NanoLC Ultra[®] System

for TripleTOF^{$^{\text{TM}}$} 5600, QTRAP[®] 5500 and 4000 QTRAP[®] Systems

System Integration Test





Document Number: D5030175 A Release Date: March 2012 This document is provided to customers who have purchased AB Sciex equipment to use in the operation of such AB Sciex equipment. This document is copyright protected and any reproduction of this document or any part of this document is strictly prohibited, except as AB Sciex may authorize in writing.

Software that may be described in this document is furnished under a license agreement. It is against the law to copy, modify, or distribute the software on any medium, except as specifically allowed in the license agreement. Furthermore, the license agreement may prohibit the software from being disassembled, reverse engineered, or decompiled for any purpose.

Portions of this document may make reference to other manufacturers and/or their products, which may contain parts whose names are registered as trademarks and/or function as trademarks of their respective owners. Any such use is intended only to designate those manufacturers' products as supplied by AB Sciex for incorporation into its equipment and does not imply any right and/or license to use or permit others to use such manufacturers' and/or their product names as trademarks.

AB Sciex makes no warranties or representations as to the fitness of this equipment for any particular purpose and assumes no responsibility or contingent liability, including indirect or consequential damages, for any use to which the purchaser may put the equipment described herein, or for any adverse circumstances arising therefrom.

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

The trademarks mentioned herein are the property of AB Sciex Pte. Ltd. or their respective owners. Eksigent is a division of AB Sciex. LLC.

AB SCIEX[™] is being used under license.





Eksigent 5875 Arnold Road, Dublin, CA 94568. AB Sciex LP is ISO 9001 registered. © 2012 AB SCIEX. Printed in Canada.

Contents

Foreword	. 5
General Safety Information Symbols and Conventions Safety Instructions Qualified Personnel Equipment Use and Modification Mains Supply Environmental Conditions Instrument Disposal (Waste Electrical and Electronic Equipment) Regulatory Compliance Additional Documentation Technical Support	.5 .6 .8 .9 .9 .10 .10 .11
Chapter 1 LC/MS System Configuration Test— AB SCIEX TripleTOF™ 5600 System	13
Create the Methods and Batch for the System Functional Test Create the Acquisition Methods and Batch—Functional Test Create the LC Methods Create the Acquisition Method in the Analyst® TF Software Create the Methods and Batch for the System Performance Test Create the Acquisition Methods and Batch—Performance Test Create the LC Method Specify the Acquisition Method in the Analyst TF Software Prepare the System for Testing Prepare the Solution and Dilution Condition the System Verify System Readiness Perform the System Functional Test Troubleshoot Peak Problems Create a Backup of the EKSettings.reg File Test Results—TripleTOF 5600 Instruments	14 14 21 25 25 26 31 34 35 36 37 42 46 49 50
Chapter 2 LC/MS System Configuration Test— AB SCIEX QTRAP [®] 5500 System	51
Create the Methods and Batch for the System Functional Test Create the Acquisition Methods and Batch—Functional Test Create the LC Methods Create the Acquisition Method in the Analyst® Software Create the Methods and Batch for the System Performance Test Create the Acquisition Methods and Batch—Performance Test Create the Acquisition Methods and Batch—Performance Tests Create the LC Methods Specify the Acquisition Method in the Analyst Software Prepare the System for Testing Prepare the Solution and Dilution	52 54 59 63 63 64 69 73 73

Condition the System .74 Verify System Readiness .74 Perform the System Functional Test .76 Perform the System Performance Test .78 Troubleshoot Peak Problems .80 Create a Backup of the EKSettings.reg File .82 Test Results—QTRAP 5500 Instruments .83
Chapter 3 LC/MS System Configuration Test— AB SCIEX 4000 QTRAP [®] System
Create the Methods and Batch for the System Functional Test 86 Create the Acquisition Method and Batch—Functional Test 86 Create the LC Methods 88 Create the Acquisition Method in the Analyst® Software 93 Create the Methods and Batch for the System Performance Test 97 Create the Acquisition Methods and Batch—Performance Test 97 Create the Acquisition Methods and Batch—Performance Test 97 Create the Acquisition Method in the Analyst Software 103 Prepare the LC Methods 98 Create the Acquisition Method in the Analyst Software 103 Prepare the System for Testing 107 Prepare the Solution and Dilution 107 Condition the System 108 Verify System Readiness 108 Perform the System Functional Test 110 Perform the System Performance Test 112 Troubleshoot Peak Problems 114 Create a Backup of the EKSettings.reg File 116 Test Results—4000 QTRAP Instruments 117
Appendix A System Calibration
Prepare the [Glu ¹]-Fibrinopeptide B Dilution .119 Edit the Calibration Reference Table for [Glu1]-Fibrinopeptide B .120 Calibrate the TOF MS Scan Mode .121 Calibrate the TOF MS/MS for High Sensitivity and High Resolution .122 Product Ion Modes .122

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.



