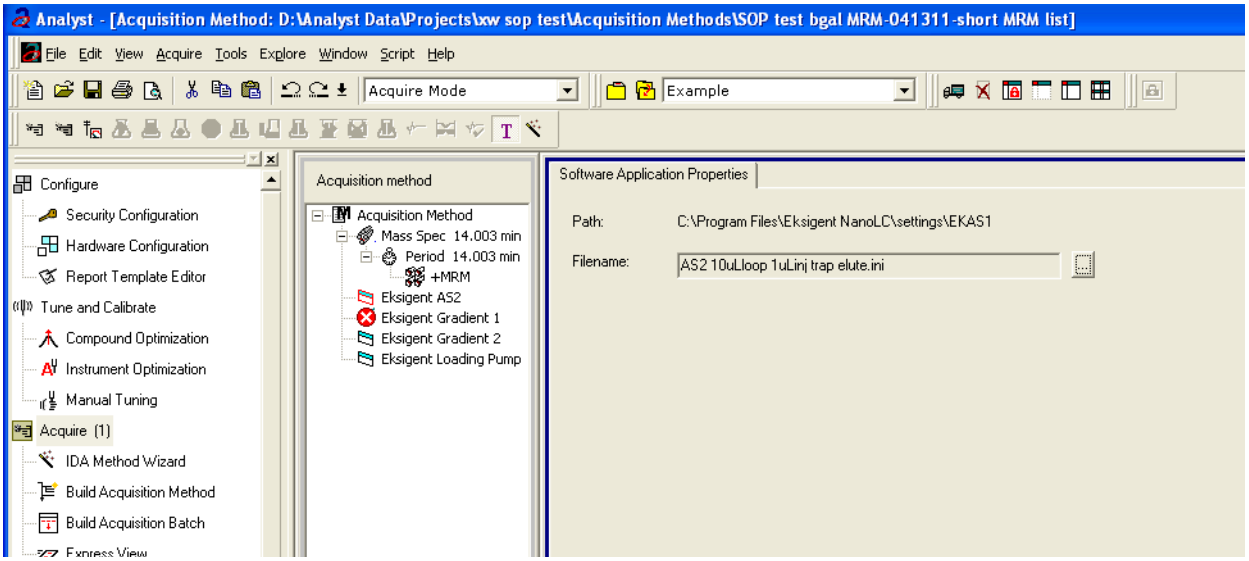


**Figure 2-9 Software Application Properties tab—autosampler filename**

3. Click **Eksigent Gradient 2**, and then select the gradient 2 pump method, CH2 15min 400nL min nanoflex trap.ini or CH2 15min 400nL min trap.ini, depending on the installation.

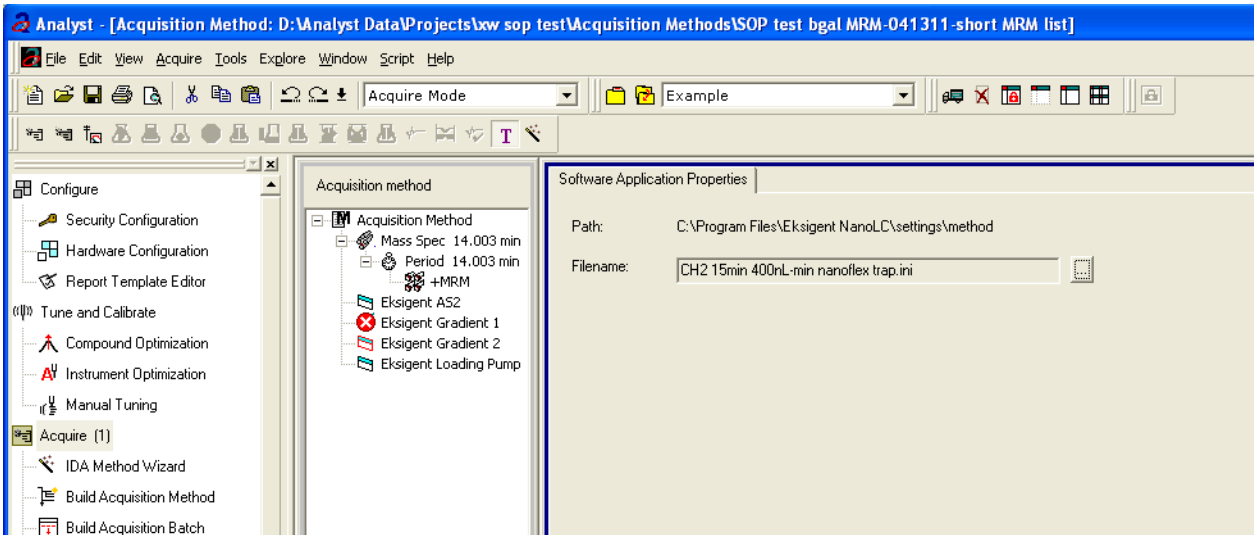


**Figure 2-10 Software Application Properties tab—gradient 2 filename**

4. Click **Eksigent Loading Pump**, and then select the loading pump method, Load Pump 2min Trap Wash.ini.

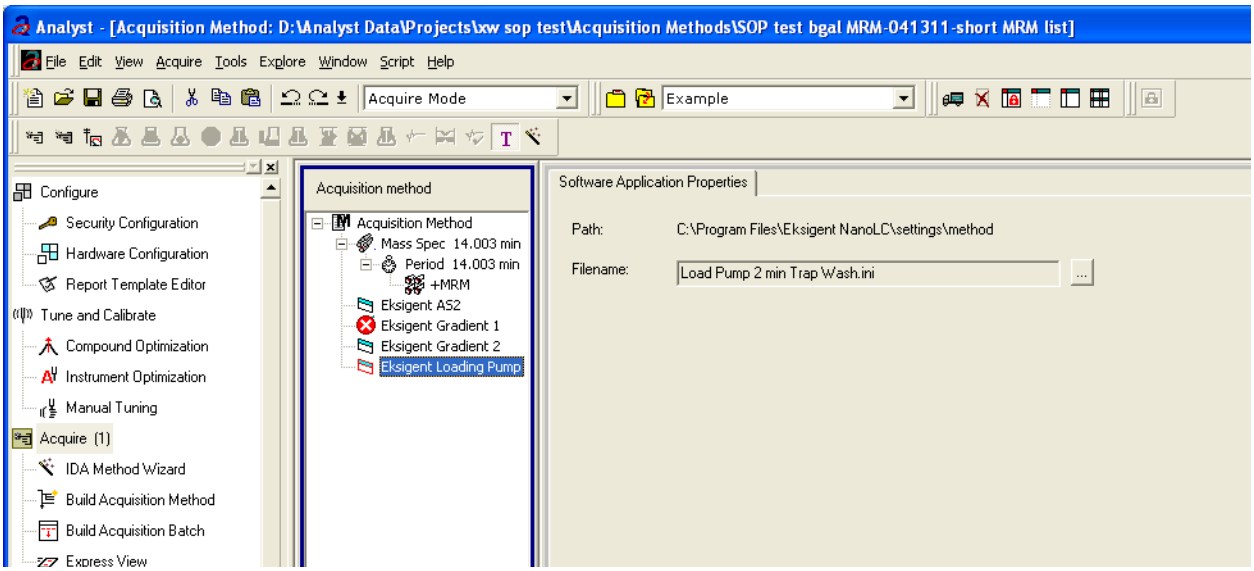


**Figure 2-11 Software Application Properties tab—loading pump filename**

5. Save the method as “System Functional Test”.

# Create the Methods and Batch for the System Performance Test

This section provides tests for the NanoLC Ultra system that indicate of the performance level of the instrument for proteomics applications such as protein identification and quantification.

Perform these tests when the mass spectrometer is known to be operating well and meeting performance specifications. If the NanoLC system has been idle for two weeks or more, then calibrate the system. Refer to the appendix, System Calibration, for more information.

The expected test duration is 60 minutes using the NanoSpray ion source. Repeat the test until you have consistent peak shape and intensity (approximately 180 minutes).

## Create the Acquisition Methods and Batch—Performance Tests

1. Plumb the autosampler valve with a 10 µL sample loop.
2. In the **AS1/AS2 Autosampler** window, click **Method Editor**.
3. Create the autosampler method for a trap-elute configuration, as shown in Figure 2-12 or Figure 2-13, depending on the installation.

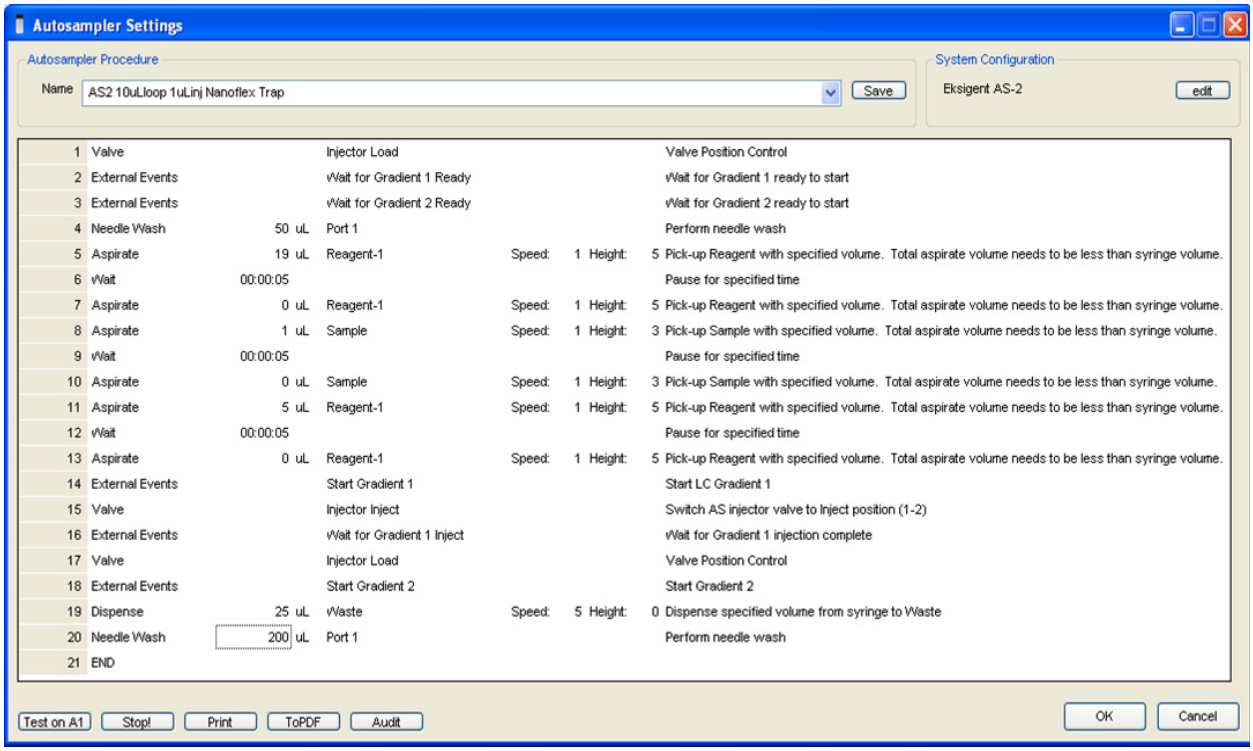


**Figure 2-12 Autosampler Settings dialog—trap-elute configuration (Nanoflex system)**

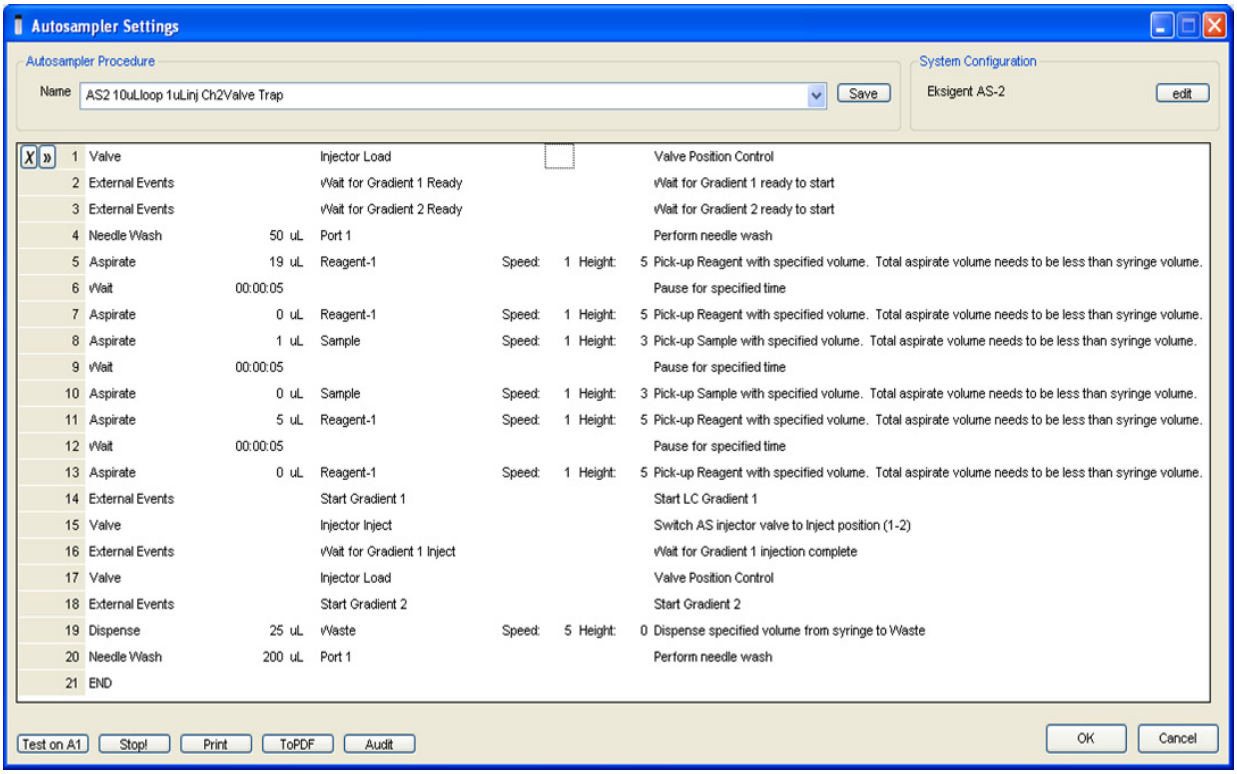


**Figure 2-13 Autosampler Settings dialog—trap-elute configuration (column)**

4. In the **Name** field, specify the name of this method as AS2 10uLloop 1uLinj Nanoflex Trap or AS2 10uLloop 1uLinj Ch2Valve Trap, depending on the installation.
5. Click **Save**.

# Create the LC Methods

The aqueous channel for each pump (Channel A) will be filled with Buffer A. The organic channel (Channel B) will be filled with Buffer B. For the Loading Pump, Buffer A is always used. Typical buffers are shown in Table 2-4.

**Table 2-4 Typical Buffer Mixtures**

| Buffer | Mixture | Channel |
|---|---|---|
| Buffer A | 100% water:0.1% formic acid | Channel A |
| Buffer B | 100% acetonitrile:0.1% formic acid | Channel B |

In the method below, the loading pump will be the pump with the microflow module.

## Create the Pump Method in the Eksigent Control Software

1. Ensure that **Loading Pump** is selected as the channel (top, right corner of window).
2. Click **LC Methods**.
3. In the **Name** field, type **Load Pump 10 min Trap Wash**, and then click **Save**.

4. On the **Gradient Table** tab, revise the method for the loading pump (the loading pump will be the pump with the microflow module), as shown in Figure 2-14.

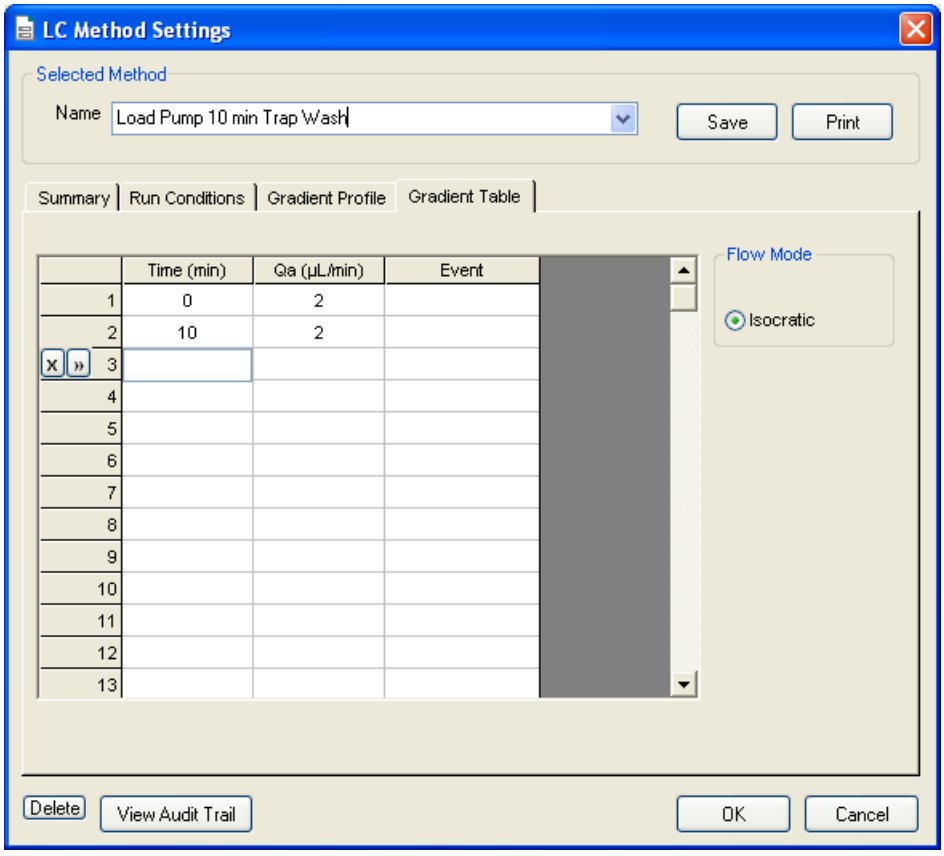


**Figure 2-14 LC Method Settings dialog—Gradient Table tab**

5. On the **Run Conditions** tab, specify conditions as shown in Figure 2-15.

**Note:** For a trap-elute configuration, the Sample Injection method should be Standard.

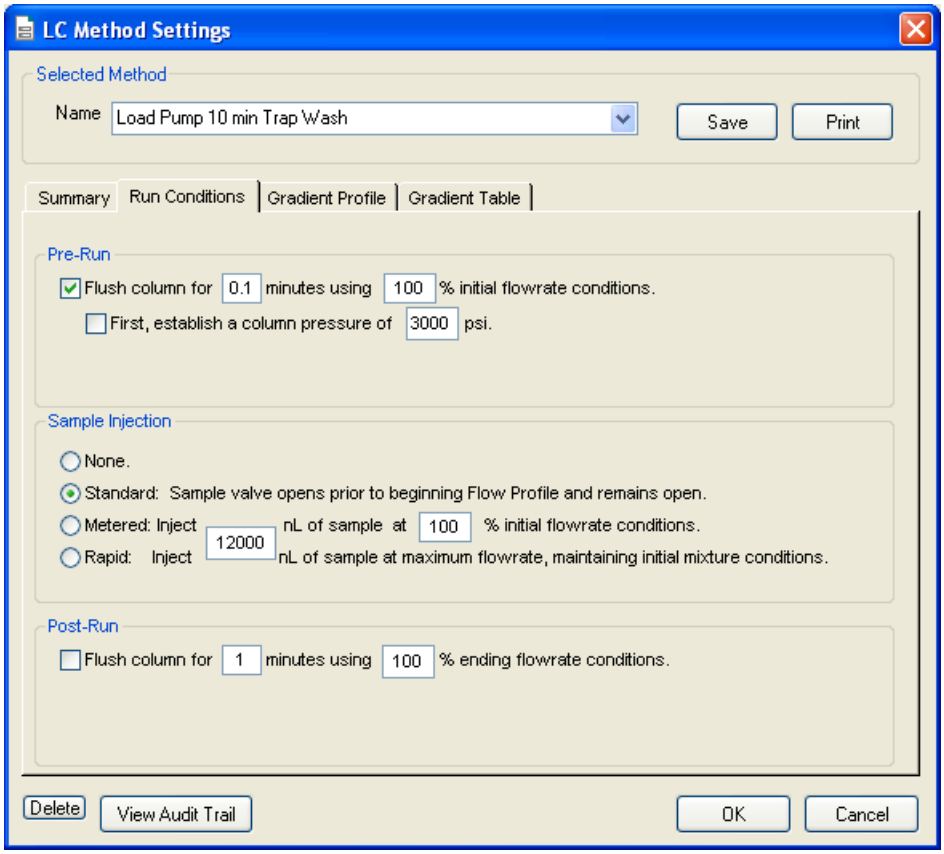


**Figure 2-15 LC Method Settings dialog—Run Conditions tab**

6. Click **Save**.

## Create the Gradient Method in the Eksigent Control Software

For the analytical gradient (typically on the Gradient 2 pump with the nanoflow module), create the gradient method.

1. Ensure that **Gradient 2** is selected as the channel (top, right corner of window).
2. Click **LC Methods**.
3. If this is a Nanoflex system installation, then in the **Name** field, type **CH2 45min 300nLmin Nanoflex trap**, and click **Save**.

   Or

   If this is a column installation, then in the **Name** field, type **CH2 45min 300nLmin column trap**, and click **Save**.
4. On the **Gradient Table** tab, specify the method, as shown in Figure 2-16 or Figure 2-17, depending on the installation.