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 μ L 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.

🛛 Aut	osampler Settings							
Autos	sampler Procedure						System Configuration	
Na	AS2 10uLloop 1ul	Linj Nanoflex Trap				v Save	Eksigent AS-2	edit
						J	<u></u>	
	1 Valve		Injector Load			Valve Position Control		
	2 External Events		Wait for Gradient 1 Ready			Wait for Gradient 1 ready to start		
	3 External Events		Wait for Gradient 2 Ready			Wait for Gradient 2 ready to start		
	4 Needle Wash	50 uL	Port 1			Perform needle wash		
	5 Aspirate	19 uL	Reagent-1	Speed:	1 Height:	5 Pick-up Reagent with specified volume. Total	aspirate volume needs to be less than syringe v	olume.
	6 v/Vait	00:00:05				Pause for specified time		
	7 Aspirate	0 uL	Reagent-1	Speed:	1 Height:	5 Pick-up Reagent with specified volume. Total	aspirate volume needs to be less than syringe v	olume.
	8 Aspirate	1 uL	Sample	Speed:	1 Height:	3 Pick-up Sample with specified volume. Total a	spirate volume needs to be less than syringe vo	lume.
	9 v/Vait	00:00:05				Pause for specified time		
	10 Aspirate	0 uL	Sample	Speed:	1 Height:	3 Pick-up Sample with specified volume. Total a	spirate volume needs to be less than syringe vo	lume.
	11 Aspirate	5 uL	Reagent-1	Speed:	1 Height:	5 Pick-up Reagent with specified volume. Total	aspirate volume needs to be less than syringe v	olume.
	12 vVait	00:00:05				Pause for specified time		
	13 Aspirate	0 uL	Reagent-1	Speed:	1 Height:	5 Pick-up Reagent with specified volume. Total	aspirate volume needs to be less than syringe v	olume.
	14 External Events		Start Gradient 1			Start LC Gradient 1		
	15 Valve		Injector Inject			Switch AS injector valve to Inject position (1-2)	
	16 External Events		Wait for Gradient 1 Inject			Wait for Gradient 1 injection complete		
	17 Valve		Injector Load			Valve Position Control		
	18 External Events		Start Gradient 2			Start Gradient 2		
	19 Dispense	25 uL	Waste	Speed:	5 Height:	0 Dispense specified volume from syringe to We	aste	
	20 Needle Wash	200 uL	Port 1			Perform needle wash		
	21 END							
Test o	on A1 Stop! (Print ToPD	F Audit				ок	ancel

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

Autosar	npler Settings						
Autosamp	er Procedure						System Configuration
Name	AS2 10uLloop 1uLinj Chi	2∀alve Trap				Save	Eksigent AS-2 edit
X » 1	Valve		Injector Load	1		Valve Position Control	
2	External Events		Wait for Gradient 1 Ready			Wait for Gradient 1 ready to start	
3	External Events		Wait for Gradient 2 Ready			Wait for Gradient 2 ready to start	
4	Needle Wash	50 uL	Port 1			Perform needle wash	
5	Aspirate	19 uL	Reagent-1	Speed:	1 Height:	5 Pick-up Reagent with specified volume. Total a	spirate volume needs to be less than syringe volume.
6	rVait	00:00:05				Pause for specified time	
7	Aspirate	0 uL	Reagent-1	Speed:	1 Height:	5 Pick-up Reagent with specified volume. Total a	spirate volume needs to be less than syringe volume.
8	Aspirate	1 uL	Sample	Speed:	1 Height:	3 Pick-up Sample with specified volume. Total as	spirate volume needs to be less than syringe volume.
9	v∿ait	00:00:05				Pause for specified time	
10	Aspirate	0 uL	Sample	Speed:	1 Height:	3 Pick-up Sample with specified volume. Total as	spirate volume needs to be less than syringe volume.
11	Aspirate	5 uL	Reagent-1	Speed:	1 Height:	5 Pick-up Reagent with specified volume. Total a	spirate volume needs to be less than syringe volume.
12	v/vait	00:00:05				Pause for specified time	
13	Aspirate	0 uL	Reagent-1	Speed:	1 Height:	5 Pick-up Reagent with specified volume. Total a	spirate volume needs to be less than syringe volume.
14	External Events		Start Gradient 1			Start LC Gradient 1	
15	Valve		Injector Inject			Switch AS injector valve to Inject position (1-2)	(
16	External Events		Wait for Gradient 1 Inject			Wait for Gradient 1 injection complete	
17	Valve		Injector Load			Valve Position Control	
18	External Events		Start Gradient 2			Start Gradient 2	
19	Dispense	25 uL	Waste	Speed:	5 Height:	0 Dispense specified volume from syringe to Was	ste
20	Needle Wash	200 uL	Port 1			Perform needle wash	
21	END						
Test on A1	Stop! Pri	nt ToPDf	Audit				OK Cancel

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.

Name [.oad Pump 2 min '	Trap Wash		*	Save Print
Summary	Run Conditions	Gradient Profile	Gradient Table]	
					Elow Mode
	Time (min)	Qa (µL/min)		Event	
X » 1	0	3.5			
2	2	3.5			Olsocratic
3					
4					
5					
6					
7					
8					
9					
10					
11					
12					-
13					•
			1		



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.

LC Method	Settings
- Selected Metl	bd
Name Loa	l Pump 2 min Trap Wash 🔗 🔗 🔗 🖓 Save 🛛 Print
Summary R	n Conditions Gradient Profile Gradient Table
-Pre-Run	
Flush c	umn for 0.1 minutes using 100 % initial flowrate conditions.
- Firs	establish a column pressure of 3000 psi.
-Sample Injec	on
None.	Sample value opens prior to beginning Flow Profile and remains open
O Metere	Inject nL of sample at 100 % initial flowrate conditions.
O Rapid:	Inject 500 nL of sample at maximum flowrate, maintaining initial mixture conditions.
-Post-Run	
Flush c	umn for 1 minutes using 100 % ending flowrate conditions.
Delete)	

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.

Name C	:H2 15min 400nL_	min nanoflex trap		*		Save Print
ımmary	Run Conditions	Gradient Profile	Gradient Table]		
	Time (min)	% A	%В	Event		Flow Mode
) » 1	0	85	15	AUX3 TTL Low		 Conserved flow
2	0	85	15	AUX4 TTL High		
3	0.1	85	15	AUX3 TTL High		Profile Editor
4	5	60	40			Total flowrate:
5	6	20	80			400 nL/min
6	8	20	80			
7	9	95	5			
8	15	95	5	AUX4 TTL Low		
9						
10						
11						
12						
13					-	

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.

Name C	H2 15MIN 400NL	PERMIN TRAP		~		Save Print
ummary	Run Conditions	Gradient Profile	Gradient Table			
	Time (min)	% A	% B	Event	•	Flow Mode
()» 1	0	85	15	Valve Inject		Conserved flow
2	5	60	40			O Independent flow
3	6	20	80			Profile Editor
4	8	20	80			Total flowrate:
5	9	95	5			400 nL/min
6	15	95	5	Valve Load		
7						
8						
9						
10						
11						
12						
13					-	

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.

LC Method Settings
Selected Method
Name CH2 15min 400nl-min nanoflex trap Save Print
Summary Run Conditions Gradient Profile Gradient Table
Pre-Run Flush column for 0.1 minutes using 100 % initial flowrate conditions. First, establish a column pressure of 3000 psi.
Sample Injection
⊙ None.
Standard: Sample valve opens prior to beginning Flow Profile and remains open.
Meteret: Inject nL of sample at 100 % initial flowrate conditions. Seciet: Inject 500 nl of cample at maximum flowrate, mainteigns initial mixture conditions
Chapte. Press
Post-Run
Flush column for 1 minutes using 100 % ending flowrate conditions.
Delete View Audit Trail OK Cancel

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**.





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.

Method Wizard	
🛒 Choose Methods 🔽 Ion Source	e Parameters 🛷 TOF MS - MS/MS
Ion Source Parameters	🕜 Help
Ion Source: Nan	ospray
Curtain Gas (CUR):	20
Ion Source Gas 1 (GS1):	5
Ion Source Gas 2 (GS2):	0
Ion Spray Voltage Floating (ISVF)): 2300
Interface Heater Temperature (IH	T): 150

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.

🤨 Method Wizard								
🐙 Choose Methods	👎 Ion Source Paran	neters 🛷 TOF MS - MS/MS						
TOF MS - MS	TOF MS - MS/MS							
Start TOF Mass (D	a):	400						
Stop TOF Mass (D	a):	1800						
TOF Accumulation	Time (s):	0.250015						
MS2 Start Mass (E)a):	100						
MS2 Stop Mass (D	a):	1800						
MS2 Accumulation	Time (s) (per precurs	or): 0.5						
Cycle Time (s):		0.8						
Mass Spec Acquis	ition Duration (min):	14.001						
Product of (Da)	m/z Collision En (V)	ergy Collision Energy Spread (V)						
729.3652	45	3						
ack	Next 🖒 🛛	/ Finish 🗱 Cancel						

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 μ L 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.