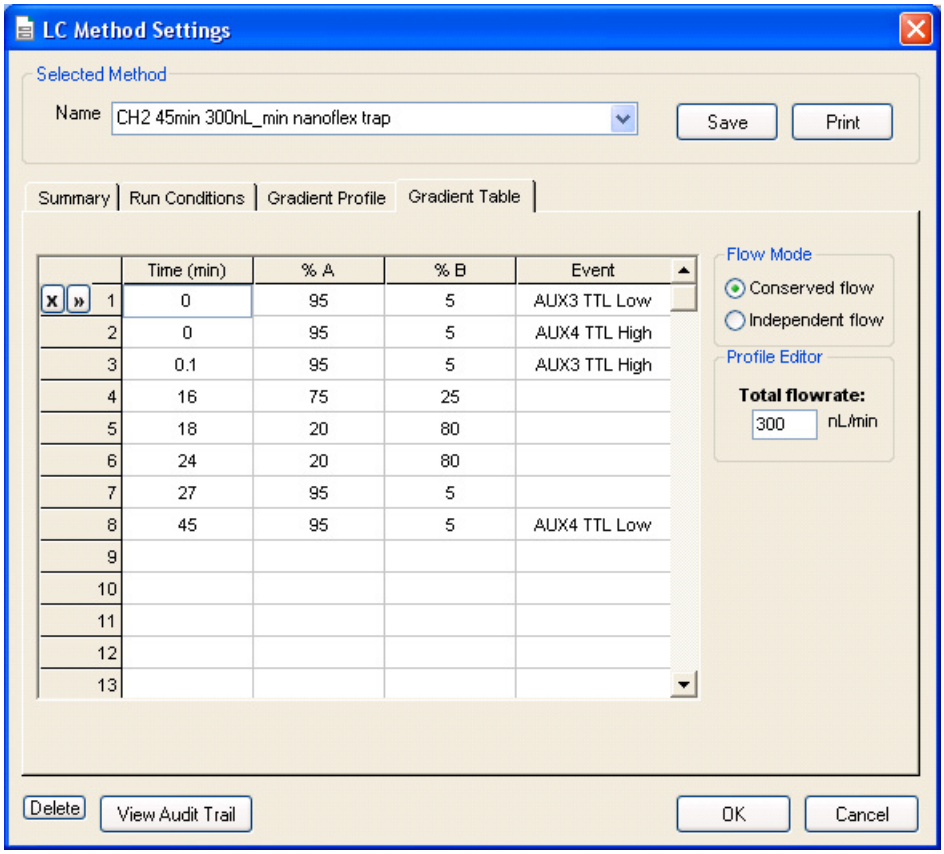


**Figure 2-16 LC Method Settings dialog—Gradient Table tab (Nanoflex system)**

**Note:** The events shown above verify the correct switching of the Nanoflex valve. The signals at Time 0 will move the Nanoflex valve to the Inject position and the signals at Time 45 will move the Nanoflex valve back to the Load position.

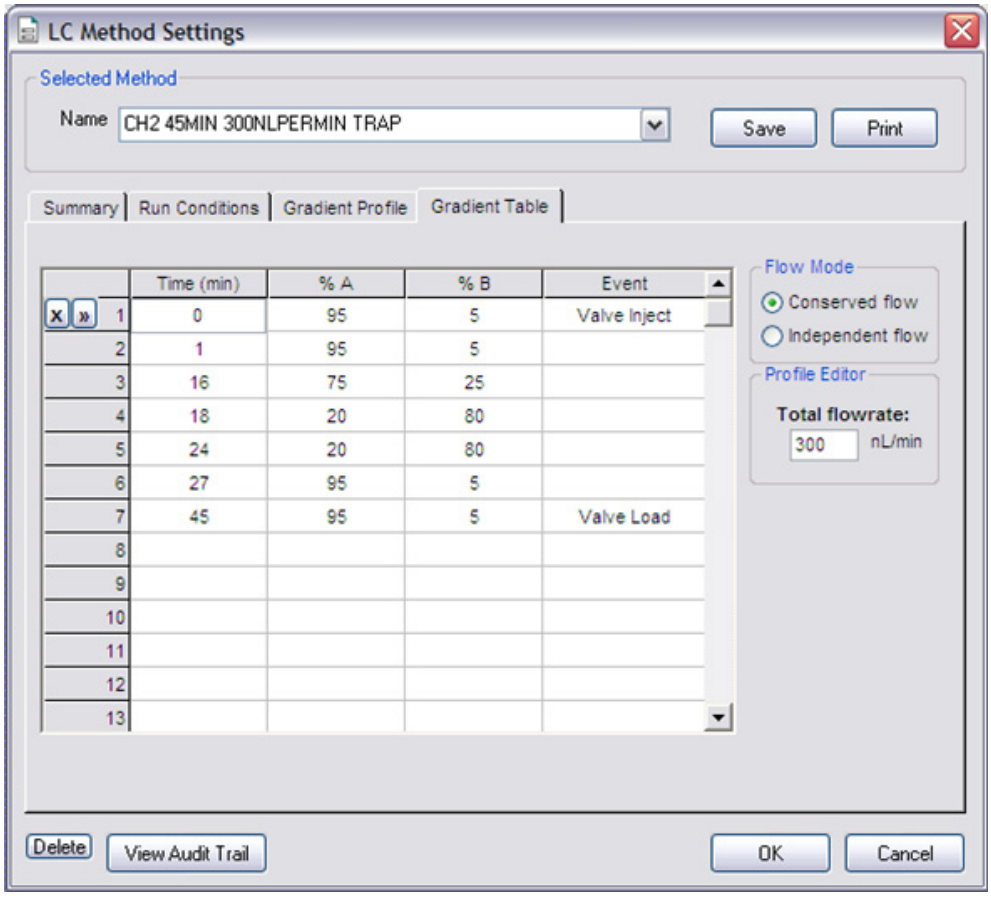


**Figure 2-17 LC Method Settings dialog—Gradient Table tab (column)**

5. On the **Run Conditions** tab, specify the method as shown in Figure 2-18.

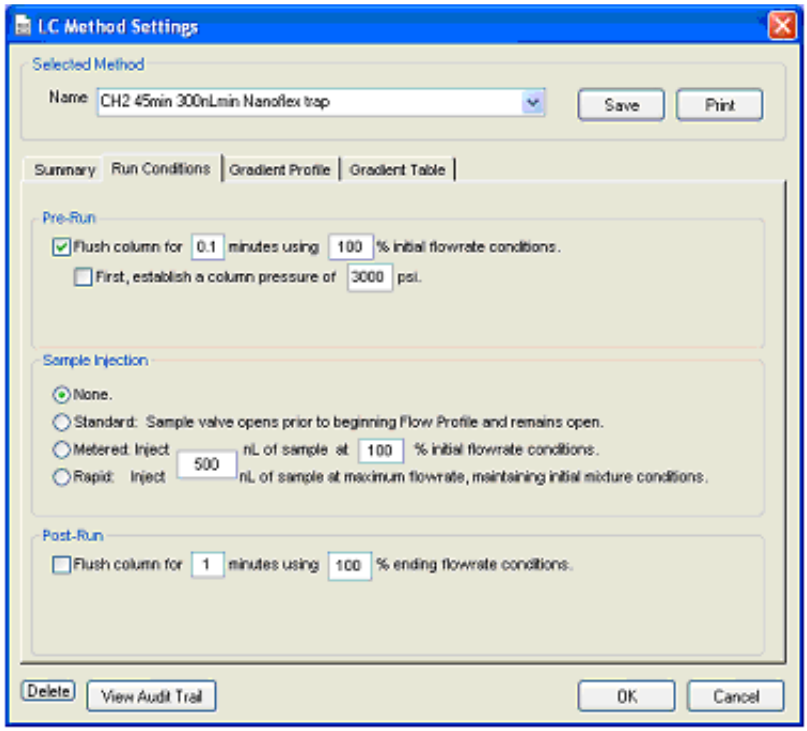


**Figure 2-18 LC Method Settings dialog—Run Conditions tab (Nanoflex system)**

6. Click **Save**.

# Specify the Acquisition Method in the Analyst Software

1. Create the acquisition method. See Table 2-5 for details.

**Note:** The acquisition time should be shorter than the LC run time.

**Table 2-5 Key Parameters**

| Parameter | Value |
|---|---|
| **MS** | |
| Scan Type | MRM Scan |
| Polarity | Positive |
| MCA | Off |
| Q1/Q3 Masses and CE | See Table 2-6. |
| Acquisition time | 40 min |
| **Advanced MS** | |
| Q1 Resolution | Unit |
| Q3 Resolution | Unit |

**Table 2-5 Key Parameters (Continued)**

| Parameter | Value |
|---|---|
| **Source/Gas**** | |
| Curtain Gas (CUR) | 20 |
| CAD Gas | HIGH |
| IonSpray Voltage (IS) | 2300V |
| Ion Source Gas 1 (GS1) | 2-15 |
| Interface Heater | 150°C |
| **Compound** | |
| Declustering Potential (DP) | 70 |

** Source/Gas parameters may vary between systems and spray tip. Determine the best value for the system you are working with. Ensure the spray tip position is optimized before creating the acquisition method.

2. Enter the MRM transitions from Table 2-6.

**Note:** In the Analyst MRM transition table, verify that the additional CE (collision energy) column is added to the table view by right-clicking the table and selecting CE from the menu.

**Table 2-6 MRM Transitions for Beta-Galactosidase**

| Q1 | Q3 | Dwell | ID | CE |
|---|---|---|---|---|
| 433.9 | 723.4 | 50 | BG_ELNYGPHQWR | 30 |
| 450.7 | 524.3 | 50 | BG_FNDDFSR | 28 |
| 503.2 | 760.3 | 50 | BG_YSQQQLMETSHR | 27 |
| 528.9 | 855.4 | 50 | BG_RDWENPGVTQLNR | 25 |
| 542.3 | 636.4 | 50 | BG_GDFQFNISR | 26 |
| 550.3 | 871.4 | 50 | BG_IDPNAWVER | 27 |
| 567.1 | 932.5 | 50 | BG_DVSLLHKPTTQISDFHVATR | 30 |
| 607.9 | 685.4 | 50 | BG_ITDSLAVVLQR | 39 |
| 671.3 | 755.5 | 50 | BG_VDEDQPFPAVPK | 33 |
| 697.9 | 821.5 | 50 | BG_LPSEFDLSAFLR | 35 |
| 714.9 | 884.5 | 50 | BG_DWENPGVTQLNR | 32 |
| 729.4 | 832.5 | 50 | BG_APLDNDIGVSEATR | 48 |
| 871.9 | 915.5 | 50 | BG_LSGQTIEVTSEYLFR | 40 |
| 879.4 | 664.3 | 50 | BG_VNWLGLGPQENYPDR | 40 |

3. Save the method as “System Performance Test”.

## Add LC Information to the Acquisition Method

1. Click **Acquisition Method** in the left pane, and then select **LC Sync** as the **Synchronization Mode**.

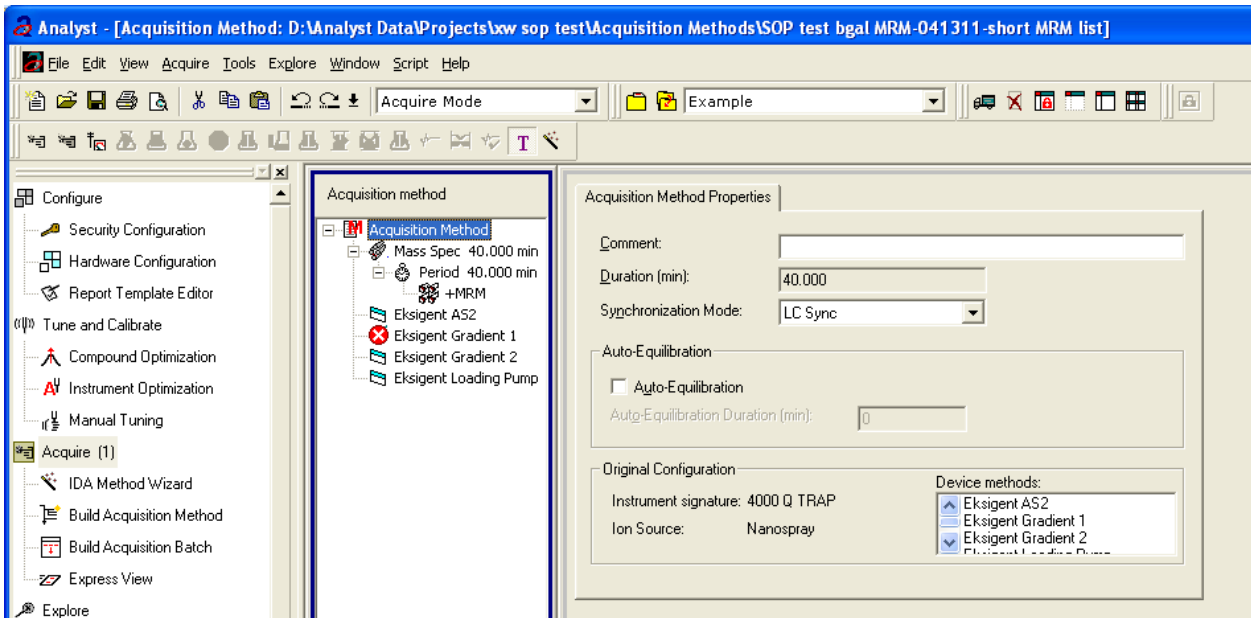


**Figure 2-19 Acquisition Method Properties tab—synchronization mode**

2. Click **Eksigent AS2** and then select the autosampler method, AS2 10µLinj Nanoflex trap elute.ini or AS2 10µLinj trap elute.ini, depending on the installation.

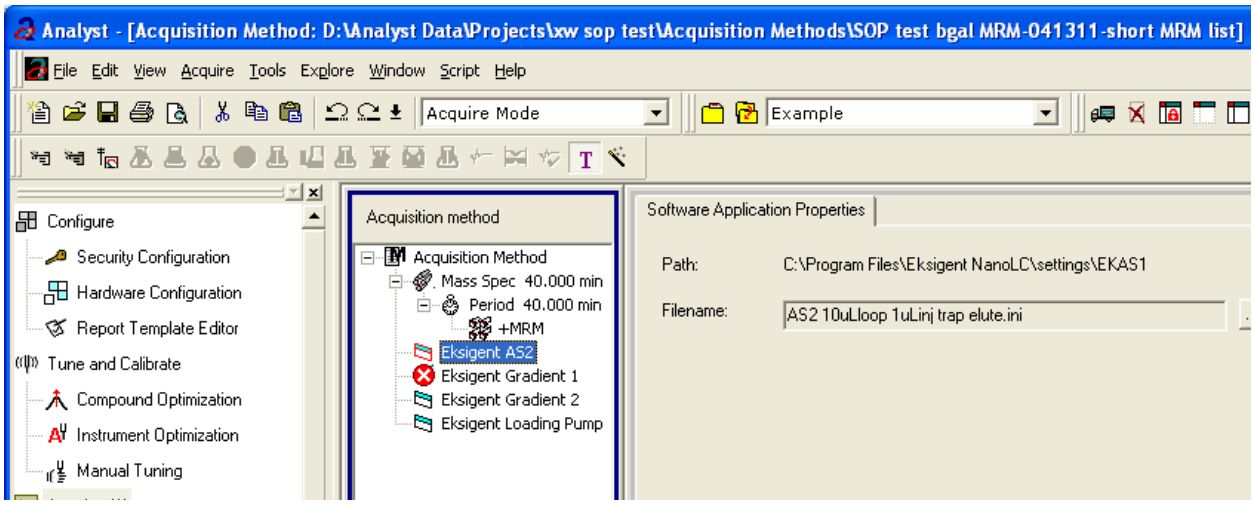


**Figure 2-20 Software Application Properties tab—autosampler method**

3. Click **Eksigent Gradient 2** and then select the gradient pump method, CH2 45min 300nLmin Nanoflex trap.ini or CH2 45min 300nLmin column trap.ini, depending on the installation.

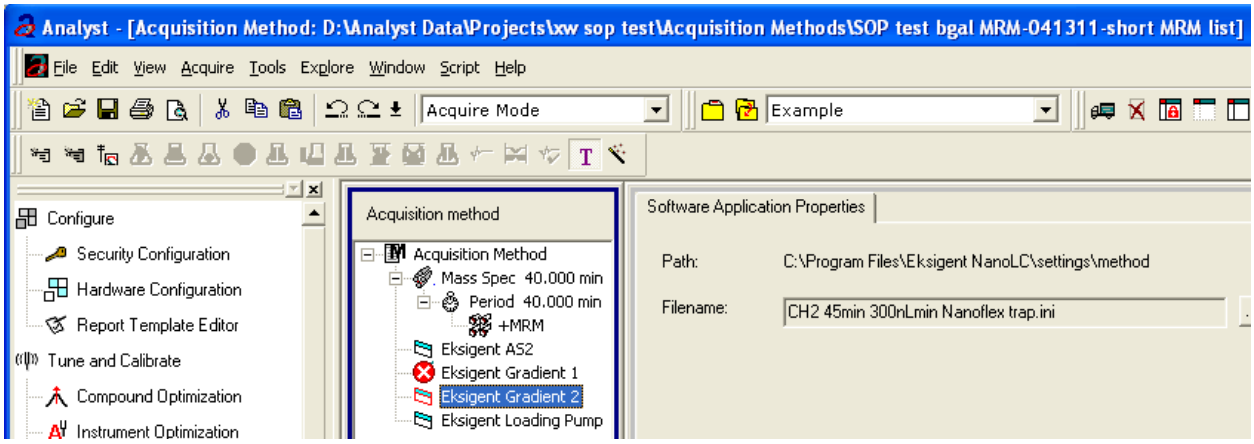


**Figure 2-21 Software Application Properties tab—gradient 2 method**

4. Click **Eksigent Loading Pump** and then select the loading pump method, Load Pump 10 min Trap Wash.ini.

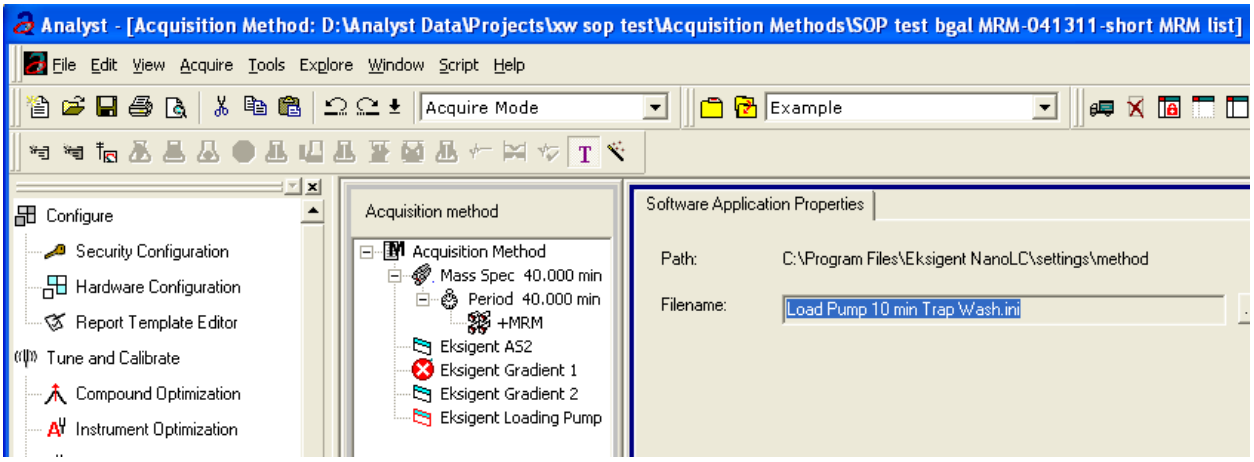


**Figure 2-22 Software Application Properties tab—loading pump method**

5. Save the method as “System Performance Test”.

# Prepare the System for Testing

Plumb the system in trap-elute configuration to perform the pre-column desalting workflow.

## Prepare the Solution and Dilution

Prepare the Beta-Galactosidase stock solution from the Beta-Galactosidase vial provided in the LC/MS Peptide/Protein Mass Standards Kit as described below. This will produce a stock solution of 1 pmol/µL.

1. Add 625.0 µL of Buffer A (100% water:0.1% formic acid) to the Beta-Galactosidase vial.
2. Vortex the vial for at least 30 seconds.
3. Using a centrifuge, spin the vial to bring the liquid down to the bottom of the vial before opening.
4. Repeat step 2 and step 3 to confirm dissolution.
5. Aliquot the stock solution (1 pmol/µL concentration) into 50 µL volumes and freeze for future use.

**Note:** Solutions can be stored at 4°C for up to 3 days after thawing.

### Prepare the Dilution for Functional Evaluation

1. Combine 40 µL of Buffer A (100% water:0.1% formic acid) with 10 µL of the Beta-Galactosidase protein digest stock solution in a clean vial. A 1 µL injection of a 200 fmol/µL solution will be performed.
2. Vortex the vial for at least 30 seconds to properly mix the solution. This is a 1/5 dilution and will give a final concentration of 200 fmol/µL.
3. Transfer the solution to the autosampler vial and make sure there is no bubble on the bottom of the vial.

### Prepare the Dilution for Performance Evaluation

1. Prepare the solution as described above. A 1 µL injection of a 10 fmol/µL solution will be performed.
2. Prepare 500 µL of the working solution of Beta-Galactosidase.
   - Combine 495 µL of Buffer A (100 water:0.1% formic acid) with 5 µL of the Beta-Galactosidase protein digest stock solution in a clean vial.
   - Vortex the vial for at least 30 seconds to properly mix the solution. This is a 1/100 dilution and will give a final concentration of 10 fmol/µL.
   - Transfer the solution to the autosampler vial and make sure there is no bubble on the bottom of the vial.

## Condition the System

A trap and column typically require 2 to 3 runs with 200 fmol of protein digest for conditioning.

- Verify that the trap and analytical column are well conditioned with protein digest injections before performing this test.

## Verify System Readiness

Ensure the NanoLC Ultra system is meeting performance specifications.

1. Connect effluent from the NanoLC Ultra system to the NanoSpray ion source and verify that the spray is stable by monitoring the background signal in the Analyst software.
2. Equilibrate the LC/MS system with the starting conditions of the method outlined above.
3. Ensure the spray is stable.
4. Double-click **Manual Tune** in the left **Navigation** bar.
5. Enter the key parameters from Table 2-7 and then click **Start** to begin acquisition.

**Table 2-7 Key Parameters**

| Parameter | Value |
|---|---|
| **MS** | |
| Scan type | Q1 Scan |
| Polarity | Positive |
| MCA | Off |
| Start Mass | 400 |
| Stop Mass | 1000 |
| Run Time | 2 min |
| **Source/Gas** | |
| Curtain Gas (CUR) | 20-25 |
| IonSpray Voltage (IS) | 2100-2400 V |
| Ion Source Gas 1 (GS1) | 2-15 |
| Interface Heater | 150ºC |
| **Compound** | |
| Declustering Potential | 70 |

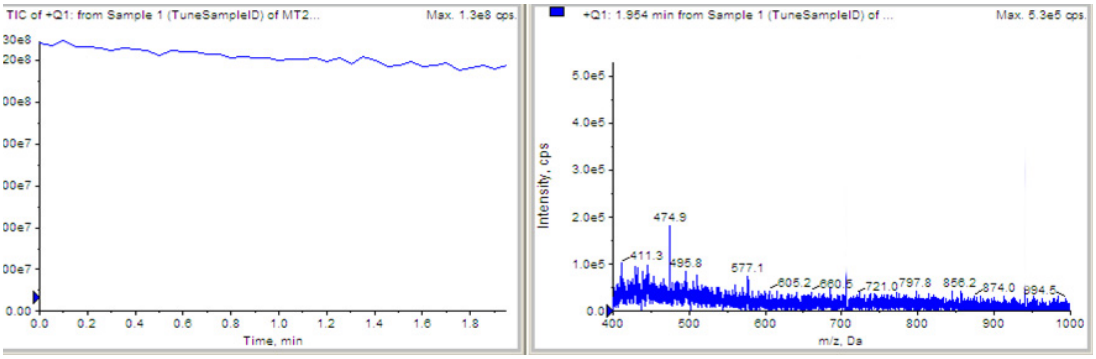


**Figure 2-23 Q1 MS scan for background noise**

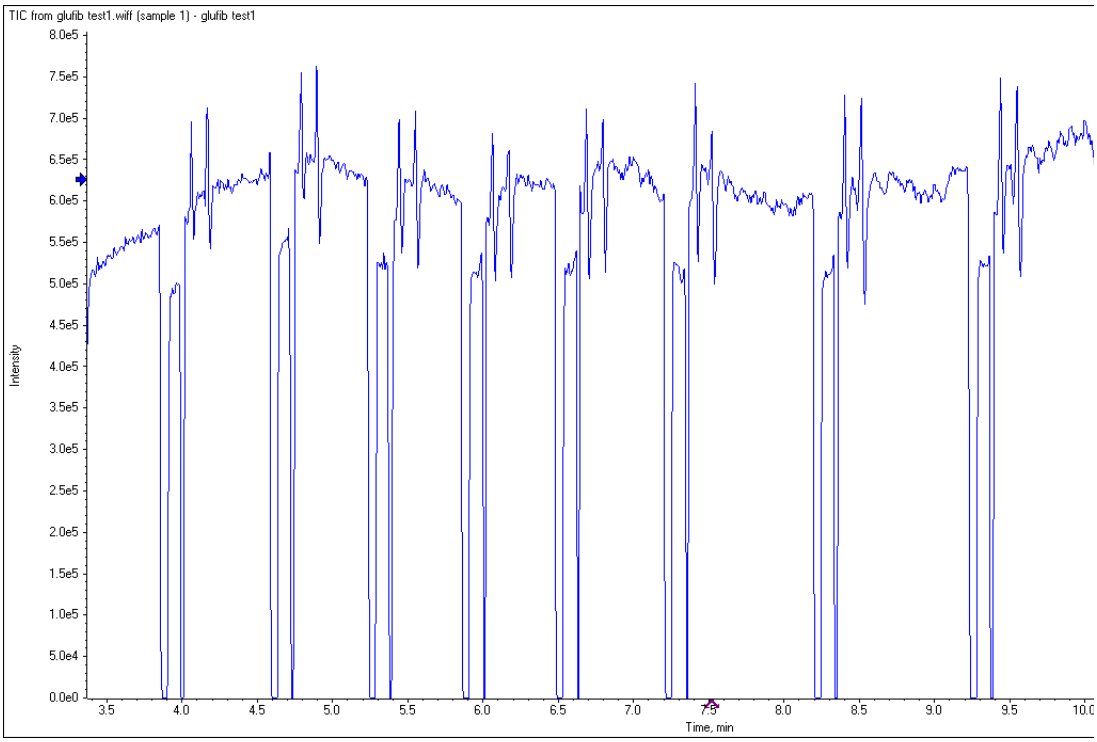


**Figure 2-24 Unstable spray induced by air bubbles**

6. Ensure that the spray is still stable by monitoring the background signal with a Q1 MS scan.
   - Stable spray appears as shown in Figure 2-23.
   - Unstable spray appears as shown in Figure 2-24 (typical unstable spray induced by air bubbles).
   - If the spray is not stable, retune the NanoSpray ion source by infusion. Refer to the *NanoSpray® Ion Source Operator Guide* for more information.

# Perform the System Functional Test

Create the LC/MS acquisition batch, run the batch and then verify the results.

## Create the LC/MS Acquisition Batch in the Analyst Software

1. Double-click **Build Acquisition Batch** in the left **Navigation** bar.
2. Build the acquisition batch, as shown in Figure 2-25.
   i. In the **Acquisition** group, select the acquisition method from the list.
   ii. Click **Add Set**, and then click **Add Samples**.

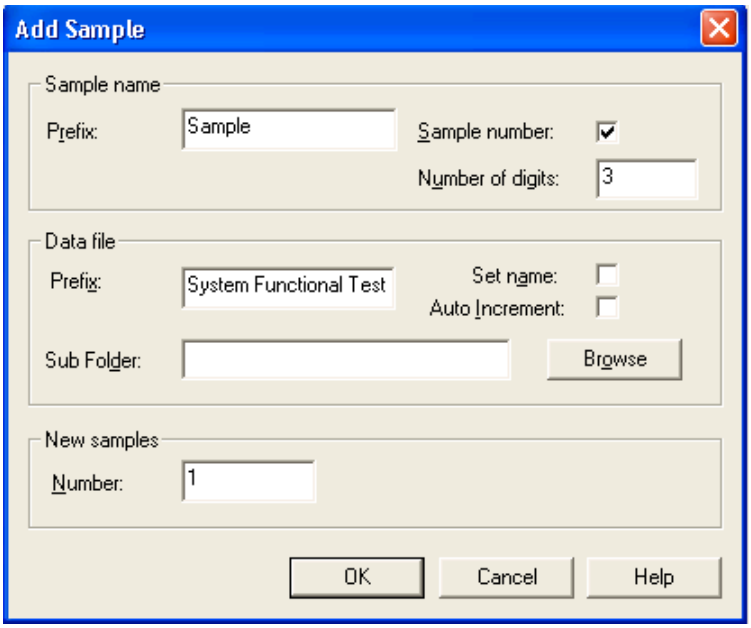


**Figure 2-25 Add Samples dialog—System Functional Test**

3. Click **OK**.
4. Save the data file as QTRAP 5500 system LC MRM BGal functional status check <date>.
5. On the **Location** tab, specify the location of the Beta-Galactosidase sample in the autosampler.

## Run the Batch

1. On the **Submit** tab, click **Submit**.
2. In the **View** menu, click **Sample Queue**.
3. In the **Acquire** menu, click **Start Sample**.

## Verify the Results

1. After the experiment has finished, open the sample from the data file in the **Explore** window.

   Figure 2-26 shows typical data for the instrument.

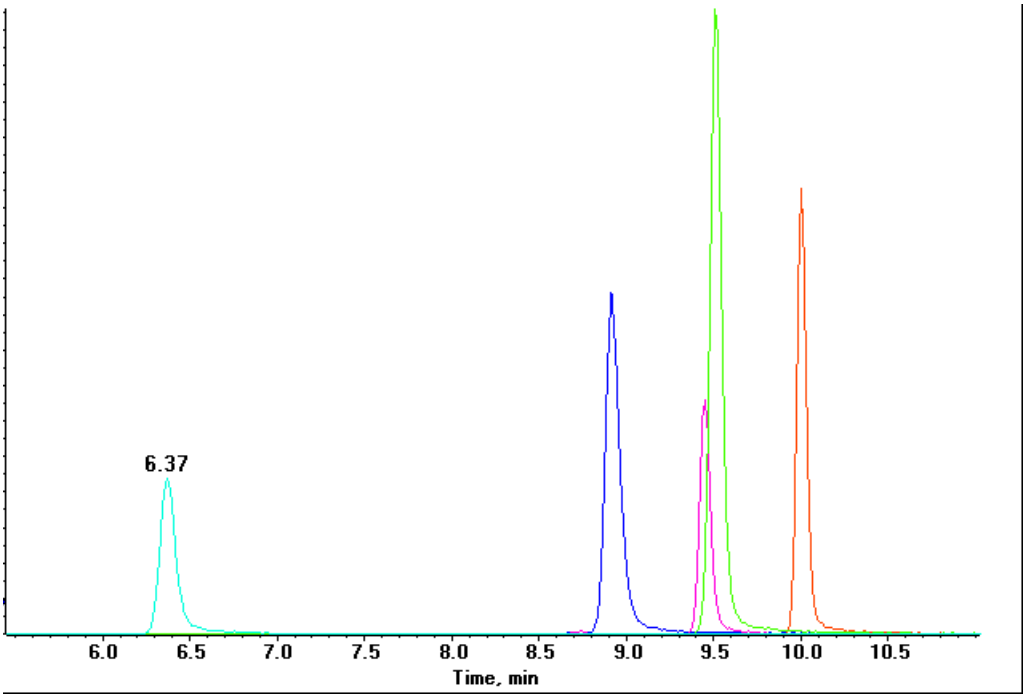


**Figure 2-26 MRM XICs of 5 peptides from Beta-Galactosidase NanoLC Ultra pre-column desalting run (200 fmol on column)**

2. Repeat the acquisition until you have consistent peak shape and peak intensity. If required, refer to Troubleshoot Peak Problems for more information.

**Note:** This test is not a specification. Use this example to confirm injection and peak shape of each MRM transition.

# Perform the System Performance Test

Create the LC/MS acquisition batch, run the batch and then verify the results.

## Create the LC/MS Acquisition Batch in the Analyst Software

1. Double-click **Build Acquisition Batch** in left **Navigation** bar.
2. Build the acquisition batch, as shown in Figure 2-27.
   i. In the **Acquisition** group, select the acquisition method from the list.
   ii. Click **Add Set**, and then click **Add Samples**.

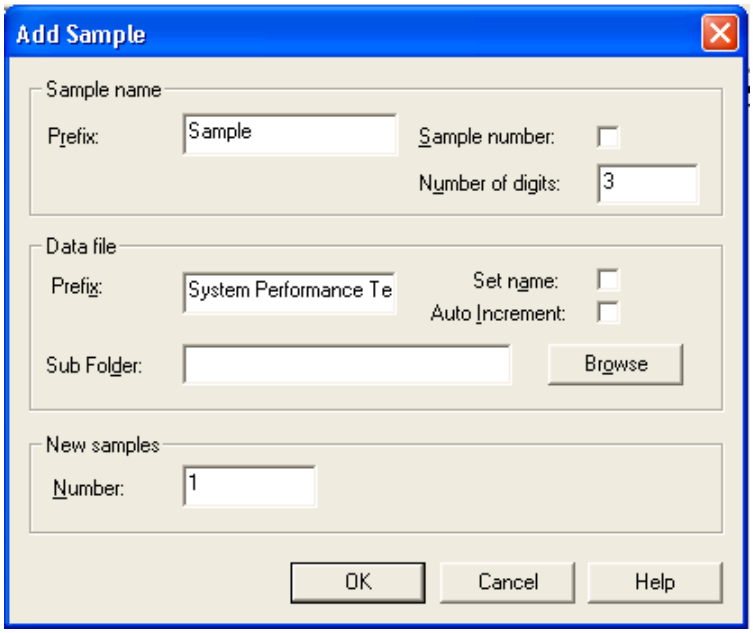


**Figure 2-27 Add Sample dialog—system performance test**

3. Click **OK**.
4. Save the data file as 5500 QTRAP system LC MRM BGal Performance Test <date>.
5. On the **Location** tab, specify the location of the Beta-Galactosidase sample in the autosampler.

## Run the Batch

1. On the **Submit** tab, click **Submit**.
2. In the **View** menu, click **Sample Queue**.
3. In the **Acquire** menu, click **Start Sample**.

## Verify the Results

1. After the experiment has finished, open the sample from the data file in the **Explore** window.
2. Right-click the TIC for the MRM experiment, and then click **Extract Ions**.

3. In the **Extract Ions** dialog, select the 5 MRMs from Table 2-8, and then click **OK**. Figure 2-28 shows typical data for the instrument.

**Table 2-8 MRM Transitions for Beta-Galactosidase**

| Q1 | Q3 | ID |
|---|---|---|
| 503.2 | 760.3 | BG_YSQQQLMETSHR |
| 542.3 | 636.4 | BG_GDFQFNISR |
| 671.3 | 755.5 | BG_VDEDQPFPAVPK |
| 714.9 | 884.5 | BG_DWENPGVTQLNR |
| 729.4 | 832.5 | BG_APLDNDIGVSEATR |

4. Record the peak areas of the specified MRM transitions in the section, Test Results—QTRAP 5500 Instruments on page 83.

**Note:** Most XICs should have peak widths of no more than 0.2 minute half height. Some peaks will be narrower and some broader.

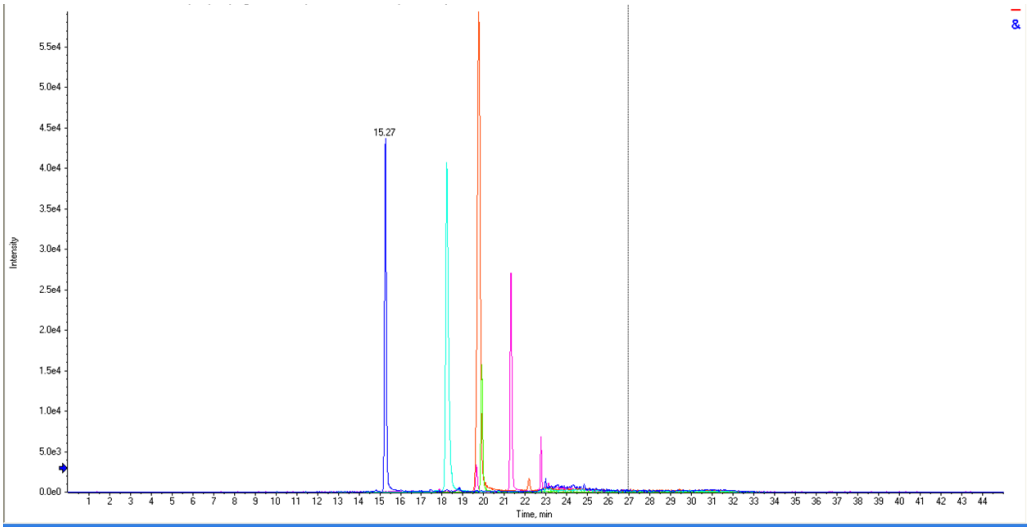


**Figure 2-28 MRM XICs of 5 peptides from Beta-Galactosidase NanoLC Ultra pre-column desalting run (10 fmol on column)**

5. Record the retention times of the chosen peaks. This will vary with each system. Time of elution of the first peak (approximately 14 -16 minutes, as shown in Figure 2-28) indicates dead volume of the system. Minimize dead volume where possible.
6. Repeat the acquisition until you have consistent peak shape, retention time, and peak intensity (a minimum of 3 times).

   For new columns, this may require that you repeat the acquisition 10 or more times in order to obtain consistent peak shape, retention time, and peak intensity. If required, refer to Troubleshoot Peak Problems for more information.
7. Record the results for each acquisition.
8. Record the average peak area of the acquisitions in the section, Test Results—QTRAP 5500 Instruments on page 83.

9. Ensure that the average peak area meets the minimum requirement specified in the section, Test Results—QTRAP 5500 Instruments on page 83.

# Troubleshoot Peak Problems

This section provides information for troubleshooting peak related problems such as broad or tailing peak widths, lack of separation between peaks, and low peak area.

### Peak widths are too broad or are tailing

- Inspect all connections in the flow path to verify that there are no dead volumes.
- Look at connections post-column and around trap column. A small increase in peak width is often seen when a trap column is used.

**Caution: Potential Instrument Damage: If using the Nanoflex system and problems persist, do not attempt to troubleshoot the fittings connected to the chip.**

### No separation between the peaks

- Ensure that both pumps are delivering the correct amount of solvent.
- Ensure that the pressure spike upon injection is not too severe in the high-flow channel (less than 300 psi change in pressure).
- Large pressure change upon injection suggests an air bubble has been introduced to the sample loop or is present in the trap column plumbing.

**Note:** The overall separation of the chromatography itself will often be less than direct injection. Components that elute comparably on the trap and analytical column will not re-resolve on the analytical column and, as a result, spread out or bunch together.

### Peak intensity or peak area is too low

- Verify the performance of the mass spectrometer and the ion source spray using the infusion tests in the *Nanospray® Ion Source Operator Guide*.
- Verify that the trap and analytical column are well conditioned with protein digest injections before performing this test. A trap typically requires 2 to 3 runs with 200 fmol of protein digest on the column for conditioning.
- Verify that the correct amount of sample has been withdrawn from the autosampler vial.
- Perform a direct injection with a protein digest on the analytical column to determine if the problem is related to the trap.
- If the first LC peak does not elute for a long time, inspect the system for dead volume before the trap.
- If the early eluting peaks are not visible or are very low in intensity, this could mean that trapping efficiency is low. Replace the trap.

**Tip!** Minimize tubing length wherever possible and make sure all tubing for the nanoflow path has an inner diameter of approximately 25 µm i.d.

- If the late eluting peaks are not visible or are very low in intensity, this is usually a sign that the column is getting old. In rare cases, it could mean that the Beta-Galactosidase standard is degraded. See Figure 2-29 for an example of a scan with an older column.

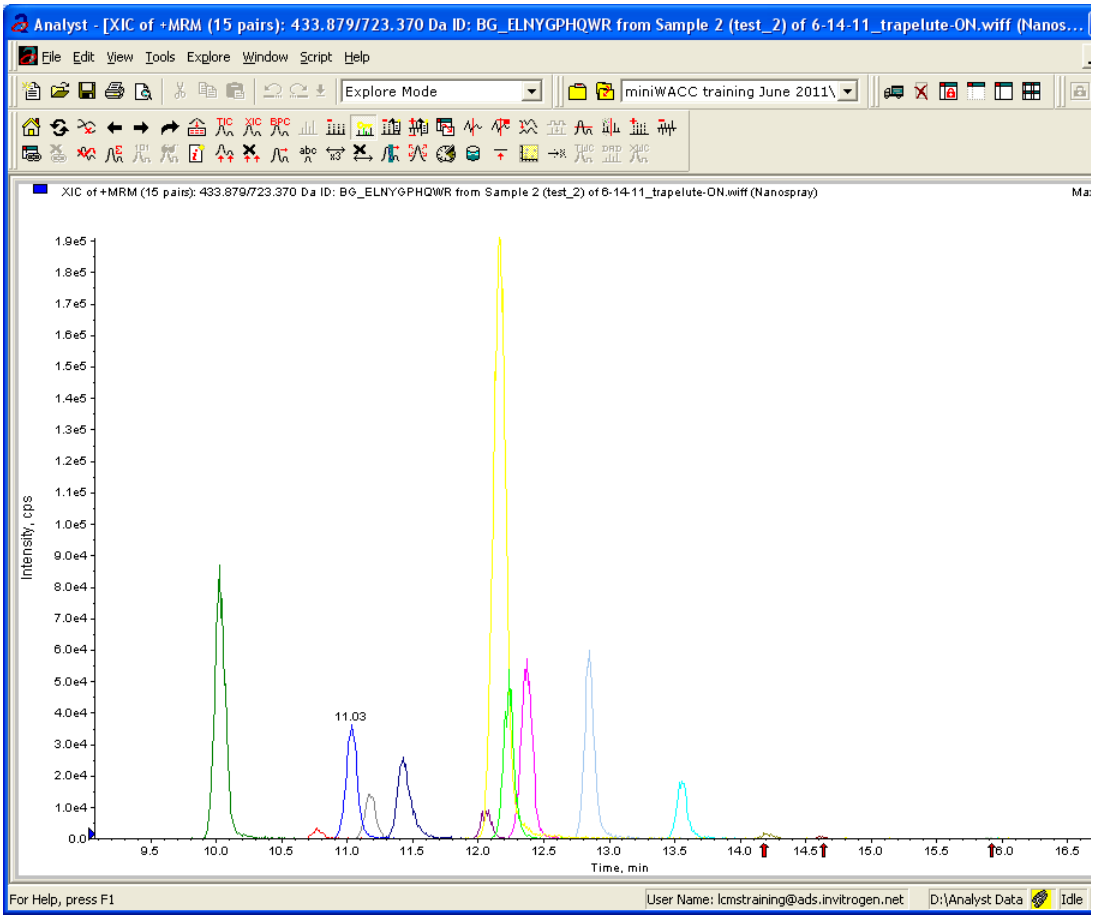


**Figure 2-29 Extraction of all peaks—late eluting peaks not present**

- Always monitor the column and trap pressure over time; increasing pressure often indicates increasing blockage, probably at the Nanospray ion source tip. If when the connection between the column and the ion source head is unfastened and the pressure changes quickly, this means that the tip is getting clogged and should be changed.
- For better long-term column lifetime, verify that there is at least a 30% change in pressure observed during the high organic flush of the column. Increase the duration of the high organic flush until a good pressure change is observed. This time might increase for the trap column configuration relative to the direct injection configuration.

- Figure 2-30 shows a minimal pressure change upon injection and a 30% pressure decrease during the high organic flush.

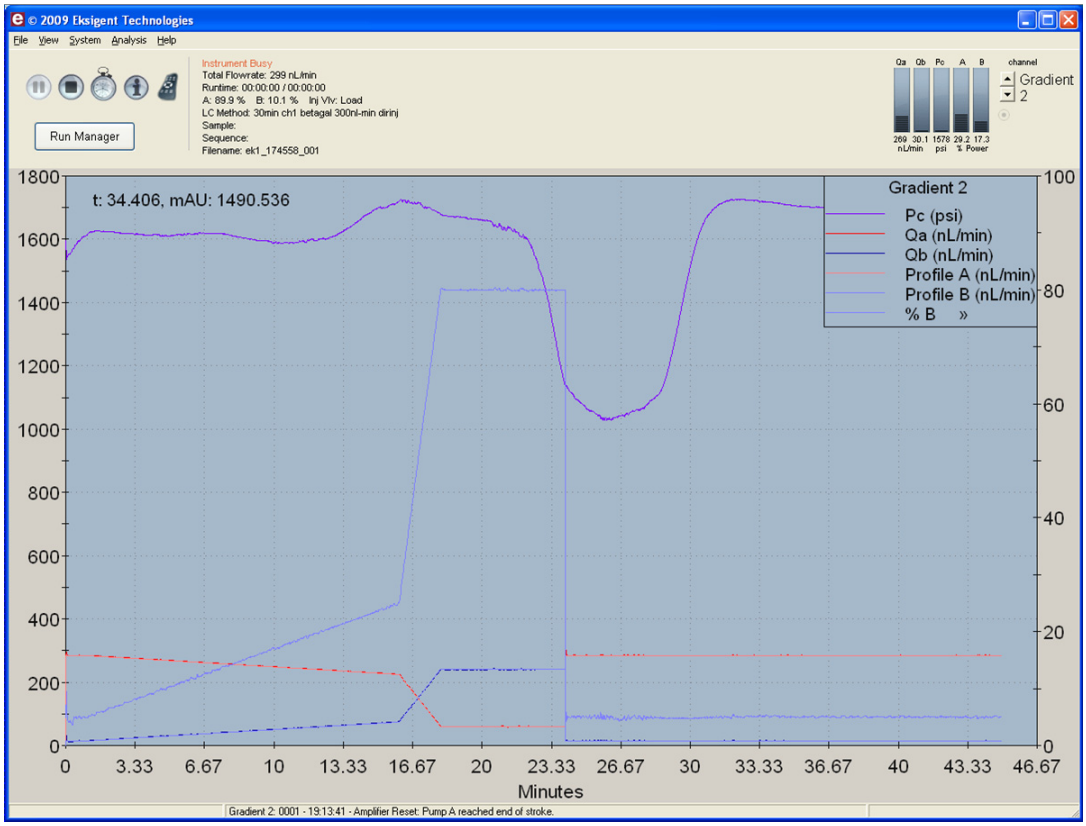


**Figure 2-30 Good pressure profile for a direct injection NanoLC Ultra system run**

# Create a Backup of the EKSettings.reg File

The EKSettings.reg file can be used to re-establish the system settings derived on installation if they are lost. Create a copy of the REG file upon completion of these tests.