Autos	ampler Settings							
Autosar	pler Procedure						System Configuration	
Name	AS2 10uLloop 1uLi	nj Nanoflex Trap				Save	Eksigent AS-2	edit
	1 Valve		Injector Load			Valve Position Control		
	2 External Events		Wait for Gradient 1 Ready			Wait for Gradient 1 ready to start		
	3 External Events		Wait for Gradient 2 Ready			Wait for Gradient 2 ready to start		
	4 Needle Wash	50 uL	Port 1			Perform needle wash		
	5 Aspirate	19 uL	Reagent-1	Speed:	1 Height:	5 Pick-up Reagent with specified volume. Total	aspirate volume needs to be less	than syringe volume.
	6 v/vait	00:00:05				Pause for specified time		
	7 Aspirate	0 uL	Reagent-1	Speed:	1 Height:	5 Pick-up Reagent with specified volume. Total	aspirate volume needs to be less	than syringe volume.
	8 Aspirate	1 uL	Sample	Speed:	1 Height:	3 Pick-up Sample with specified volume. Total	aspirate volume needs to be less th	nan syringe volume.
	9 vVait	00:00:05				Pause for specified time		
1	0 Aspirate	0 uL	Sample	Speed:	1 Height:	3 Pick-up Sample with specified volume. Total	aspirate volume needs to be less th	nan syringe volume.
1	1 Aspirate	5 uL	Reagent-1	Speed:	1 Height:	5 Pick-up Reagent with specified volume. Total	l aspirate volume needs to be less	than syringe volume.
1	2 v/vait	00:00:05				Pause for specified time		
1	3 Aspirate	0 uL	Reagent-1	Speed:	1 Height:	5 Pick-up Reagent with specified volume. Total	aspirate volume needs to be less	than syringe volume.
1	4 External Events		Start Gradient 1			Start LC Gradient 1		
1	5 Valve		Injector Inject			Switch AS injector valve to Inject position (1-	2)	
1	6 External Events		Wait for Gradient 1 Inject			Wait for Gradient 1 injection complete		
1	7 Valve		Injector Load			Valve Position Control		
1	8 External Events		Start Gradient 2			Start Gradient 2		
1	9 Dispense	25 uL	Waste	Speed:	5 Height:	0 Dispense specified volume from syringe to W	laste	
1	0 Needle Wash	200 uL	Port 1			Perform needle wash		
1	1 END							
Test on a	Test on A1 Stop! Print ToPDF Audit OK Cancel							

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.

🖹 LC Method Settings 🛛 🔀								
Selected	Method							
Name	Name Load Pump 10 min Trap Wash Save Print							
Summary	Run Conditions	Gradient Profile	Gradient Table					
						Flow Mode		
	Time (min)	Qa (µL/min)	Event					
	1 0	2						
	2 10	2				Visocialic		
x »	3							
	4							
	5							
	6							
	7							
	8							
	9							
1	0							
1	1							
1	2							
1	3							
Delete	Delete View Audit Trail OK Cancel							

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.

🖹 LC Method Settings 🛛 🛛 🔀
Selected Method
Name Load Pump 10 min Trap Wash 🗸 Save Print
Summary Run Conditions Gradient Profile Gradient Table
Pre-Run
✓ Flush column for 0.1 minutes using 100 % initial flowrate conditions.
First, establish a column pressure of 3000 psi.
Sample Injection
None.
Standard: Sample valve opens prior to beginning Flow Profile and remains open. Metered: Inject
Rapid: Inject 12000 nL of sample at maximum flowrate, maintaining initial mixture conditions.
Delete View Audit Trail OK Cancel

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.

Name C	H2 45min 300nL_	_min nanoflex tra	ар	~	Save	Print
ummary]	Run Conditions	Gradient Profil	e Gradient Tal	ble		
	Time (min)	% A	%B	Event	Flow Mod	le
x » 1	0	95	5	AUX3 TTL Low	Conse	rved flow
2	0	95	5	AUX4 TTL High	OIndepe	endent flow
3	0.1	95	5	AUX3 TTL High	Profile Ed	itor
4	16	75	25		Total fl	owrate:
5	18	20	80		300	nL/min
6	24	20	80			
7	27	95	5			
8	45	95	5	AUX4 TTL Low		
9						
10						
11						
12						
13					-	

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.

Name	CH2 45MIN 300NL	PERMIN TRAP		~	Save Print
ummary	Run Conditions	Gradient Profile	Gradient Table		
	Time (min)	% A	% B	Event	Flow Mode
()) 1	1 0	95	5	Valve Inject	Conserved flow
2	2 1	95	5		O Independent flow
	3 16	75	25		Profile Editor
4	18	20	80		Total flowrate:
4	5 24	20	80		300 nL/min
(3 27	95	5		
7	7 45	95	5	Valve Load	
8	3				
9	9				
10	0				
11	1				
12	2				
13	3				•

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.

🖬 LC Method Settings 🛛 🛛 🔀
Selected Method
Name CH2 45min 300nLmin Nanoflex trap Save Print
Summary Run Conditions Gradient Profile Gradient Table
, Pre-Run
Flush column for 0.1 minutes using 100 % initial flowrate conditions.
First, establish a column pressure of 3000 psi.
Sample Injection
None.
Standard: Sample valve opens prior to beginning Flow Profile and remains open.
Metered: Inject nL of sample at 100 % initial flowrate conditions. Soo nL of sample at maximum flowrate, maintaining initial mixture conditions.
Post-Run
Flush column for 1 minutes using 100 % ending flowrate conditions.
Delete View Audit Trail OK Cancel

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.

Kethod Wizard						
Choose Methods 👎 Ion Source P	arameters 🛷 TOF MS - MS/MS					
Ion Source Parameters	🕜 Help					
Ion Source: Nanos	pray					
Curtain Gas (CUR):	20					
Ion Source Gas 1 (GS1):	5					
Ion Source Gas 2 (GS2):	0					
Ion Spray Voltage Floating (ISVF):	2300					
Interface Heater Temperature (IHT):	150					

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.

🔨 Method Wizard								
¥	😸 Choose Methods 🔁 Ion Source Parameters 🛷 TOF MS - MS/MS							
	TOF MS - MS/MS							
	s	tart TOF Mass (D	a):	-	400			
	s	top TOF Mass (D	a):	[1800]		
	Т	OF Accumulation	Time (s):		0.250015			
	м	IS2 Start Mass (E)a):	[100			
	м	IS2 Stop Mass (D	a):	[1800			
	м	IS2 Accumulation	Time (s) (per precursor):	0.5			
	с	ycle Time (s):			0.8			
	м	lass Spec Acquis	ition Durati	on (min):	44.002			
		Product of (Da)	m/z Co (V	ollision Energy)	Collision Energy Spread (V)			
		729.3652	45		3			
		·						
🔁 Back 🛛 Next 🖙 🚽 🗸 Finish 🛛 💥 Cancel								



- 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/ μ L.

- 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/ μ L concentration) into 50 μ L 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

- 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.

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

Table 1-3 Key Parameters



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.

Add Sample			
- Sample name - P <u>r</u> efix:	Sample	<u>S</u> ample number: N <u>u</u> mber of digits:	▼ 3
Data file Prefi <u>x</u> :	System Functional Test	Set n <u>a</u> me: Auto <u>I</u> ncrement:	
Sub Fol <u>d</u> er: New samples - <u>N</u> umber:	1		Browse
	OK	Cancel	Help

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

	Specify XIC I	Ranges		×
Γ	Center	Width	Compound	^
	503.2368	0.05	YSQQQLMETSHR	
	542.2645	0.05	GDFQFNISR	
	671.33789	0.05	VDEDQPFPAVPK	E
	714.84692	0.05	DWENPGVTQLNR	
	729.36517	0.05	APLDNDIGVSEATR	

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.

Add Sample			
⊢ Sample name - P <u>r</u> efix:	Sample	<u>S</u> ample number: N <u>u</u> mber of digits:	3
Data file Prefi <u>x</u> : Sub Fol <u>d</u> er:	System Performance Te	Set n <u>a</u> me: Auto Increment:	Browse
New samples- <u>N</u> umber:	1		
	OK	Cancel	Help

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 lons 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**.
 - 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.

•	Specify XIC Rang	jes		×			
	Center	Width	Compound	^			
	433.87915	0.05	ELNYGPHQWR				
	450.69595	0.05	FNDDFSR	1			
	503.2368	0.05	YSQQQLMETSHR				
	528.93408	0.05	RDWENPGVTQLNR	1			
	542.2645	0.05	GDFQFNISR				
	550.28015	0.05	IDPNAWVER				
	567.0551	0.05	DVSLLHKPTTQISDFHVATR				
	607.8588	0.05	ITDSLAWLQR				
	671.33789	0.05	VDEDQPFPAVPK	1			
	697.8694	0.05	LPSEFDLSAFLR	1			
	714.84692	0.05	DWENPGVTQLNR	1			
	729.36517	0.05	APLDNDIGVSEATR	1			
	871.9516	0.05	LSGQTIEVTSEYLFR	1			
	879.4339	0.05	VNWLGLGPQENYPDR	1			
				1			
				~			
			OK Cancel				

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: _____

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?	

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

Notes

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 μ L 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.