1. In the Eksigent control software, on the **System** menu, click **Instrument Configuration**.
2. Click **Export Settings**.

   A backup of the REG file is created.
3. Navigate to the system settings folder (for example, C:\Program Files\Eksigent NanoLC\settings).
4. Copy the previous_settings.reg file to another location, separate from the host computer.

# Test Results—QTRAP 5500 Instruments

For QTRAP 5500 instruments, complete this table with the results from five of the peptides from the Beta-Galactosidase digest solution stock. Ensure that the peak area is below specification.

Most XICs should have peak widths of no more than 0.2 minute half height. Some peaks will be narrower and some broader. Additionally, verify that the peaks elute within 5 minutes of each other.

Beta-Gal Lot Number: _____

**Table 2-9 LC/MS Specification Test—QTRAP 5500 Instruments**

| Q1 | Q3 | Dwell | Peptide ID | CE | Spec. (peak area) | Actual |
|---|---|---|---|---|---|---|
| 503.2 | 760.3 | 50 | BG_YSQQQLMETSHR | 27 | 2.0E+04 | |
| 542.3 | 636.4 | 50 | BG_GDFQFNISR | 26 | 5.0E+05 | |
| 671.3 | 755.5 | 50 | BG_VDEDQPFPAVPK | 33 | 5.0E+05 | |
| 714.9 | 884.5 | 50 | BG_DWENPGVTQLNR | 32 | 7.0E+04 | |
| 729.4 | 832.5 | 50 | BG_APLDNDIGVSEATR | 48 | 2.0E+05 | |
| | | | | | Specification Passed? | ❑ |

### Notes

| |
|---|
| |
| |
| |


eksigent®
part of AB SCIEX

# LC/MS System Configuration Test—AB SCIEX 4000 QTRAP® System

# 3

This chapter describes the steps for preparing and performing LC/MS system configuration tests for the NanoLC Ultra® system configured with the cHiPLC® Nanoflex system (or external ChromXP column) and the AB SCIEX 4000 QTRAP® system.

**Note:** The tests in this chapter are written for Gradient 2 as the low-flow channel. If this is not true for your system (for example, if you have a 1D or 1D+ system), then make the appropriate changes throughout the tests.

The tests in this chapter are divided as follows:

- Fast test to condition the column and determine the functional status of the system. See Create the Methods and Batch for the System Functional Test for details.

  Perform these tests after completing the NanoSpray® ion source infusion tests in order to first confirm the spray performance of the tip. Refer to the *NanoSpray® Ion Source Operator Guide* for more information.

- Longer test to determine the performance level of the instrument for proteomics applications such as protein identification and quantification. See Create the Methods and Batch for the System Performance Test for details.

  These tests can be used as a measure of the NanoLC Ultra system performance in isolation of performance of the other components. Results from these initial tests can become the baseline performance for the system and can be performed regularly and used as a system quality control test in the future.

Approximate time required:

1. Create the methods and batch: 45 minutes
2. Prepare the system for testing: 3-4 hours
3. Perform the test.
   i. System functional test: 90 minutes
   ii. System performance test: 180 minutes

Recommended solvents can be ordered from VWR:

- Burdick and Jackson acetonitrile with 0.1% formic acid, P/N BJLC441-1.0
- Burdick and Jackson water with 0.1% formic acid, P/N BJLC452-1.0

Required materials for a Nanoflex system installation:

- Reverse phase cHiPLC column (75 µm x 15 cm ChromXP C18-CL 3 µm 120 Å)
- cHiPLC trap (200 µm x 0.5 mm ChromXP C18-CL 3 µm 120 Å)
- LC/MS Peptide/Protein Mass Standards Kit (P/N 4368624)

**Note:** Ensure that the Nanoflex system is in the Load position before beginning these tests.

Required materials for an external column installation:

- Reversed phase ChromXP nanoLC column (75 µm ID x 15 cm, ChromXP C18 3 µm 120 Å, P/N 805-00120)
- ChromXP nanoLC Trap column (350 µm ID x 0.5 mm, ChromXP C18 3 µm 120 Å, P/N 5016752)
- LC/MS Peptide/Protein Mass Standards Kit (P/N 4368624)

**Note:** After successfully completing the tests, create a backup of the EKSettings.reg file. See Create a Backup of the EKSettings.reg File for more information.

# Create the Methods and Batch for the System Functional Test

This section describes a test for the NanoLC Ultra system to condition the column and determine the functional status.

Perform these tests when the mass spectrometer is known to be operating well and meeting performance specifications.

**Note:** The steps in this section do not constitute a NanoLC Ultra system performance test. See Create the Methods and Batch for the System Performance Test.

The expected test duration is 30 minutes using the NanoSpray® ion source. Repeat the test until you have consistent peak shape and intensity (approximately 90 minutes).

## Create the Acquisition Method and Batch—Functional Test

**Note:** Use Gradient 2 for the autosampler method. Gradient 2 is the nanoflow module for LC configuration.

1. Plumb the autosampler valve with a 10 µL sample loop.
2. In the **AS1/AS2 Autosampler** status window, click **Method Editor**.
3. Create the autosampler method for a trap-elute configuration, as shown in Figure 3-1 or Figure 3-2, depending on the installation.

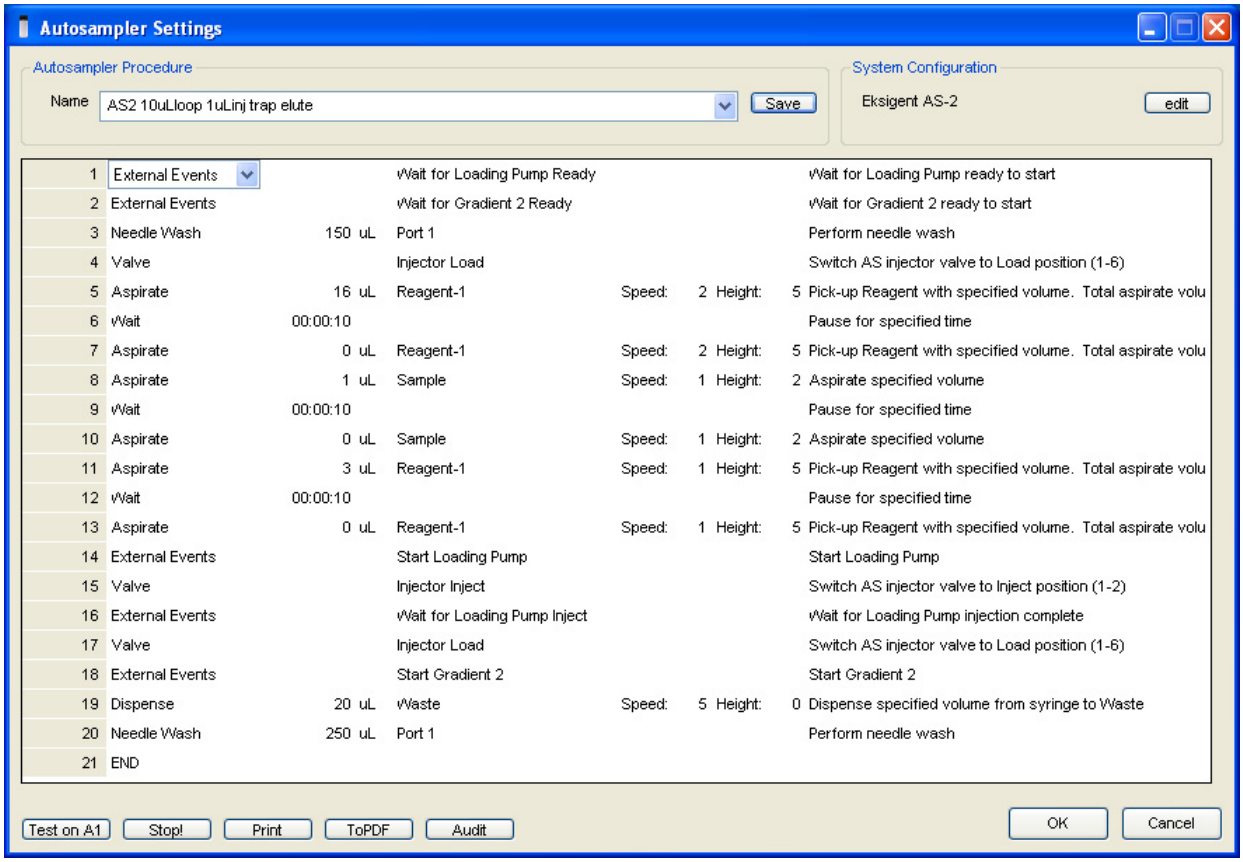


**Figure 3-1 Autosampler Settings dialog—trap-elute configuration (Nanoflex system)**

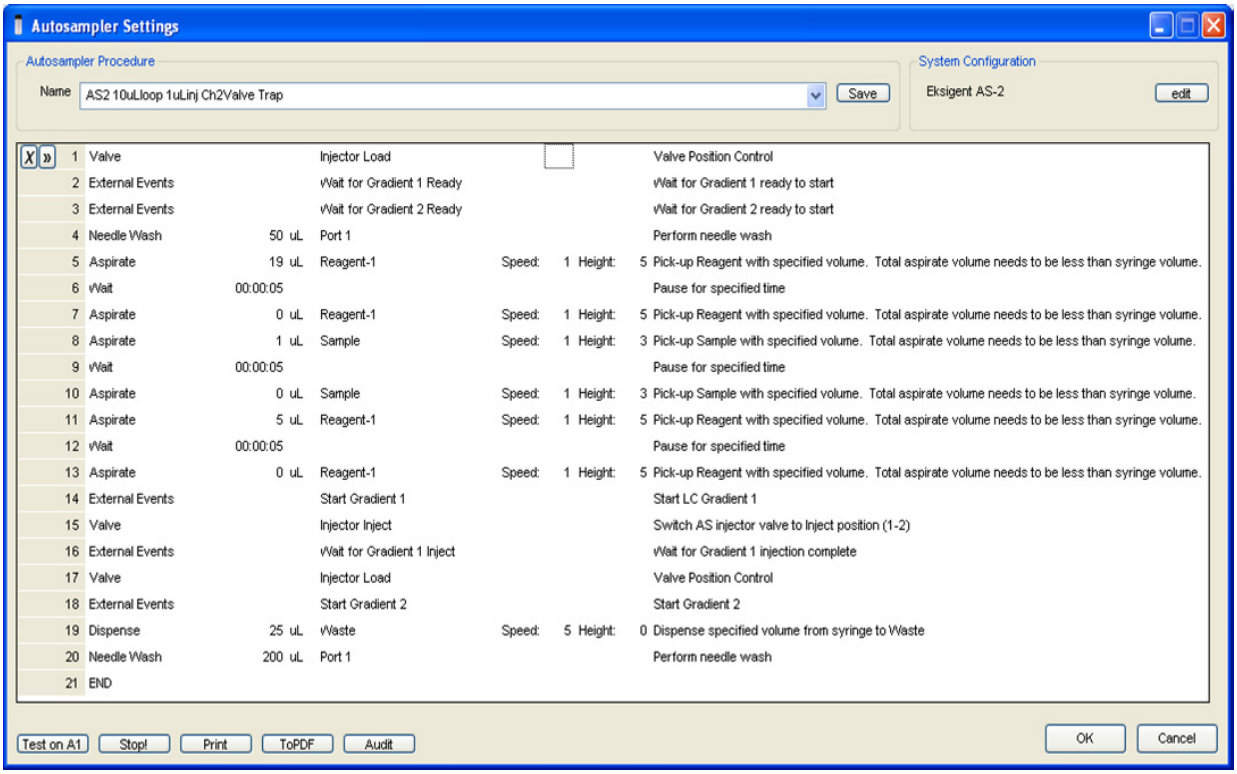


**Figure 3-2 Autosampler Settings dialog—trap-elute configuration (column)**

4. In the **Name** field, specify the name of this method as AS2 10uLloop 1uLinj Nanoflex trap elute or AS2 10uLloop 1uLinj Ch2Valve Trap, depending on the installation.
5. Click **Save**.

# Create the LC Methods

The aqueous channel for each pump (Channel A) will be filled with Buffer A. The organic channel (Channel B) will be filled with Buffer B. For the Loading Pump, Buffer A is always used. Typical buffer mixtures are shown in Table 3-1.

**Table 3-1 Typical Buffer Mixtures**

| Buffer | Mixture | Channel |
|---|---|---|
| Buffer A | 100% water:0.1% formic acid | Channel A |
| Buffer B | 100% acetonitrile:0.1% formic acid | Channel B |

In the method below, the loading pump will be the pump with the microflow module.

## Create the Pump Method in the Eksigent® Control Software

1. Ensure that **Loading Pump** is selected as the channel (top, right corner of window).
2. Click **LC Methods**.
3. In the **Name** field, type **Load Pump 2 min Trap Wash**, and then click **Save**.

4. On the **Gradient Table** tab, revise the method for the loading pump (the loading pump will be the pump with the microflow module), as shown in Figure 3-3.

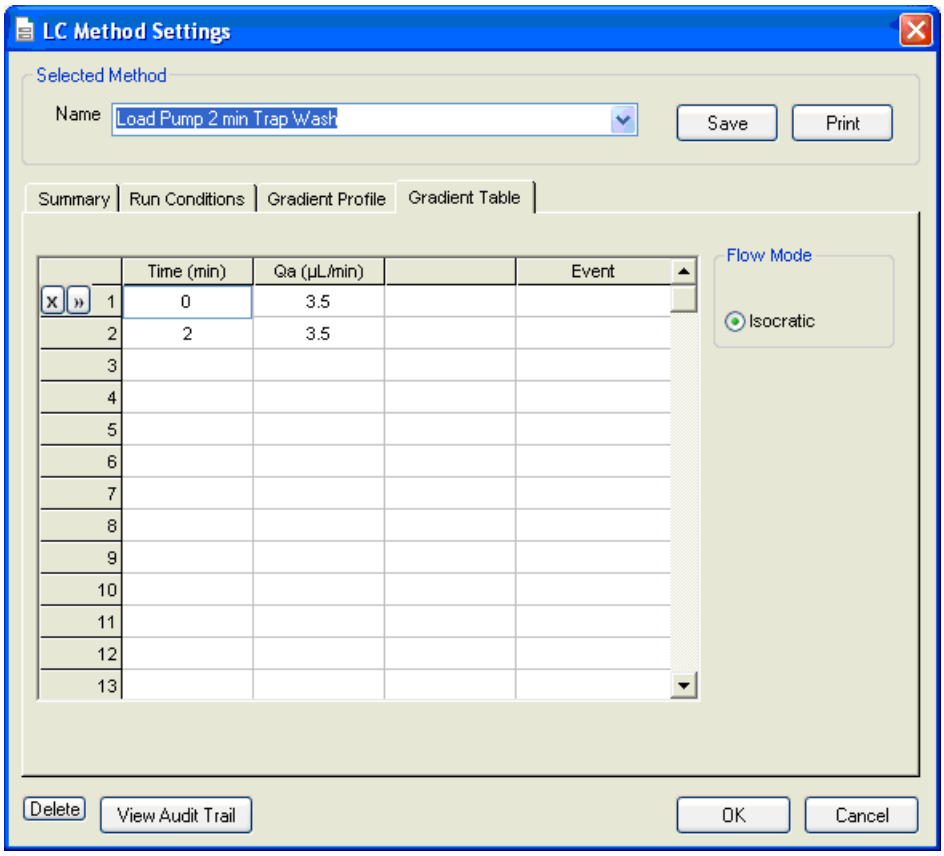


**Figure 3-3 LC Method Settings dialog—Gradient Table tab**

5. On the **Run Conditions** tab, specify conditions as shown in Figure 3-4.

**Note:** For a trap-elute configuration, the Sample Injection method should be Standard.

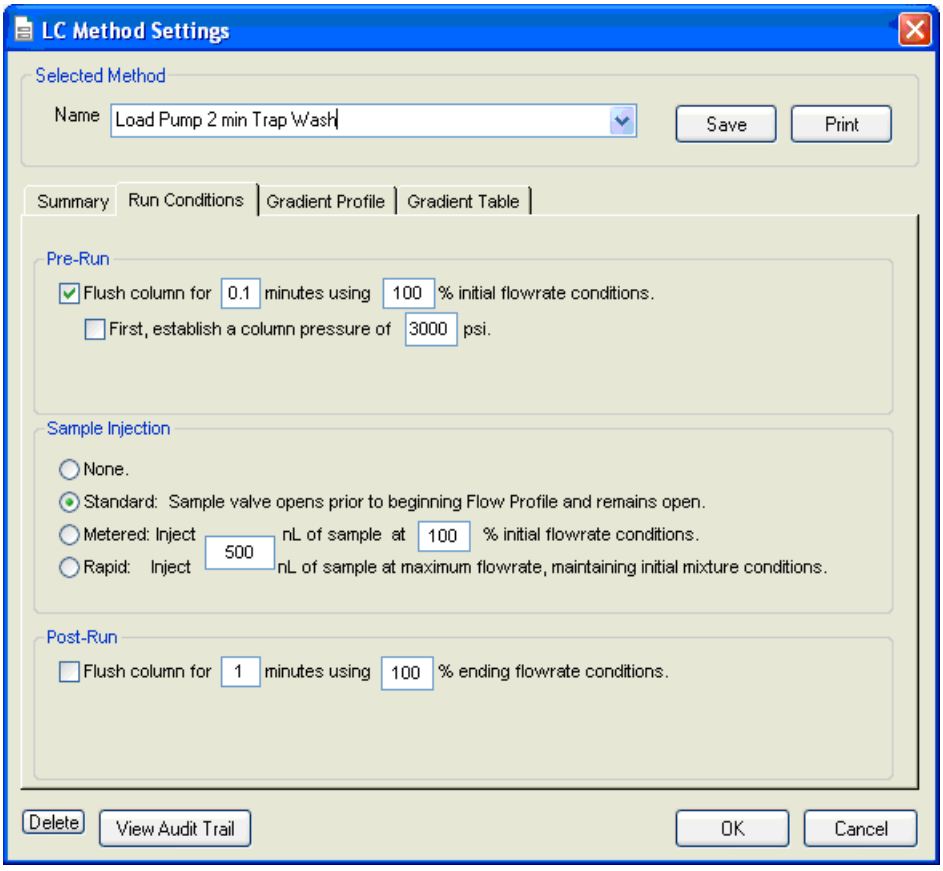


**Figure 3-4 LC Method Settings dialog—Run Conditions tab**

6. Click **Save**.

## Create the Gradient Method in the Eksigent Control Software

For the analytical gradient (typically, the Gradient 2 pump with the nanoflow module), create the gradient method.

1. Ensure that **Gradient 2** is selected as the channel (top, right corner of window).
2. Click **LC Methods**.
3. If the installation includes a Nanoflex system, then in the **Name** field, type **CH2 15min 400nLmin nanoflex trap**, and click **Save**.

   Or

   If the installation includes an external column, then in the **Name** field, type **CH2 15min 400nLmin trap**, and click **Save**.
4. On the **Gradient Table** tab, specify the method as shown in Figure 3-5 or Figure 3-6, depending on the installation.

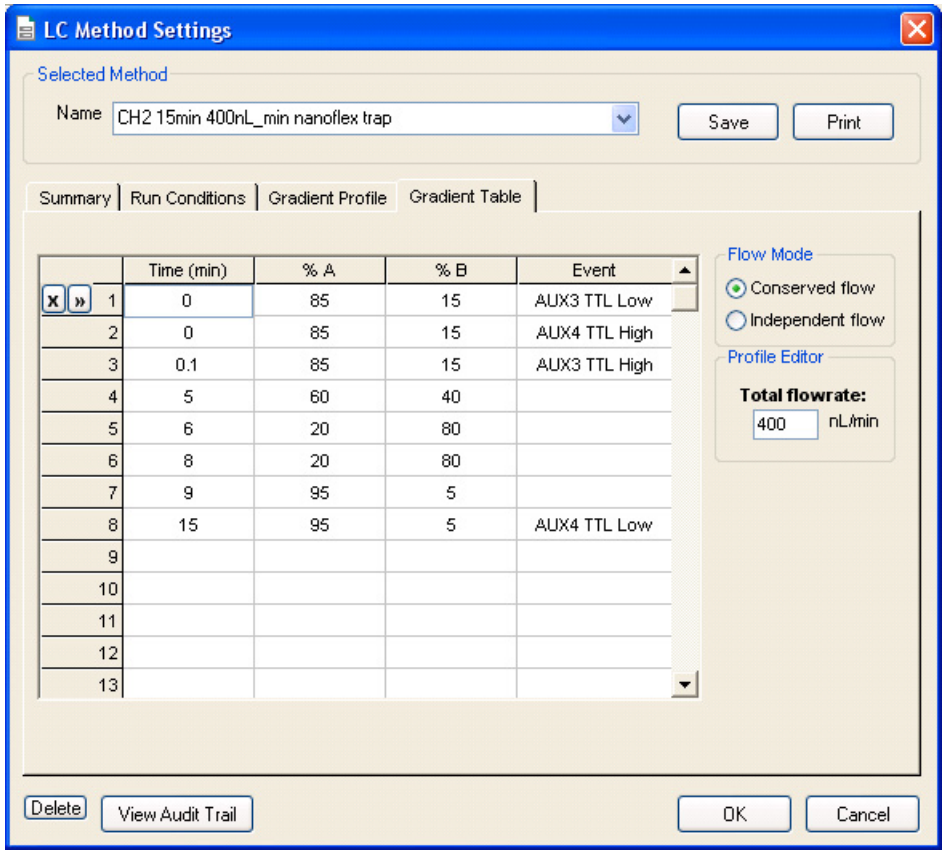


**Figure 3-5 LC Method Settings dialog—Gradient Table tab (Nanoflex system)**

**Note:** The events shown verify the correct switching of the Nanoflex valve. The signals at Time 0 will move the Nanoflex valve to the Inject position and the signals at Time 15 will move the Nanoflex valve back to the Load position.