Autosa	mpler Settings							
Autosamp	ler Procedure						System Configuration	
Name	AS2 10uLloop 1uLinj	Nanoflex Trap				Save	Eksigent AS-2	edit
1	Valve		Injector Load			Valve Position Control		
2	External Events		Wait for Gradient 1 Ready			Wait for Gradient 1 ready to start		
3	External Events		Wait for Gradient 2 Ready			Wait for Gradient 2 ready to start		
4	Needle Wash	50 uL	Port 1			Perform needle wash		
5	Aspirate	19 uL	Reagent-1	Speed:	1 Height:	5 Pick-up Reagent with specified volume. Total a	aspirate volume needs to be less th	nan syringe volume.
6	vVait	00:00:05				Pause for specified time		
7	Aspirate	0 uL	Reagent-1	Speed:	1 Height:	5 Pick-up Reagent with specified volume. Total a	aspirate volume needs to be less th	an syringe volume.
8	Aspirate	1 uL	Sample	Speed:	1 Height:	3 Pick-up Sample with specified volume. Total as	spirate volume needs to be less tha	an syringe volume.
9	vVait	00:00:05				Pause for specified time		
10	Aspirate	0 uL	Sample	Speed:	1 Height:	3 Pick-up Sample with specified volume. Total as	spirate volume needs to be less tha	an syringe volume.
11	Aspirate	5 uL	Reagent-1	Speed:	1 Height:	5 Pick-up Reagent with specified volume. Total a	aspirate volume needs to be less th	an syringe volume.
12	vVait	00:00:05				Pause for specified time		
13	Aspirate	0 uL	Reagent-1	Speed:	1 Height:	5 Pick-up Reagent with specified volume. Total a	aspirate volume needs to be less th	an syringe volume.
14	External Events		Start Gradient 1			Start LC Gradient 1		
15	Valve		Injector Inject			Switch AS injector valve to Inject position (1-2))	
16	External Events		Wait for Gradient 1 Inject			Wait for Gradient 1 injection complete		
17	Valve		Injector Load			Valve Position Control		
18	External Events		Start Gradient 2			Start Gradient 2		
19	Dispense	25 uL	vVaste	Speed:	5 Height:	0 Dispense specified volume from syringe to Wa	ste	
20	Needle Wash	200 uL	Port 1			Perform needle wash		
21	END							
Test on A1	Stop	Print ToPDF	F Audit				ок	Cancel

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

Autosa	mpler Settings						X
Autosam	pler Procedure					System Configuration	
Name	AS2 10uLloop 1uLi	inj Ch2Valve Trap				Save Eksigent AS-2 edit	
				28			
X» 1	Valve		Injector Load			Valve Position Control	٦
:	2 External Events		Wait for Gradient 1 Ready			Wait for Gradient 1 ready to start	
:	B External Events		Wait for Gradient 2 Ready			Wait for Gradient 2 ready to start	
4	Needle Wash	50 uL	Port 1			Perform needle wash	
4	5 Aspirate	19 uL	Reagent-1	Speed:	1 Height:	5 Pick-up Reagent with specified volume. Total aspirate volume needs to be less than syringe volume	s.
	S v/vait	00:00:05				Pause for specified time	
1	Aspirate	0 uL	Reagent-1	Speed:	1 Height:	5 Pick-up Reagent with specified volume. Total aspirate volume needs to be less than syringe volume	£.
8	B Aspirate	1 uL	Sample	Speed:	1 Height:	3 Pick-up Sample with specified volume. Total aspirate volume needs to be less than syringe volume.	
9	9 v/vait	00:00:05				Pause for specified time	
10	Aspirate	0 uL	Sample	Speed:	1 Height:	3 Pick-up Sample with specified volume. Total aspirate volume needs to be less than syringe volume.	
11	Aspirate	5 uL	Reagent-1	Speed:	1 Height:	5 Pick-up Reagent with specified volume. Total aspirate volume needs to be less than syringe volume	£.
12	2 v/vait	00:00:05				Pause for specified time	
13	B Aspirate	0 uL	Reagent-1	Speed:	1 Height:	5 Pick-up Reagent with specified volume. Total aspirate volume needs to be less than syringe volume	s.
14	External Events		Start Gradient 1			Start LC Gradient 1	
15	5 Valve		Injector Inject			Switch AS injector valve to Inject position (1-2)	
16	External Events		Wait for Gradient 1 Inject			Wait for Gradient 1 injection complete	
17	Valve		Injector Load			Valve Position Control	
18	B External Events		Start Gradient 2			Start Gradient 2	
15	9 Dispense	25 uL	√√aste	Speed:	5 Height:	0 Dispense specified volume from syringe to Waste	
20	Needle Wash	200 uL	Port 1			Perform needle wash	
21	END						
							-
Test on A	1 Stop!	Print ToPDF	Audit			OK Cancel	

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

 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.