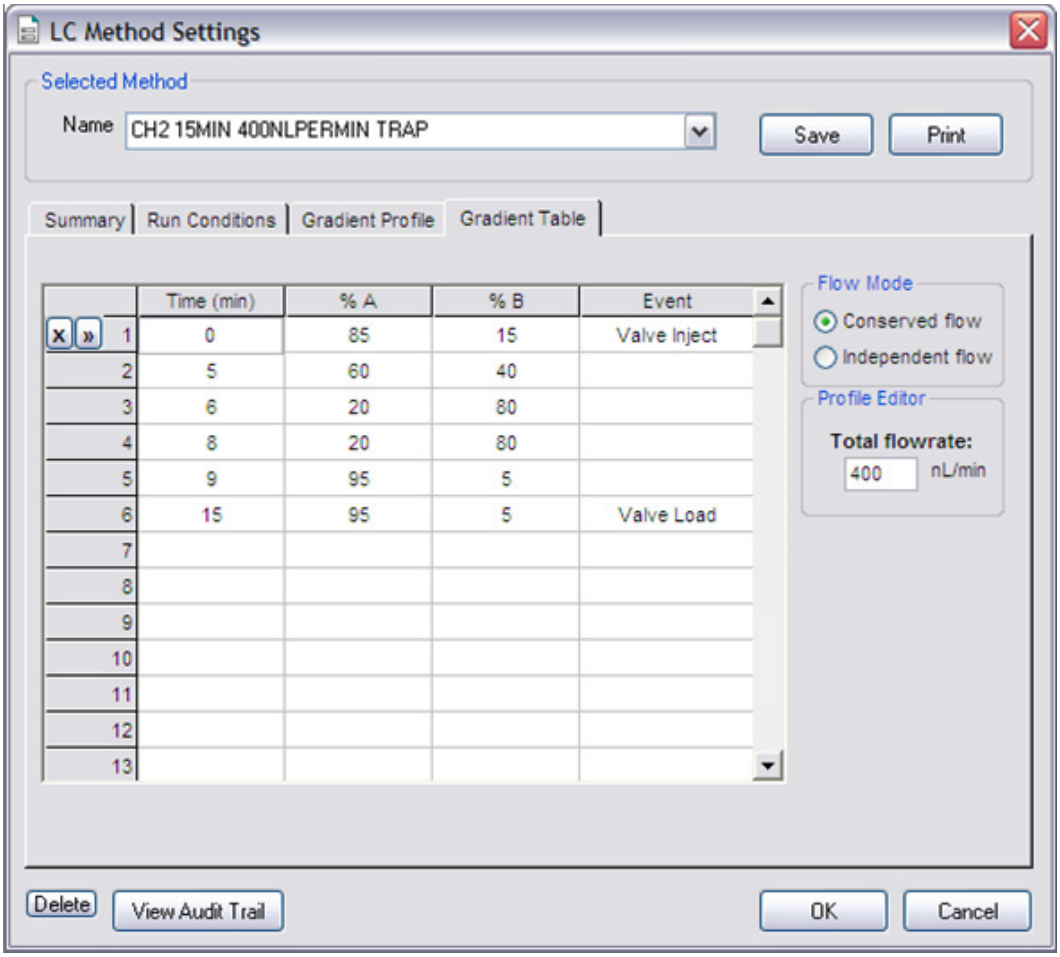


**Figure 3-6 LC Method Settings dialog—Gradient Table tab (column)**

5. On the **Run Conditions** tab, specify the method as shown in Figure 3-7.

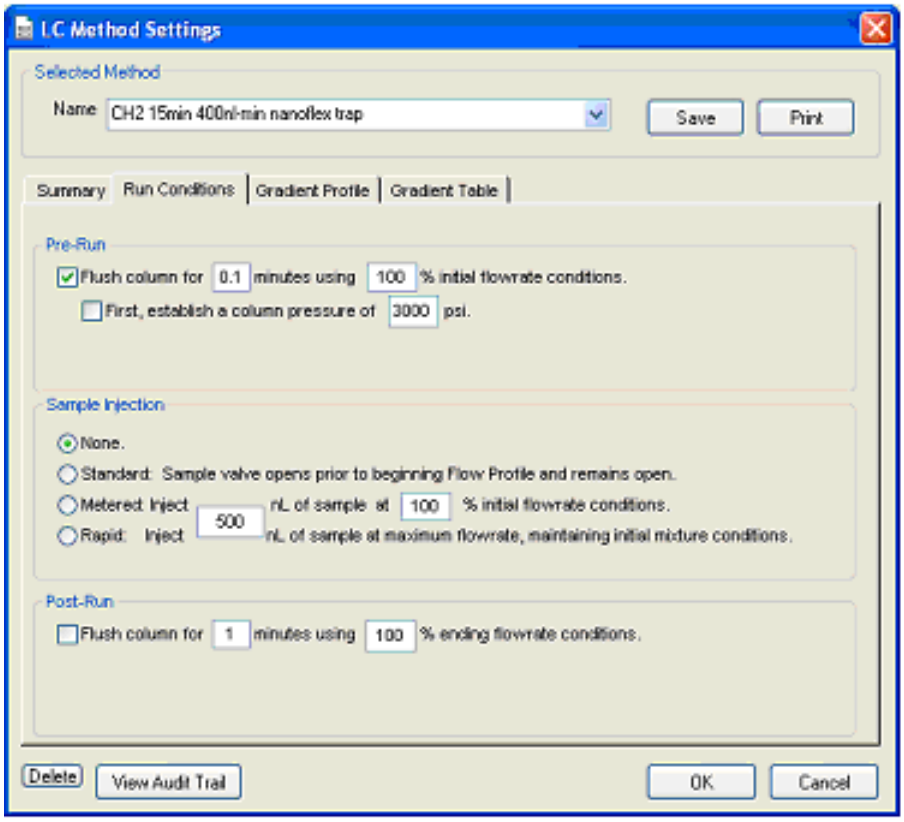


**Figure 3-7 LC Method Settings dialog—Run Conditions tab (Nanoflex system)**

6. Click **Save**.

# Create the Acquisition Method in the Analyst® Software

1. Double-click **Build Acquisition Method** on the left **Navigation** bar to create an acquisition method.
2. Specify the key parameters, as shown in Table 3-2.

**Note:** The acquisition time should be shorter than the LC run time.

**Table 3-2 Key Parameters**

| Parameter | Value |
|---|---|
| **MS** | |
| Scan Type | MRM Scan |
| Polarity | Positive |
| Q1/Q3 Masses and CE | See Table 3-3. |
| Acquisition time | 14 min |

**Table 3-2 Key Parameters (Continued)**

| Parameter | Value |
|---|---|
| **Advanced MS** | |
| Q1 Resolution | Unit |
| Q3 Resolution | Unit |
| **Source/Gas**** | |
| Curtain Gas (CUR) | 20 |
| CAD Gas | HIGH |
| IonSpray Voltage (IS) | 2300V |
| Ion Source Gas 1 (GS1) | 2-15 |
| Interface Heater | 150°C |
| **Compound** | |
| Declustering Potential (DP) | 70 |

** Source/Gas parameters may vary between systems and spray tip. Determine the best value for the system you are working with. Ensure the spray tip position is optimized before creating the acquisition method.

3. Enter the MRM transitions from Table 3-3.

**Note:** In the Analyst MRM transition table, verify that the additional CE (collision energy) column is added to the table view by right-clicking the table and selecting CE from the menu that displays.

**Table 3-3 MRM Transitions for Beta-Galactosidase**

| Q1 | Q3 | Dwell | ID | CE |
|---|---|---|---|---|
| 503.2 | 760.3 | 50 | BG_YSQQQLMETSHR | 27 |
| 542.3 | 636.4 | 50 | BG_GDFQFNISR | 26 |
| 671.3 | 755.5 | 50 | BG_VDEDQPFPAVPK | 33 |
| 714.9 | 884.5 | 50 | BG_DWENPGVTQLNR | 32 |
| 729.4 | 832.5 | 50 | BG_APLDNDIGVSEATR | 48 |

## Add LC Information to the Acquisition Method

1. Click **Acquisition Method** in the left pane, and then select **LC Sync** as the **Synchronization Mode**.

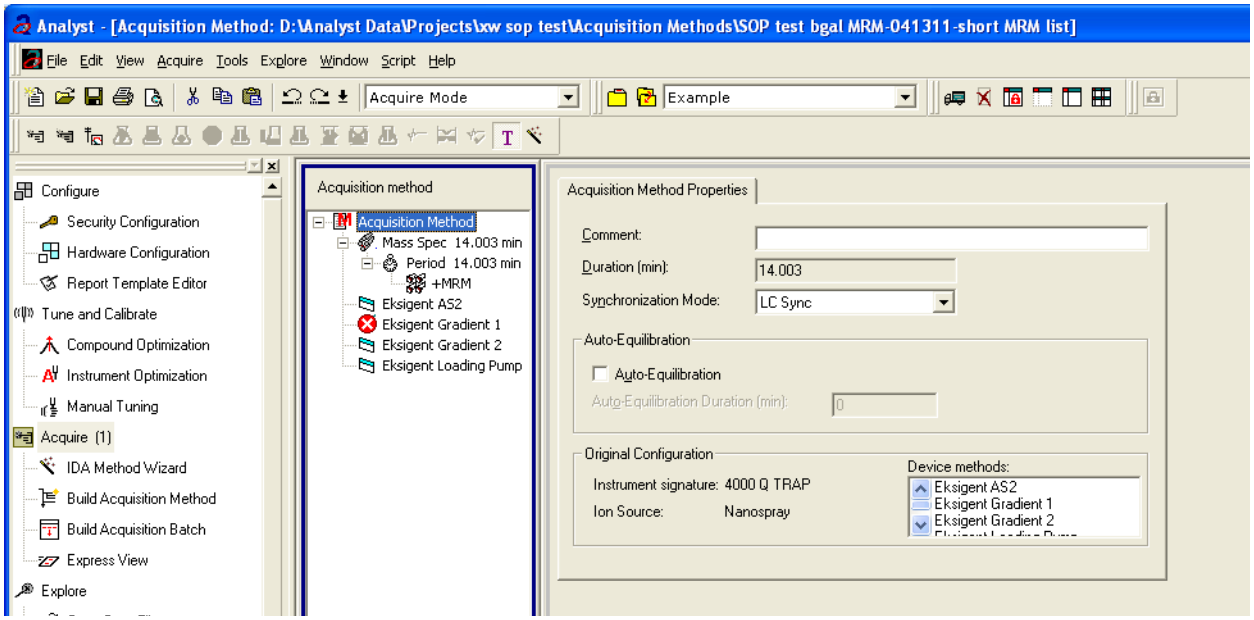


**Figure 3-8 Acquisition Method Properties tab—synchronization mode**

2. Click **Eksigent AS2** and then select the autosampler method, AS2 10uLloop 1uLinj Nanoflex trap elute.ini or AS2 10uLloop 1uLinj Ch2Valve Trap.ini, depending on the installation.

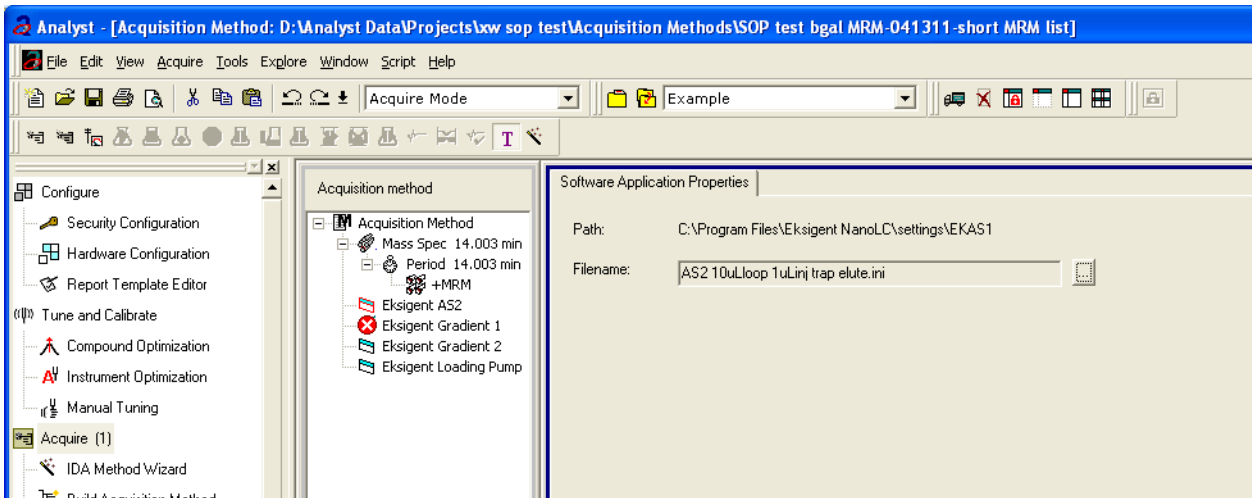


**Figure 3-9 Software Application Properties tab—autosampler filename**

3. Click **Eksigent Gradient 2**, and then select the gradient 2 pump method, CH2 15min 400nL min nanoflex trap.ini or CH2 15min 400nL min column trap.ini.

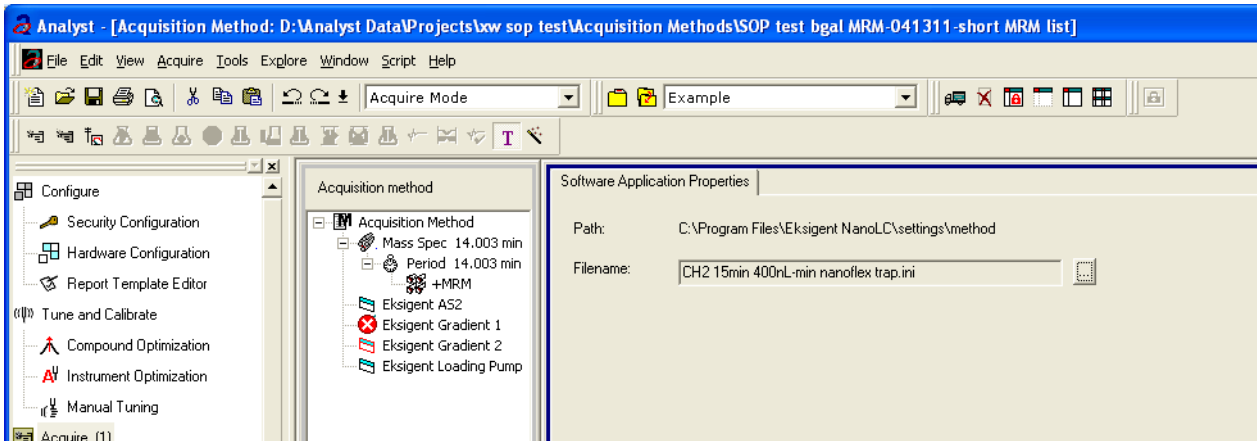


**Figure 3-10 Software Application Properties tab—gradient 2 filename**

4. Click **Eksigent Loading Pump**, and then select the loading pump method, Load Pump 2 min Trap Wash.ini.

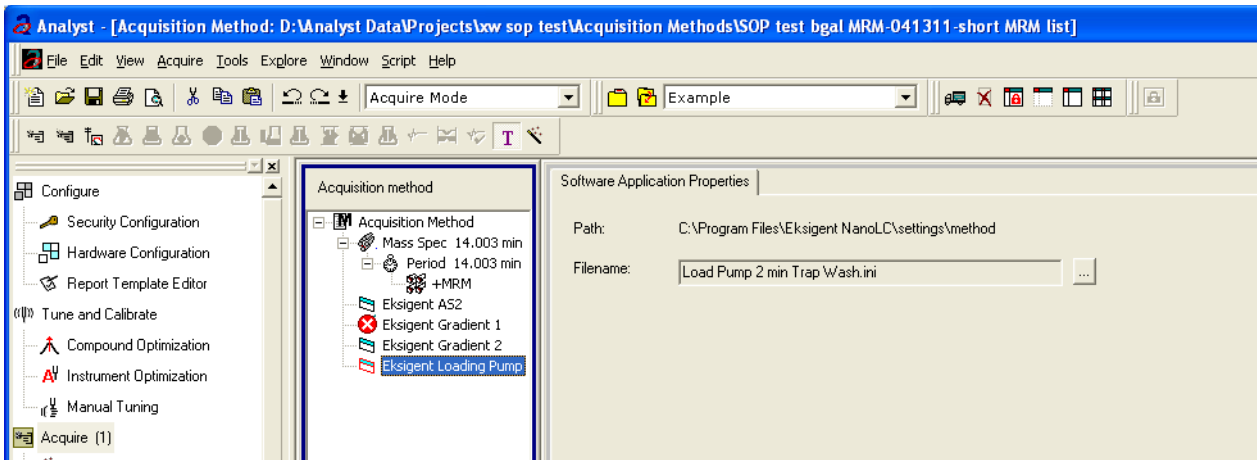


**Figure 3-11 Software Application Properties tab—loading pump filename**

5. Save the method as “System Functional Test”.

# Create the Methods and Batch for the System Performance Test

This section provides tests for the NanoLC Ultra system that indicate the performance level of the instrument for proteomics applications such as protein identification and quantification.

Perform these tests when the mass spectrometer is known to be operating well and meeting performance specifications.

The expected duration of the performance tests is 60 minutes using the NanoSpray ion source. Repeat the test until you have consistent peak shape and peak intensity (approximately 180 minutes).

## Create the Acquisition Methods and Batch— Performance Tests

1. Plumb the autosampler valve with a 10 µL sample loop.
2. In the **AS1/AS2 Autosampler** window, click **Method Editor**.
3. Create the autosampler method for a trap-elute configuration, as shown in Figure 3-12.

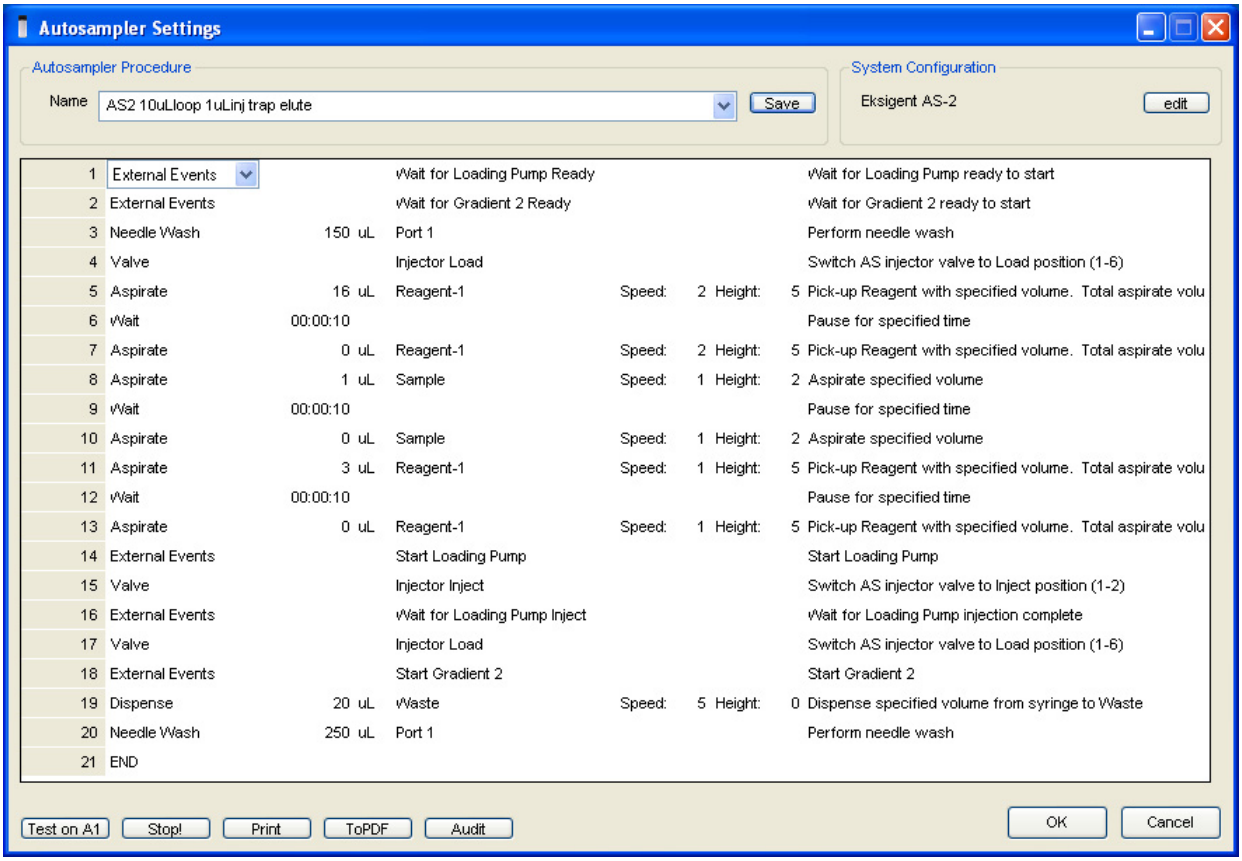


**Figure 3-12 Autosampler Settings dialog—trap-elute configuration (Nanoflex system)**

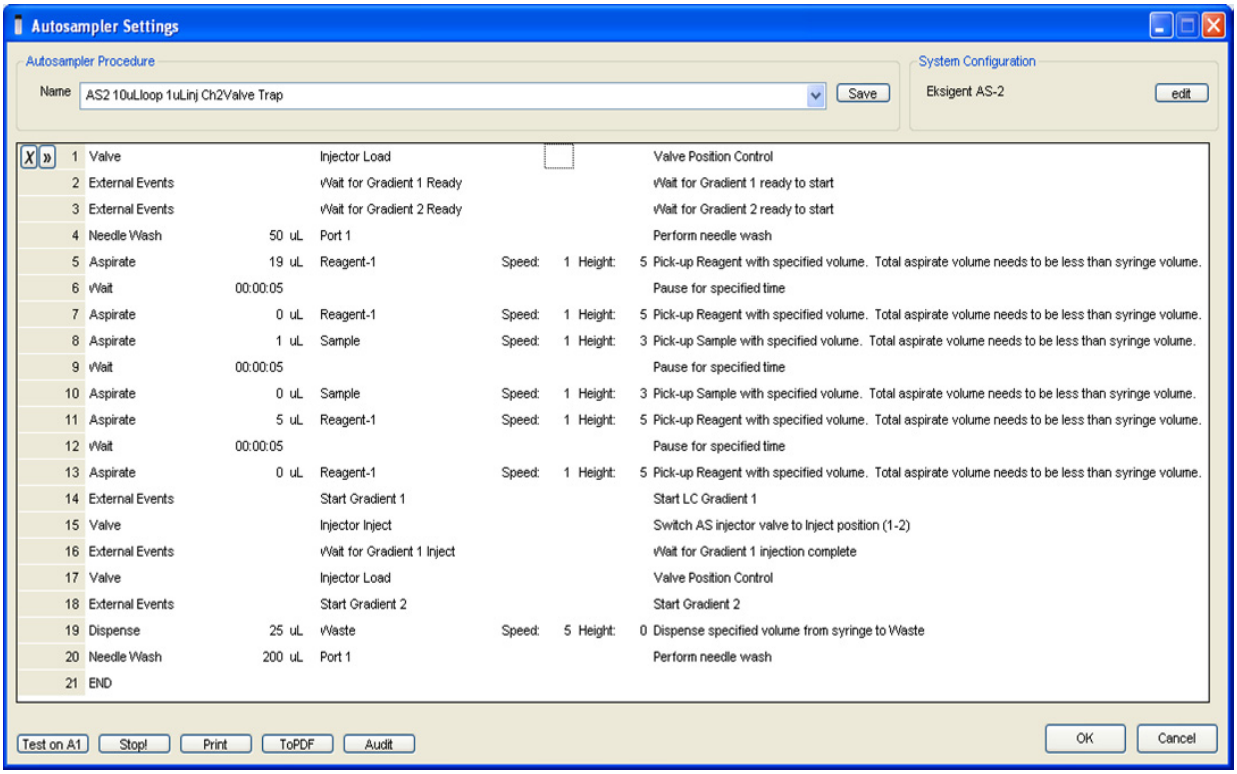


**Figure 3-13 Autosampler Settings dialog—trap-elute configuration (Nanoflex system or column installation)**

4. In the **Name** field, specify the name of this method as AS2 10uLloop 1uLinj trap elute or AS2 10uLloop 1uLinj Ch2Valve Trap, depending on the installation.
5. Click **Save**.

# Create the LC Methods

The aqueous channel for each pump (Channel A) will be filled with Buffer A. The organic channel (Channel B) will be filled with Buffer B. For the Loading Pump, Buffer A is always used. Typical buffers are shown in Table 3-4.

**Table 3-4 Typical Buffer Mixtures**

| Buffer | Mixture | Channel |
|---|---|---|
| Buffer A | 100% water:0.1% formic acid | Channel A |
| Buffer B | 100% acetonitrile:0.1% formic acid | Channel B |

In the method below, the loading pump will be the pump with the microflow module.

## Create the Pump Method in the Eksigent Control Software

1. Ensure that **Loading Pump** is selected as the channel (top, right corner of window).
2. Click **LC Methods**.

3. In the **Name** field, type **Load Pump 10 min Trap Wash**, and then click **Save**.
4. On the **Gradient Table** tab, revise the method for the loading pump (the loading pump will be the pump with the microflow module), as shown in Figure 3-14.

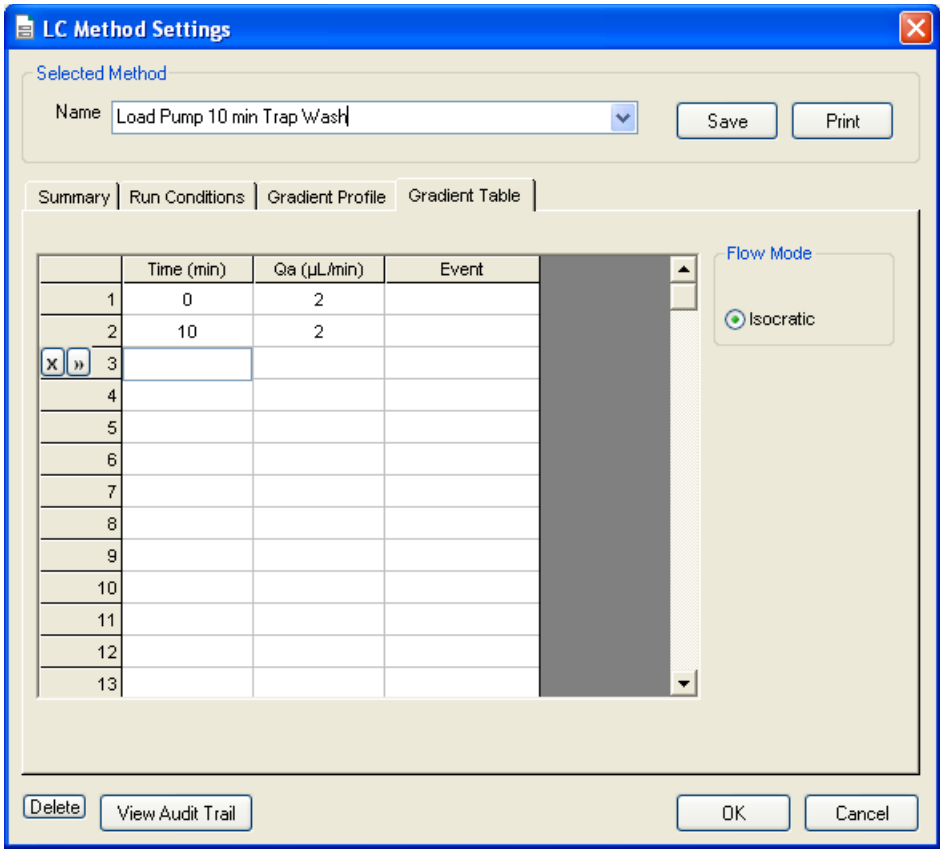


**Figure 3-14 LC Method Settings dialog—Gradient Table tab**

5. On the **Run Conditions** tab, specify conditions as shown in Figure 3-15.

**Note:** For a trap-elute configuration, the Sample Injection method should be Standard.

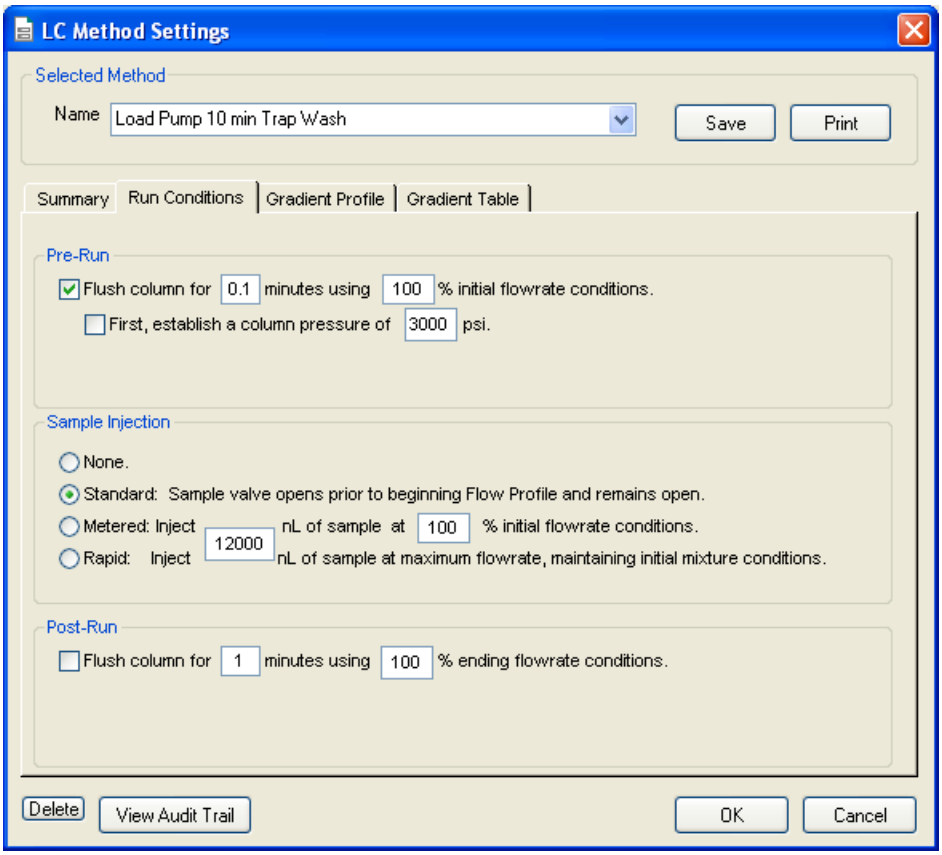


**Figure 3-15 LC Method Settings dialog—Run Conditions tab**

6. Click **Save**.

## Create a Gradient Method in the Eksigent Control Software

For the analytical gradient (typically on the Gradient 2 pump with the nanoflow module), create the gradient method.

1. Ensure that **Gradient 2** is selected as the channel (top, right corner of window).
2. Click **LC Methods**.
3. If this is a Nanoflex system installation, then in the **Name** field, type **CH2 45min 300nLmin Nanoflex trap**, and click **Save**.

   Or

   If this is a column installation, then in the **Name** field, type **CH2 45min 300nLmin column trap**, and click **Save**
4. On the **Gradient Table** tab, specify the method, as shown in Figure 3-16 or Figure 3-17, depending on the installation.

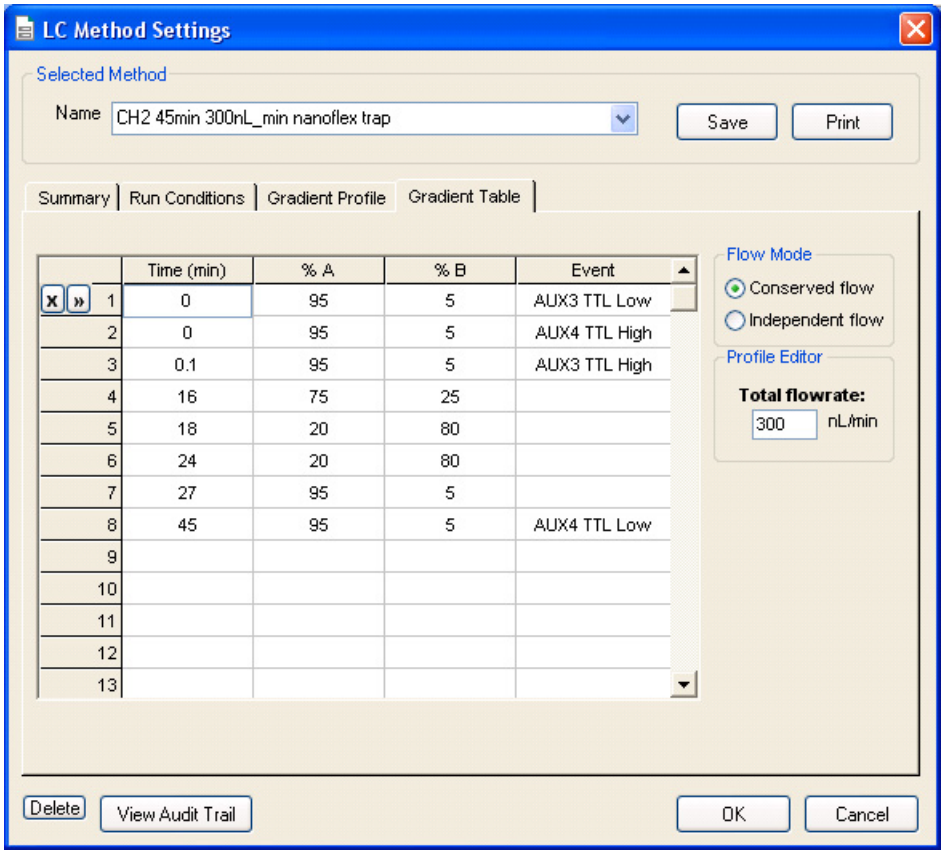


**Figure 3-16 LC Method Settings dialog—Gradient Table tab (Nanoflex system)**

**Note:** The events shown verify the correct switching of the Nanoflex valve. The signals at Time 0 will move the Nanoflex valve to the Inject position and the signals at Time 45 will move the Nanoflex valve back to the Load position.

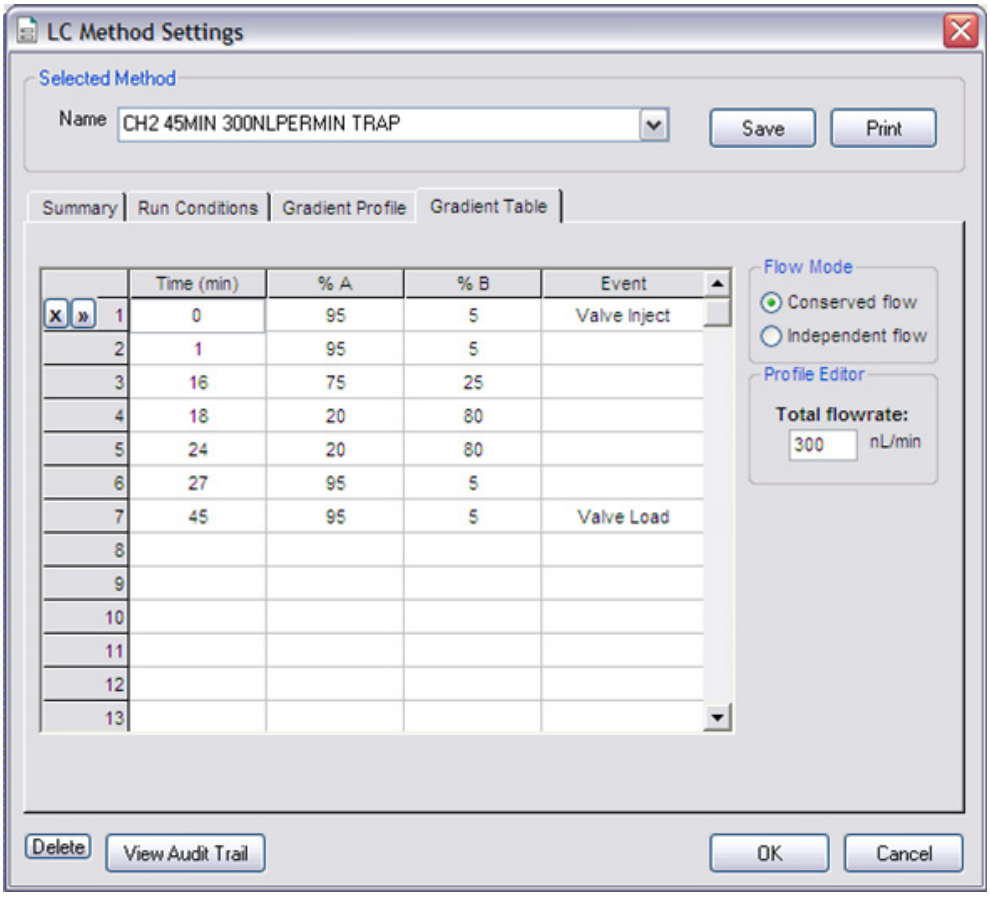


**Figure 3-17 LC Method Settings dialog—Gradient Table tab (column)**

5. On the **Run Conditions** tab, specify the method as shown in Figure 3-18.

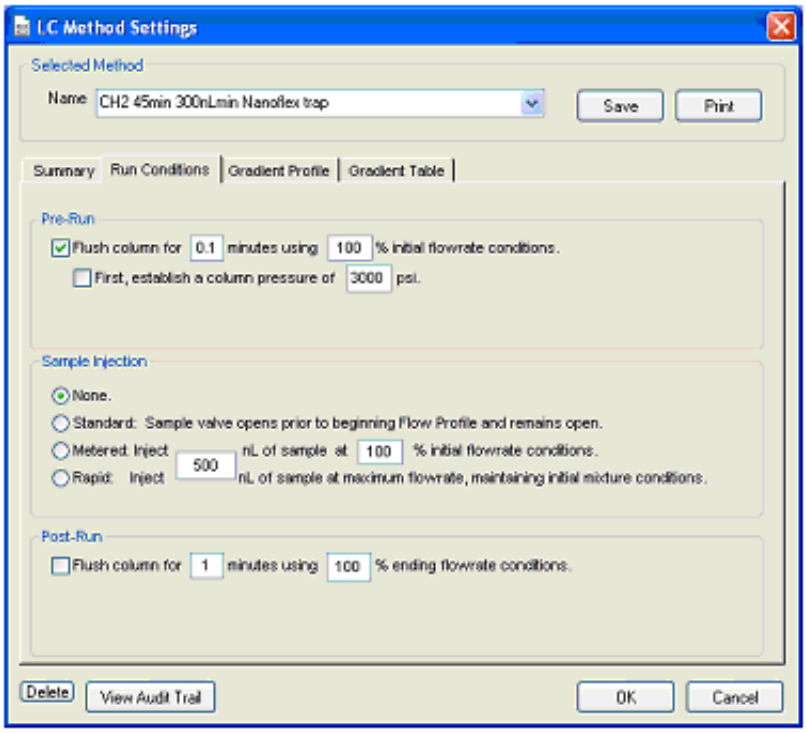


**Figure 3-18 LC Method Settings dialog—Run Conditions tab**

6. Click **Save**.

# Create the Acquisition Method in the Analyst Software

1. Create the acquisition method. See Table 3-5 for details.

**Note:** The acquisition time should be shorter than the LC run time.

**Table 3-5 Key Parameters**

| Parameter | Value |
|---|---|
| **MS** | |
| Scan Type | MRM Scan |
| Polarity | Positive |
| MCA | Off |
| Q1/Q3 Masses and CE | See Table 3-6. |
| Acquisition time | 40 min |
| **Advanced MS** | |
| Q1 Resolution | Unit |
| Q3 Resolution | Unit |
| **Source/Gas**** | |

**Table 3-5 Key Parameters (Continued)**

| Parameter | Value |
|---|---|
| Curtain Gas (CUR) | 20 |
| CAD Gas | HIGH |
| IonSpray Voltage (IS) | 2300V |
| Ion Source Gas 1 (GS1) | 3-15 |
| Interface Heater | 150°C |
| **Compound** | |
| Declustering Potential (DP) | 70 |

** Source/Gas parameters may vary between systems and spray tip. Determine the best value for the system you are working with. Ensure the spray tip position is optimized before creating the acquisition method.

2. Enter the MRM transitions from Table 3-6.

**Note:** In the Analyst MRM transition table, verify that the additional CE (collision energy) column is added to the table view by right-clicking the table and selecting CE from the menu.

**Table 3-6 MRM Transitions for Beta-Galactosidase**

| Q1 | Q3 | Dwell | ID | CE |
|---|---|---|---|---|
| 433.9 | 723.3 | 50 | BG_ELNYGPHQWR | 30 |
| 450.7 | 524.2 | 50 | BG_FNDDFSR | 28 |
| 503.2 | 760.3 | 50 | BG_YSQQQLMETSHR | 27 |
| 528.9 | 855.4 | 50 | BG_RDWENPGVTQLNR | 25 |
| 542.3 | 636.4 | 50 | BG_GDFQFNISR | 26 |
| 550.3 | 871.4 | 50 | BG_IDPNAWVER | 27 |
| 567.1 | 932.5 | 50 | BG_DVSLLHKPTTQISDFHVATR | 30 |
| 607.9 | 685.4 | 50 | BG_ITDSLAVVLQR | 39 |
| 671.3 | 755.5 | 50 | BG_VDEDQPFPAVPK | 33 |
| 697.9 | 821.5 | 50 | BG_LPSEFDLSAFLR | 35 |
| 714.9 | 884.5 | 50 | BG_DWENPGVTQLNR | 32 |
| 729.4 | 832.5 | 50 | BG_APLDNDIGVSEATR | 48 |
| 871.9 | 915.5 | 50 | BG_LSGQTIEVTSEYLFR | 40 |
| 879.4 | 664.3 | 50 | BG_VNWLGLGPQENYPDR | 40 |

3. Save the method as “System Performance Test”.

## Add LC Information to the Acquisition Method

1. Click **Acquisition Method** in the left pane, and then select **LC Sync** as the **Synchronization Mode**.

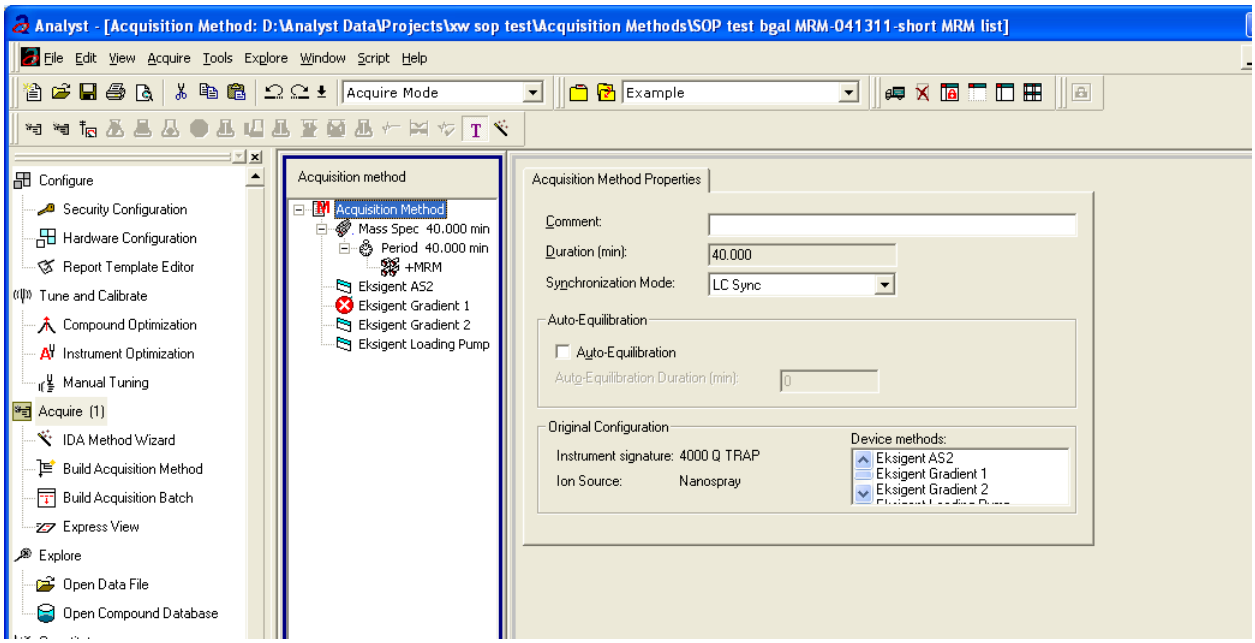


**Figure 3-19 Acquisition Method Properties tab—synchronization mode**

2. Click **Eksigent AS2** and then select the autosampler method, AS2 10µLinj Nanoflex trap.ini or AS2 10µLinj trap.ini, depending on the installation.