	LC Meth	od Settings				
	Selected M	ethod				
	Name [.oad Pump 2 min 1	Trap Wash		*	Save Print
	Summary	Run Conditions	Gradient Profile	Gradient Table	•	
						Elow Mode
		Time (min)	Qa (µL/min)		Event	
	X » 1	0	3.5			
	2	2	3.5			Isocralic
	3					
	4					
	5					
	6					
	7					
	8					
	9					
	10					
	11					
	12					
	13					▼
-						
l		/iew Audit Trail				OK Cancel

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.

E LC Method Settings
C Selected Method
Name Load Pump 2 min Trap Wash Save Print
Summary Run Conditions Gradient Profile Gradient Table
Pre-Run
✓ Flush column for 0.1 minutes using 100 % initial flowrate conditions.
First, establish a column pressure of 3000 psi.
Sample Injection
None.
Standard: Sample valve opens prior to beginning Flow Profile and remains open.
Metered: InjectnL of sample at% initial flowrate conditions.
Post-Run
Flush column for 1 minutes using 100 % ending flowrate conditions.

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.

Name C	:H2 15min 400nL_	_min nanoflex trap	<u></u>	~		Save Print
ımmary	Run Conditions	Gradient Profile	Gradient Table	•]		
	Time (min)	% A	% B	Event		Flow Mode
) 1	0	85	15	AUX3 TTL Low		 Conserved flow
2	0	85	15	AUX4 TTL High		O Independent flow
3	0.1	85	15	AUX3 TTL High		Profile Editor
4	5	60	40			Total flowrate:
5	6	20	80			400 nL/min
6	8	20	80			
7	9	95	5			
8	15	95	5	AUX4 TTL Low		
9						
10						
11						
12						
13					-	

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.

Name	CH2 15MIN 400NL	PERMIN TRAP		~	Sa	ve Print
ummary	Run Conditions	Gradient Profile	Gradient Table			
	Time (min)	% A	% B	Event		Flow Mode
() 1	0	85	15	Valve Inject		Conserved flow
2	5	60	40			Independent flow
3	6	20	80		ſ	Profile Editor
4	8	20	80			Total flowrate:
5	9	95	5			400 nL/min
6	15	95	5	Valve Load	- L	
7						
8						
9						
10						
11						
12						
13					-	



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

LC Method Settings
C Selected Method
Name CH2 15min 400nl-min nanoflex trap Save Print
Summary Run Conditions Gradient Profile Gradient Table
Pre-Fun Plush column for 0.1 minutes using 100 % initial flowrate conditions. First, establish a column pressure of 3000 psi.
Sample Injection
⊙ None.
Standard: Sample valve opens prior to beginning Flow Profile and remains open.
Metered: Inject 500 nL of sample at 100 % initial flowrate conditions. Rapid: Inject 500 nL of sample at maximum flowrate, maintaining initial mixture conditions.
Post-Run
Flush column for 1 minutes using 100 % ending flowrate conditions.
Delete View Audit Trail OK Cancel

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			

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

Table 2-2 Key Parameters (Continued)

** 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.

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

Table 2-3 MRM Transitions for Beta-Galactosidase

Add LC Information to the Acquisition Method

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

a Analyst - [Acquisition Method: D:\	Analyst Data\Projects\xw sop	test\Acquisition Methods\SOP test bgal MRM-041311-short MRM list]									
Eile Edit View Acquire Iools Explore Window Script Help											
12 🖻 🖬 🎒 🖪 🐇 🖿 🛍 🗅	📔 🖆 🖶 🎒 🗟 🕺 🛍 🛍 🕰 🕰 🗄 Acquire Mode 🔄 📄 🔁 Example 🖃 🗐 🕮 🗐 📰 📰 📰 🔛										
╡╡Ĩa & & & L LL I I I I I I I I I I I I I I											
Configure Configure Configuration Compound Optimization Compoun	Acquisition method Acquisition Method Acquisition Method Period 14.003 min Period 14.	Acquisition Method Properties Comment: Duration (min): 14.003 Sygchronization Mode: LC Sync Auto-Equilibration Auto-Equilibration Auto-Equilibration Original Configuration Instrument signature: 4000 Q TRAP Ion Source: Nanospray Device methods: Eksigent Gradient 1 Eksigent Gradient 2 Finite action Process									
Quantitate											

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.