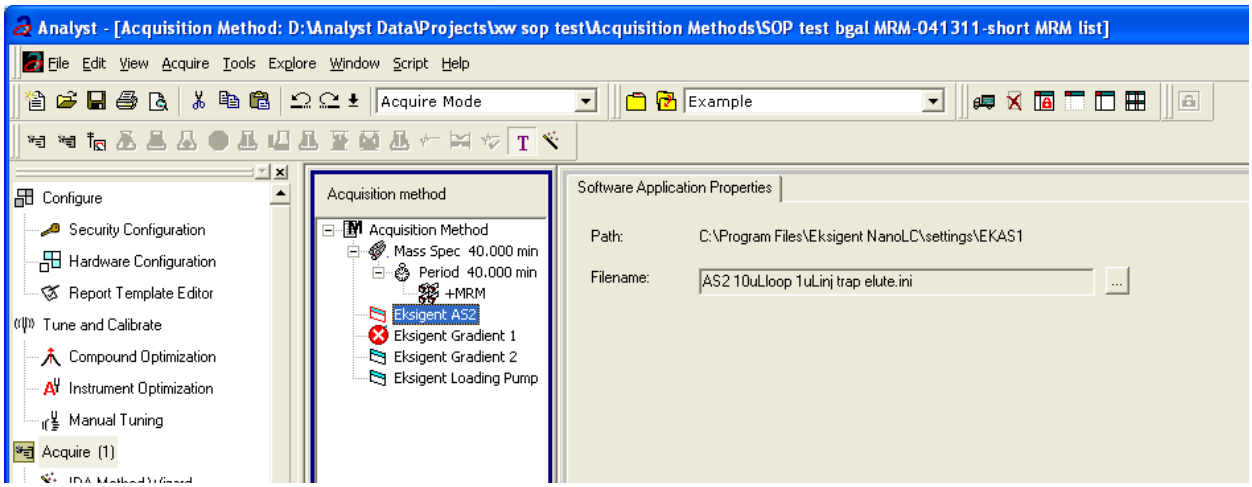


**Figure 3-20 Software Application Properties tab—autosampler method**

3. Click **Eksigent Gradient 2** and then select the gradient pump method, CH2 45min 300nLmin Nanoflex trap.ini or CH2 45min 300nLmin column trap.ini, depending on the installation.

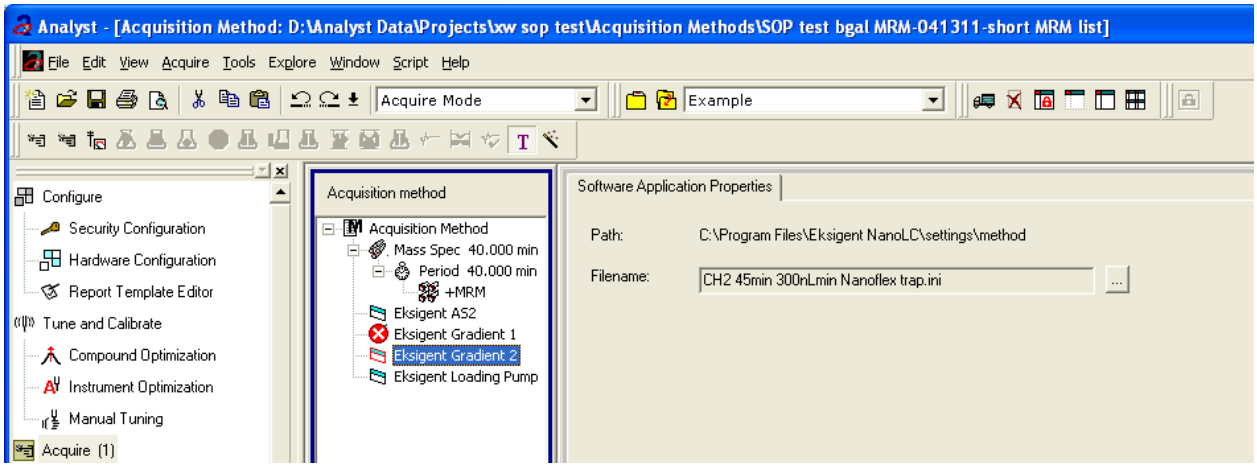


**Figure 3-21 Software Application Properties tab—gradient 2 method**

4. Click **Eksigent Loading Pump** and then select the loading pump method, Load Pump 10 min Trap Wash.ini.

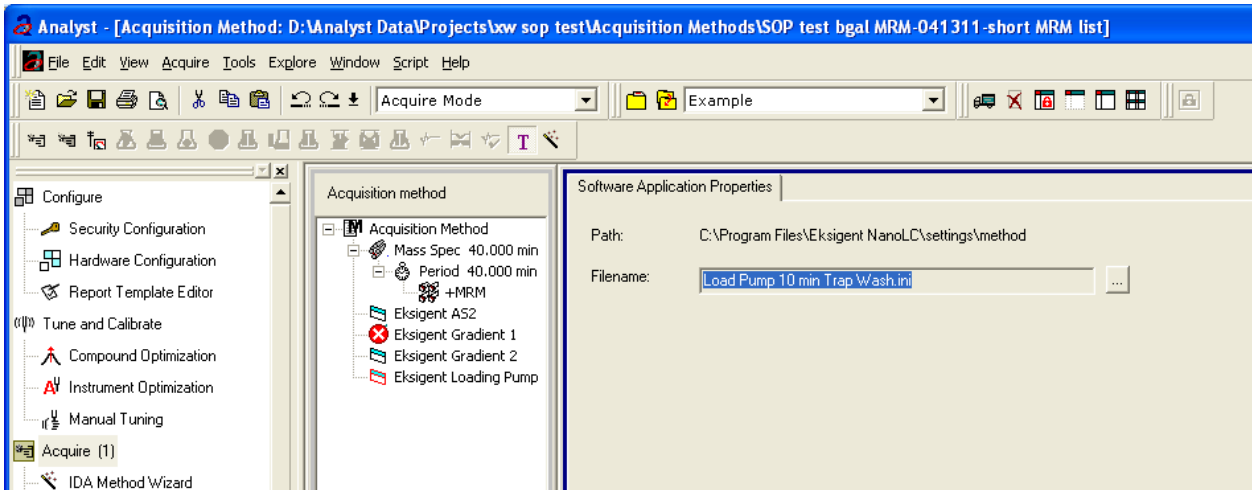


**Figure 3-22 Software Application Properties tab—loading pump method**

5. Save the method as “System Performance Test”.

# Prepare the System for Testing

Plumb the system in trap-elute configuration to perform the pre-column desalting workflow.

## Prepare the Solution and Dilution

Prepare the Beta-Galactosidase stock solution from the Beta-Galactosidase vial provided in the LC/MS Peptide/Protein Mass Standards Kit as described below. This will produce a stock solution of 1 pmol/µL.

1. Add 625.0 µL of Buffer A (100% water:0.1% formic acid) to the Beta-Galactosidase vial.
2. Vortex the vial for at least 30 seconds.
3. Using a centrifuge, spin the vial to bring the liquid down to the bottom of the vial before opening.
4. Repeat step 2 and step 3 to confirm dissolution.
5. Aliquot the stock solution (1 pmol/µL concentration) into 50 µL volumes and freeze for future use.

**Note:** Solutions can be stored at 4°C for up to 3 days after thawing.

### Prepare the Dilution for the Functional Evaluation

1. Combine 40 µL of Buffer A (100% water:0.1% formic acid) with 10 µL of the Beta-Galactosidase protein digest stock solution in a clean vial. A 1 µL injection of a 200 fmol/µL solution will be performed.
2. Vortex the vial for at least 30 seconds to properly mix the solution. This is a 1/5 dilution and will give a final concentration of 200 fmol/µL.
3. Transfer the solution to the autosampler vial and make sure there is no bubble on the bottom of the vial.

### Prepare the Dilution for the Performance Evaluation

1. Prepare the solution as described above. A 1 µL injection of a 50 fmol/µL solution will be performed.
2. Prepare 200 µL of the working solution of Beta-Galactosidase.
   - Combine 190 µL of Buffer A (100% water:0.1% formic acid) with 10 µL of the Beta-Galactosidase protein digest stock solution in a clean vial.
   - Vortex the solution for at least 30 seconds to properly mix the solution. This is a 1/20 dilution and will give a final concentration of 50 fmol/µL.
   - Transfer the solution to the autosampler vial and make sure there is no bubble on the bottom of the vial.

## Condition the System

A trap and column typically require 2 to 3 runs with 200 fmol of protein digest for conditioning.

- Verify that the trap and analytical column are well conditioned with protein digest injections before performing this test.

## Verify System Readiness

Ensure the NanoLC Ultra system is meeting performance specifications.

1. Connect effluent from the NanoLC Ultra system to the NanoSpray ion source and verify that the spray is stable by monitoring the background signal in the Analyst software.
2. Equilibrate the LC/MS system with the starting conditions of the method outlined above.
3. Ensure the spray is stable.
4. Double-click **Manual Tune** in the left **Navigation** bar.
5. Enter the key parameters from Table 3-7 and then click **Start** to begin acquisition.

**Table 3-7 Key Parameters**

| Parameter | Value |
|---|---|
| **MS** | |
| Scan type | Q1 Scan |
| Polarity | Positive |
| MCA | Off |
| Start Mass | 400 |
| Stop Mass | 1000 |
| Run Time | 2 min |
| **Source/Gas** | |
| Curtain Gas (CUR) | 20-25 |
| IonSpray Voltage (IS) | 2100-2400 V |
| Ion Source Gas 1 (GS1) | 2-15 |
| Interface Heater | 150ºC |
| **Compound** | |
| Declustering Potential | 70 |

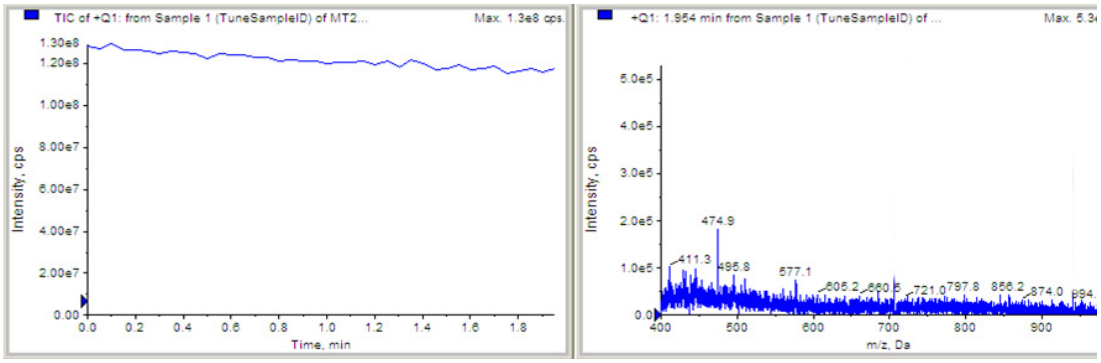


**Figure 3-23 Q1 MS scan for background noise**

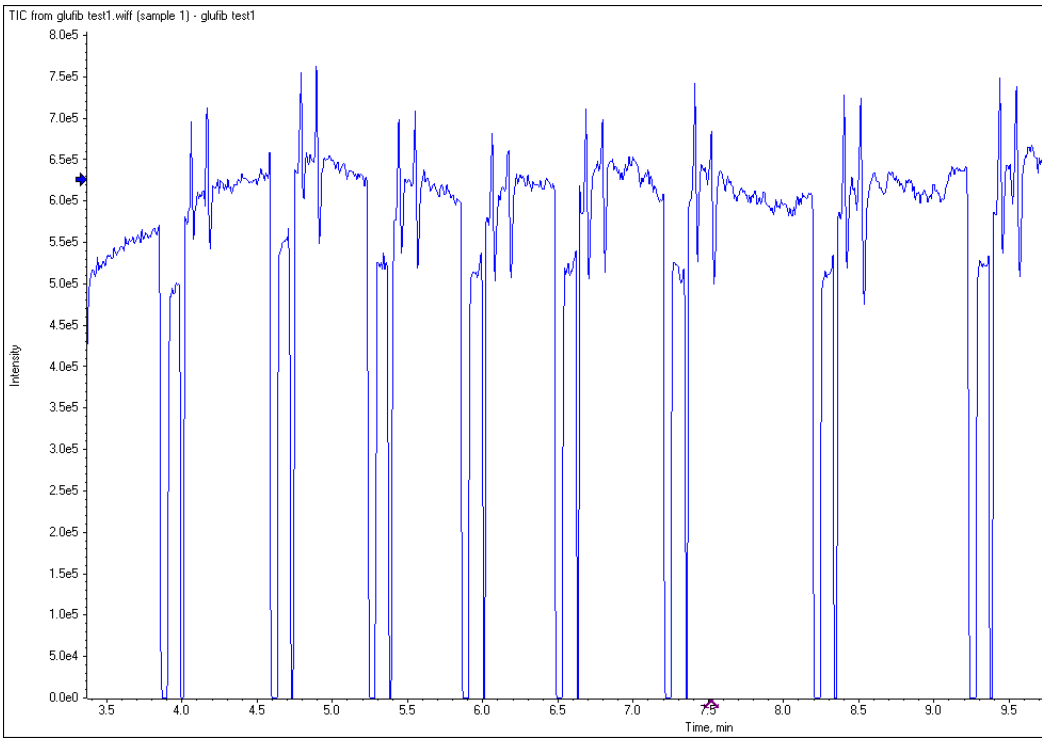


**Figure 3-24 Typical TIC of an unstable spray induced by air bubbles**

6. Ensure that the spray is still stable by monitoring the background signal with a Q1 MS scan.
   - Stable spray appears as shown in Figure 3-23.
   - Unstable spray appears as shown in Figure 3-24 (typical unstable spray induced by air bubbles).
   - If the spray is not stable, retune the NanoSpray ion source by infusion. Refer to the *NanoSpray® Ion Source Operator Guide* for more information.

# Perform the System Functional Test

Create the LC/MS acquisition batch, run the batch and then verify the results.

## Create the LC/MS Acquisition Batch in the Analyst Software

1. Double-click **Build Acquisition Batch** in the left **Navigation** bar.
2. Build the acquisition batch, as shown in Figure 3-25.
   i. In the **Acquisition** group, select the acquisition method from the list.
   ii. Click **Add Set**, and then click **Add Samples**.

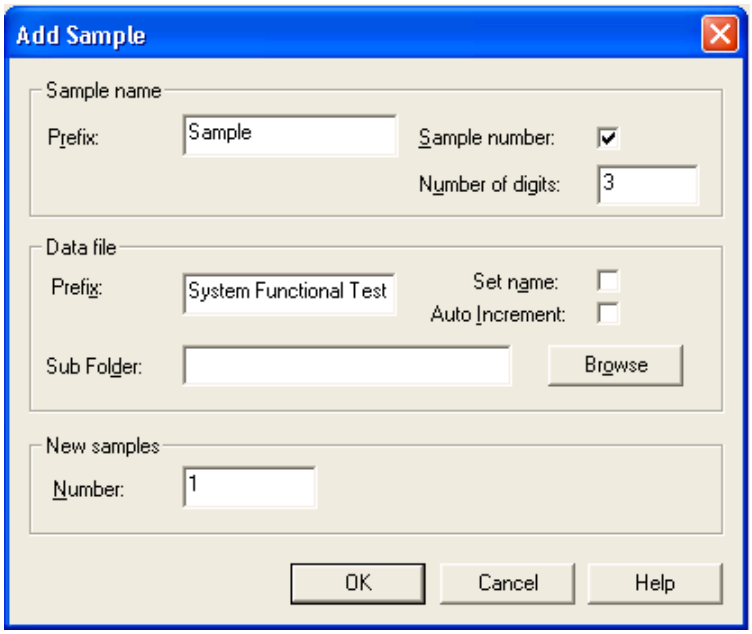


**Figure 3-25 Add Samples dialog—System Functional Test**

3. Click **OK**.
4. Save the data file as QTRAP 4000 system LC MRM BGal functional status check <date>.
5. On the **Location** tab, specify the location of the Beta-Galactosidase sample in the autosampler.

## Run the Batch

1. On the **Submit** tab, click **Submit**.
2. In the **View** menu, click **Sample Queue**.
3. In the **Acquire** menu, click **Start Sample**.

## Verify the Results

1. After the experiment has finished, open the sample from the data file in the **Explore** window.

   Figure 3-26 shows typical data for the instrument.

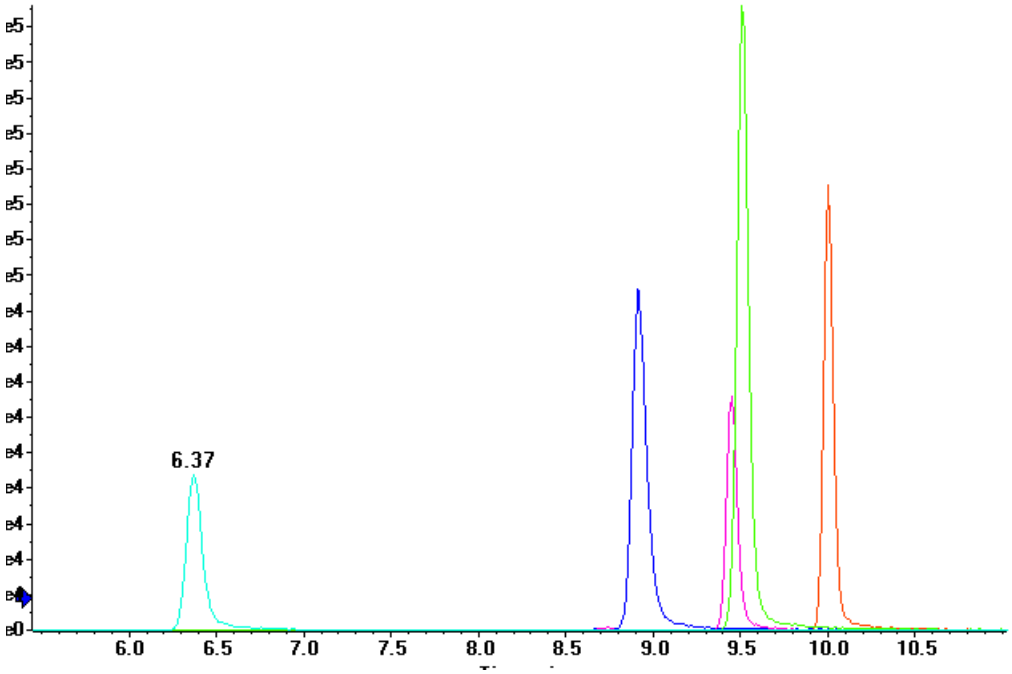


**Figure 3-26 MRM XICs of 5 peptides from Beta-Galactosidase NanoLC Ultra pre-column desalting run (200 fmol on column)**

2. Repeat the acquisition until you have consistent peak shape and peak intensity. If required, refer to Troubleshoot Peak Problems for more information.

**Note:** This test is not a specification. Use this example to confirm injection and peak shape of each MRM transition.

# Perform the System Performance Test

Create the LC/MS acquisition batch, run the batch and then verify the results.

## Create the LC/MS Acquisition Batch in the Analyst Software

1. Double-click **Build Acquisition Batch** in left **Navigation** bar.
2. Build the acquisition batch, as shown in Figure 3-27.
   i. In the **Acquisition** group, select the acquisition method from the list.
   ii. Click **Add Set**, and then click **Add Samples**.

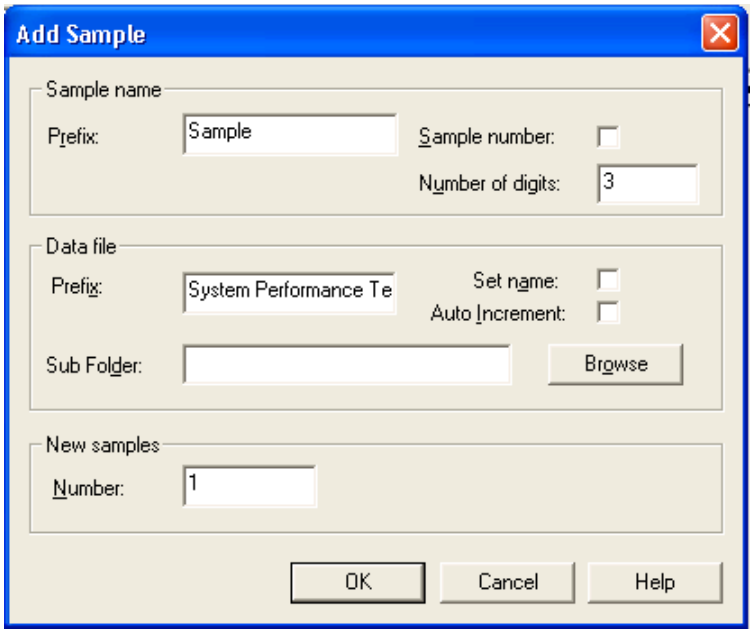


**Figure 3-27 Add Sample dialog—System Performance Test**

3. Click **OK**.
4. Save the data file as 4000 QTRAP system LC MRM BGal Performance Test <date>.
5. On the **Location** tab, specify the location of the Beta-Galactosidase sample in the autosampler.

## Run the Batch

1. On the **Submit** tab, click **Submit**.
2. In the **View** menu, click **Sample Queue**.
3. In the **Acquire** menu, click **Start Sample**.

## Verify the Results

1. After the experiment has finished, open the sample from the data file in the **Explore** window.
2. Right-click the TIC for the MRM experiment, and then click **Extract Ions**.

3. In the **Extract Ions** dialog, select the 5 MRMs from Table 3-8, and then click **OK**. Figure 3-28 shows typical data for the instrument.

**Table 3-8 MRM Transitions for Beta-Galactosidase**

| Q1 | Q3 | ID |
|---|---|---|
| 503.2 | 760.3 | BG_YSQQQLMETSHR |
| 542.3 | 636.4 | BG_GDFQFNISR |
| 671.3 | 755.5 | BG_VDEDQPFPAVPK |
| 714.9 | 884.5 | BG_DWENPGVTQLNR |
| 729.4 | 832.5 | BG_APLDNDIGVSEATR |

4. Record the peak areas of the specified MRM transitions in the section Test Results—4000 QTRAP Instruments on page 117.

**Note:** Most XICs should have peak widths of no more 0.2 minute half height. Some peaks will be narrower and some broader.

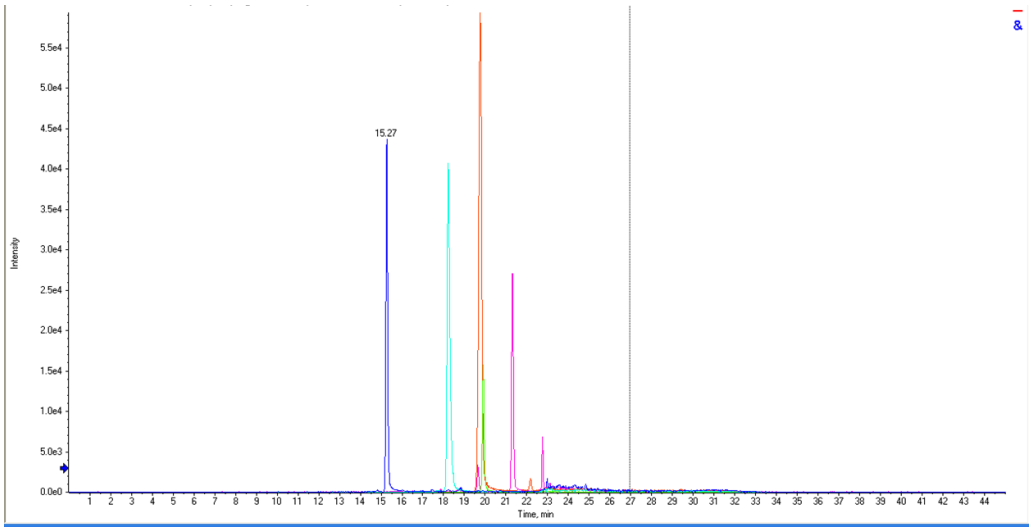


**Figure 3-28 MRM XICs of 5 peptides from Beta-Galactosidase NanoLC Ultra pre-column desalting run (10 fmol on column)**

5. Record the retention times of the chosen peaks. This will vary with each system. Time of elution of the first peak (approximately 14 to 16 minutes, as shown in Figure 3-28) indicates dead volume of the system. Minimize dead volume where possible.
6. Repeat the acquisition until you have consistent peak shape, retention time, and peak intensity (a minimum of 3 times).

   For new columns, this may require that you repeat the acquisition 10 or more times in order to obtain consistent peak shape, retention time, and peak intensity. If required, refer to Troubleshoot Peak Problems for more information.
7. Record the results for each acquisition.

8. Record the average peak area of the acquisitions in the section, Test Results—4000 QTRAP Instruments on page 117.
9. Ensure that the average peak area meets the minimum requirement specified in the section.

# Troubleshoot Peak Problems

This section provides information for troubleshooting peak related problems such as broad or tailing peak widths, lack of separation between peaks, and low peak area.

### Peak widths are too broad or are tailing

- Inspect all connections in the flow path to verify that there are no dead volumes.
- Look at connections post-column and around trap column. A small increase in peak width is often seen when a trap column is used.

**Caution: Potential Instrument Damage: If using the Nanoflex system and problems persist, do not attempt to troubleshoot the fittings connected to the chip.**

### No separation between the peaks

- Ensure that both pumps are delivering the correct amount of solvent.
- Ensure that the pressure spike upon injection is not too severe in the high-flow channel (less than 300 psi change in pressure).
- Large pressure change upon injection suggests an air bubble has been introduced to the sample loop or is present in the trap column plumbing.

**Note:** The overall separation of the chromatography itself will often be less than direct injection. Components that elute comparably on the trap and analytical column will not re-resolve on the analytical column and, as a result, spread out or bunch together.

### Peak intensity or peak area is too low

- Verify the performance of the mass spectrometer and the ion source spray using the infusion tests in the *NanoSpray® Ion Source Operator Guide*.
- Verify that the trap and analytical column are well conditioned with protein digest injections before performing this test. A trap typically requires 2 to 3 runs with 200 fmol of protein digest on the column for conditioning.
- Verify that the correct amount of sample has been withdrawn from the autosampler vial.
- Perform a direct injection with a protein digest on the analytical column to determine if the problem is related to the trap.
- If the first LC peak does not elute for a long time, inspect the system for dead volume before the trap.
- If the early eluting peaks are not visible or are very low in intensity, this could mean that trapping efficiency is low. Replace the trap.

**Tip!** Minimize tubing length wherever possible and make sure all tubing for the nanoflow path has an inner diameter of approximately 25 µm i.d.

- If the late eluting peaks are not visible or are very low in intensity, this is usually a sign that the column is getting old. In rare cases, it could mean that the Beta-Galactosidase standard is degraded. See Figure 3-29 for an example of a scan with an older column.

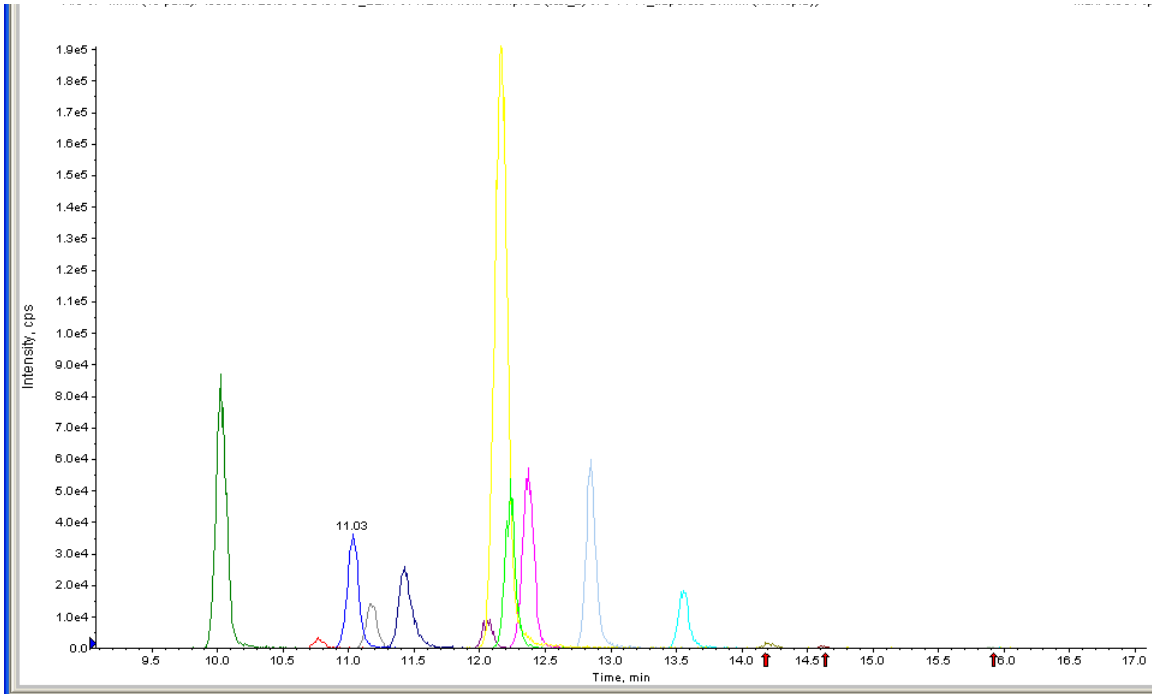


**Figure 3-29 Extraction of all peaks—late eluting peaks not present**

- Always monitor the column and trap pressure over time; increasing pressure may indicate increasing blockage, probably at the NanoSpray ion source tip. If, when the connection between the column and the ion source head is unfastened and the pressure changes quickly, then the tip is getting clogged and should be changed.
- For better long-term column lifetime, verify that there is at least a 30% drop in pressure observed during the high organic flush of the column. Increase the duration of the high organic flush until a good pressure change is observed. This time might increase for the trap column configuration relative to the direct injection configuration.

- Figure 3-30 shows a minimal pressure change upon injection and a 30% pressure decrease during the high organic flush.

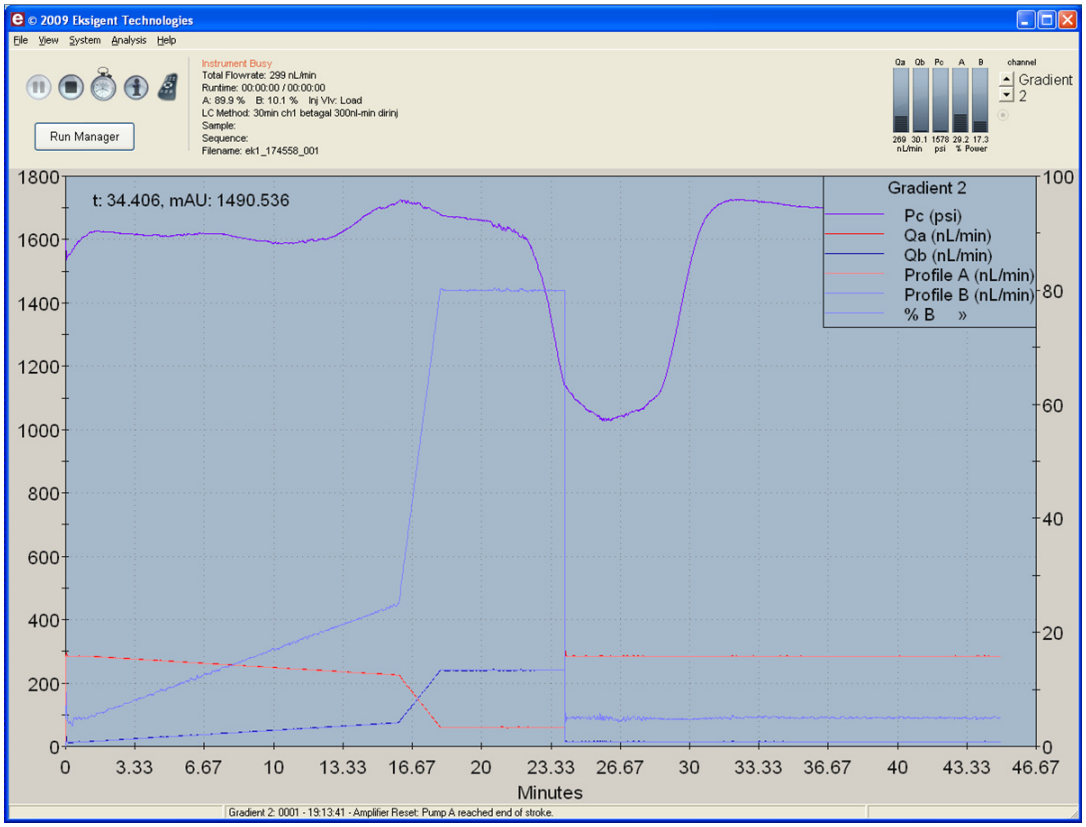


**Figure 3-30 Good pressure profile for a direct injection NanoLC Ultra system run**

# Create a Backup of the EKSettings.reg File

The EKSettings.reg file can be used to re-establish the system settings derived on installation if they are lost. Create a copy of the REG file upon completion of these tests.

1. In the Eksigent control software, on the **System** menu, click **Instrument Configuration**.
2. Click **Export Settings**.

   A backup of the REG file is created.
3. Navigate to the system settings folder (for example, C:\Program Files\Eksigent NanoLC\settings).
4. Copy the previous_settings.reg file to another location, separate from the host computer.

# Test Results—4000 QTRAP Instruments

For 4000 QTRAP instruments, complete this table with the results from five of the peptides from the Beta-Galactosidase digest solution stock. Ensure that the peak area is below specification.

Most XICs should have peak widths of no more 0.2 minute half height. Some peaks will be narrower and some broader. Additionally, verify that the peaks elute within 5 minutes of each other.

Beta-Gal Lot Number: _____

**Table 3-9 LC/MS Specification Test—4000 QTRAP Instruments**

| Q1 | Q3 | Dwell | Peptide ID | CE | Spec. (peak area) | Actual |
|---|---|---|---|---|---|---|
| 503.2 | 760.3 | 50 | BG_YSQQQLMETSHR | 27 | 2.0E+04 | |
| 542.3 | 636.4 | 50 | BG_GDFQFNISR | 26 | 5.0E+05 | |
| 671.3 | 755.5 | 50 | BG_VDEDQPFPAVPK | 33 | 5.0E+05 | |
| 714.9 | 884.5 | 50 | BG_DWENPGVTQLNR | 32 | 7.0E+04 | |
| 729.4 | 832.5 | 50 | BG_APLDNDIGVSEATR | 48 | 2.0E+05 | |
| | | | | | Specification Passed? | ❑ |

### Notes

| |
|---|
| |
| |
| |


eksigent®
part of AB SCIEX

If the NanoLC system has been idle for two weeks or more, then calibrate the system using the [Glu1]-Fibrinopeptide B, included in the LC/MS Peptide/Protein Mass Standards Kit (P/N 4368624).

**Note:** Do not infuse the tuning solution and then the [Glu1]-Fibrinopeptide B solution. Use Mobile Phase A to thoroughly flush the lines between running tests to avoid clogging the NanoSpray® tips.

# Prepare the [Glu$^1$]-Fibrinopeptide B Dilution

**Note:** Prepare the solution just before calibrating the system.

1. Add 1200 µL of Standard Diluent (0.1% formic acid, 10% acetonitrile) to the glass amber vial containing 0.1 mg [Glu$^1$]-Fibrinopeptide B.
2. Cover the vial tightly, shake it, and then vortex it for at least 2 minutes, to make sure that the peptide is fully dissolved.

   This produces a stock solution with a concentration of approximately 50 pmol/µL.

   **Note:** Peptide concentration may vary depending on the total peptide content and peptide purity of the standard solution. See the Certificate of Analysis provided by the vendor.

3. Aliquot 5 × 200 µL of the stock solution into clean tubes (included in the LC/MS Peptide/Protein Mass Standards kit) to store for future use.
4. Put 50 µL of the stock solution into a clean tube (included in the LC/MS Peptide/Protein Mass Standards kit), and then add 450 µL of Standard Diluent.
5. Vortex the tube for 30 seconds.

   This is a 1:10 dilution, providing 500 µL of a 5 pmol/µL solution.
6. Put 50 µL of the 5 pmol/µL solution into another clean tube and add 450 µL of Standard Diluent.
7. Vortex the tube for 30 seconds.

   This is a 1:10 dilution, providing 500 µL of a 500 fmol/µL solution.
8. Aliquot 50 µL of the 500 fmol/µL solution into a clean 500 µL solution tube.
9. Add 450 µL of Standard Diluent.
10. Vortex the tube for 30 seconds.

    This is the final 1:10 dilution, providing 500 µL of the final 50 fmol/µL solution, to be used for the infusion test.

11. Store the stock solution aliquots (50 pmol/µL solutions) and the diluted stock solutions (500 fmol/µL solution) of [Glu[1]]-Fibrinopeptide B in the freezer at –20°C.

# Edit the Calibration Reference Table for [Glu[1]]-Fibrinopeptide B

1. Select the **Tune and Calibrate** sidebar.
2. Click **Tools** >**Settings** >**Tuning Options**.
3. In the **Calibration** tab click the **Reference** button.
4. In the **Reference Table Editor**, select [Glu[1]]-Fibrinopeptide B in the **Name** list.
5. Add the masses in Figure A-1 to the Reference Ions for TOF MS Calibration table (left side of window).
6. Click **OK**.

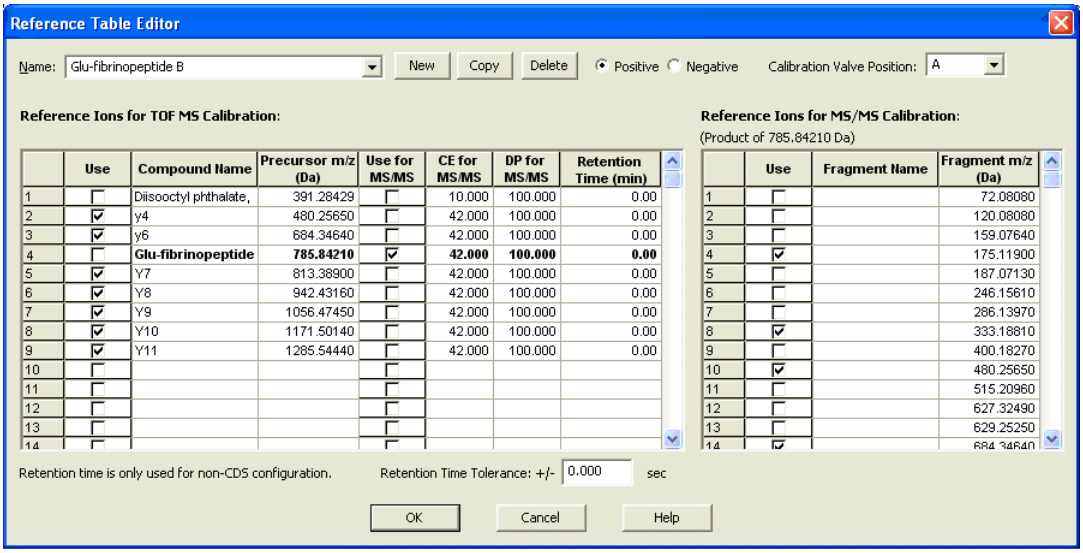


**Figure A-1 [Glu[1]]-Fibrinopeptide B reference table**

7. Click **OK** in the **Tuning Options** menu.

# Calibrate the TOF MS Scan Mode

1. In the **MS Method** window, specify the method parameters.

**Table A-1 TOF MS Method Parameters**

| Parameter | Value |
|---|---|
| **MS Parameters** | |
| Scan type | TOF MS |
| Accumulation time (sec) | 1.0 |
| Polarity | Positive |
| TOF masses (Da) | 400 to 1800 |
| Duration (min) | 0.5 |
| **Advanced MS Parameters** | |
| MCA | Off |
| Auto Adjust with mass | On |
| Q1 Transmission Window | Default (with Auto-adjust) |
| Pulsar Frequency | Default (with Auto-adjust) |
| Time bins to sum | 4 |
| Settling time | Default |
| Pause between mass ranges | Default |
| **Syringe Pump Method Parameters** | |
| Flow rate (µL/min) | 0.5 |
| Syringe Size (µL) | 100 Gastight (1.46 mm) |
| **Source/Gas Parameters** | |
| Ion Source Gas 1 (GS1) | 2 |
| Curtain Gas (CUR) | 25 |
| Interface Heater Temperature (IHT) (°C) | 150 |
| IonSpray Voltage Floating (ISVF) | 2300 |
| **Compound-Dependent Parameters** | |
| Collision energy (CE) (V) | 35 |
| **Compound Parameters** | |
| Declustering Potential | 100 |

2. Ensure spray is stable and click **Acquire** and acquire at least 30 seconds of scan data.
3. In the **TIC of +TOF MS** window (lower left) highlight 30 seconds of TIC signal to average.
4. Double-click the highlighted area.

5. In the window that appears (bottom), right-click and select **Re-Calibrate TOF** from the menu that appears.
6. In the **TOF Calibration** window, select [Glu[1]]-Fibrinopeptide B on the **Reference Table** list.
7. Ensure proper experimental masses have been identified from the infusion spectrum and match up with the reference table theoretical masses.
8. Review the **Average Error** value displayed to the right of the **Calculate New Calibrations** button.
9. Click **Calculate New Calibrations** and check the **Average Error** value has dropped to <2 ppm.
10. In the **Calibration Values** area, click **Calibrate Spectrum**.
11. In the **Save Current Calibration** area, select the **Set as Instrument Default** and **Overwrite Current File** check boxes.
12. Click **Entire File** to save new calibration for the TOF MS mode.
13. Click **Close**.

# Calibrate the TOF MS/MS for High Sensitivity and High Resolution Product Ion Modes

Complete this procedure first for high-sensitivity mode and then repeat it for high-resolution mode.

1. In the **MS Method** window, create a method from the parameters in Table A-2 below.

**Table A-2 TOF MS Method Parameters**

| Parameter | Value |
|---|---|
| **MS Parameters** | |
| Scan type | Product Ion |
| Product of | 785.8 |
| Accumulation time (sec) | 1.0 |
| Polarity | Positive |
| TOF masses (Da) | 100 to 1800 |
| High sensitivity | **On** |
| Duration (min) | 0.5 |
| **Advanced MS Parameters** | |
| MCA | Off |
| Auto Adjust with mass | On |
| Q1 Transmission Window | Default (with Auto-adjust) |
| Pulsar Frequency | Default (with Auto-adjust) |
| Time bins to sum | 4 |
| Settling time | Default |

**Table A-2 TOF MS Method Parameters (Continued)**

| Parameter | Value |
|---|---|
| Pause between mass ranges | Default |
| **Source/Gas Parameters** | |
| Ion Source Gas 1 (GS1) | as optimized |
| Curtain Gas (CUR) | as optimized |
| Interface Heater Temperature (IHT) (°C) | 150 |
| IonSpray Voltage Floating (ISVF) | as optimized |
| **Compound-Dependent Parameters** | |
| Collision energy (CE) (V) | 45 |
| **Resolution Parameters** | |
| Q1 resolution | Unit |

2. Ensure the spray is stable.
3. Click **Acquire** and acquire at least 30 seconds of scan data.
4. In the **TIC of +TOF MS** window (lower left), highlight 30 seconds of TIC signal to average
5. Double-click the area you highlighted.
6. In the window that appears (at the bottom of the screen), right-click and choose **Re-Calibrate TOF** from the menu that appears.
7. In the **TOF Calibration** window, select [$Glu^1$]-fibrinopeptide B on the **Reference Table** list.
8. Ensure proper experimental masses have been identified from the infusion spectrum and match up with the reference table theoretical masses. Review the **Average Error** value displayed to the right of the **Calculate New Calibrations** button.
9. Click **Calculate New Calibrations**.
10. Verify that the **Average Erro**r value has dropped to <2 ppm.
11. In the **Calibration Values** area, click **Calibrate Spectrum**.
12. In the **Save Current Calibration** area, select the **Set as Instrument Default** box and **Overwrite Current File** box.
13. Click **Entire File** to save new calibration for the TOF MS/MS high-sensitivity product ion mode.
14. Click **Close**.
15. Repeat this acquisition in high-resolution mode. Refer to the parameters in Table A-3 below for more information.
16. Ensure spray is stable and click **Acquire** and acquire at least 30 seconds of scan data.

17. In the **TIC of +TOF MS** window (lower left) highlight 30 seconds of TIC signal to average and double-click **Calibrate TOF** using the [Glu1]-fibrinopeptide B reference table described above.

**Table A-3 TOF MS Method Parameters**

| Parameter | Value |
|---|---|
| **MS Parameters** | |
| Scan type | Product Ion |
| Product of | 785.8 |
| Accumulation time (sec) | 1.0 |
| Polarity | Positive |
| TOF masses (Da) | 100 to 1800 |
| High resolution | **On** |
| Duration (min) | 0.5 |
| **Advanced MS Parameters** | |
| MCA | Off |
| Auto Adjust with mass | On |
| Q1 Transmission Window | Default (with Auto-adjust) |
| Pulsar Frequency | Default (with Auto-adjust) |
| Time bins to sum | 4 |
| Settling time | Default |
| Pause between mass ranges | Default |
| **Source/Gas Parameters** | |
| Ion Source Gas 1 (GS1) | as optimized |
| Curtain Gas (CUR) | as optimized |
| Interface Heater Temperature (IHT) (°C) | 150 |
| IonSpray Voltage Floating (ISVF) | as optimized |
| **Compound-Dependent Parameters** | |
| Collision energy (CE) (V) | 45 |
| **Resolution Parameters** | |
| Q1 resolution | Unit |