a Analyst - [Acquisition Method: D: Analyst Data Projects xw sop test Acquisition Methods \SOP test bgal MRM-041311-short MRM list]										
Eile Edit View Acquire Tools Explor	re <u>W</u> indow <u>S</u> cript <u>H</u> elp									
) 🏠 🚅 🖬 🍜 🖪 🐇 🖿 🛍 🖾	🖹 🖆 🖬 🎒 🔃 🏠 🖹 🛍 🛍 🗅 🗠 ± 🛛 Acquire Mode 🔄 📄 🔂 Example 💽 🗍 💭 🔛									
∬≒≒t <mark>a & & & ● &</mark> @ &	L ¥ 🖬 & + 🛏 🌾 T 🔍									
E Configure	Acquisition method	Software Application Properties								
Security Configuration Hardware Configuration Key Report Template Editor Key Tune and Calibrate Compound Optimization Key Manual Tuning Acquire (1) Key Instrument Optimization Key Manual Tuning Key Acquire (1) Key Manual Conjecture Constraint of the con	Acquisition Method Mass Spec 14.003 min Period 14.003 min Belsigent AS2 Eksigent Gradient 1 Eksigent Gradient 2 Eksigent Loading Pump	Path: C:\Program Files\Eksigent NanoLC\settings\EKAS1 Filename: AS210uLloop1uLinj trap elute.ini								

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.

👌 Analyst - [Acquisition Method: D:\Analyst Data\Projects\xw sop test\Acquisition Methods\SOP test bgal MRM-041311-short MRM list]									
🛃 Eile Edit View Acquire Iools Explore Window Script Help									
🎦 🖆 🖨 🖧 👗 🛍 🛍 🕰 🕰 Ł Acquire Mode 💽 📋 🔂 Example 💽 🚽 🗰 🕅 🗖 🖽 📗 🗎									
╡┪┪╗┹┹┹●┹╓┹포፼&ヶ⋈╭╸┰╺╴									
Configure Acquisition method Software Application Properties									
🖉 🔎 Security Configuration	Acquisition Method	Path: C:\Program Files\Eksigent NanoLC\settings\method							
	⊡	Filename: CH2 15min 400nL.min nanofley tran ini							
Template Editor	+MRM								
((W) Tune and Calibrate	Eksigent Gradient 1								
Compound Optimization	Eksigent Gradient 2								
- AY Instrument Optimization	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~								
ાં કું Manual Tuning									
🗃 Acquire (1)									
- 🌾 IDA Method Wizard									
-)12 Build Acquisition Method									
🛛 🖳 🛱 Build Acquisition Batch									

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 μ L 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.

Autosampler Pro Name AS2 1 Valv 2 Exter 3 Exter 4 Neec 5 Aspi	ve erral Events die Wash 50 uL irrate 19 uL t 00:00:05	Injector Load Wait for Gradient 1 Ready Wait for Gradient 2 Ready Port 1 Reagent-1	Speed:		Valve Position Control Wat for Gradient 1 ready to start Wat for Gradient 2 ready to start Perform needle wash
Name AS2 4	10uLloop 1uLinj Nanoflex Trap ve ernal Events ernal Events die Wash 50 uL iirate 19 uL t 00:00:05	Injector Load Wait for Gradient 1 Ready Wait for Gradient 2 Ready Port 1 Reagent-1	Speed:		Valve Position Control Wat for Gradient 1 ready to start Wat for Gradient 2 ready to start Perform needle wash
1 Valv 2 Exter 3 Exter 4 Need 5 Aspi	ve smal Events smal Events die Wash 50 uL irrate 19 uL t 00:00:05	Injector Load Wait for Gradient 1 Ready Wait for Gradient 2 Ready Port 1 Reagent-1	Speed:		Valve Position Control Wait for Gradient 1 ready to start Wait for Gradient 2 ready to start Perform needle wash
2 Exter 3 Exter 4 Need 5 Aspi	ernal Events ernal Events die Wash 50 uL wirate 19 uL t 00:00:05	vVait for Gradient 1 Ready vVait for Gradient 2 Ready Port 1 Reagent-1	Speed:		Wait for Gradient 1 ready to start Wait for Gradient 2 ready to start Perform needle wash
3 Exter 4 Need 5 Aspi	ernal Events dle Wash 50 uL virate 19 uL t 00:00:05	vVait for Gradient 2 Ready Port 1 Reagent-1	Speed:		Wait for Gradient 2 ready to start Perform needle wash
4 Need 5 Aspi	dle Wash 50 uL virate 19 uL t 00:00:05	Port 1 Reagent-1	Speed:		Perform needle wash
5 Aspi	virate 19 uL t 00:00:05	Reagent-1	Speed:		
	t 00:00:05			1 Height:	5 Pick-up Reagent with specified volume. Total aspirate volume needs to be less than syringe volume
6 vVait					Pause for specified time
7 Aspi	oirate 0 uL	Reagent-1	Speed:	1 Height:	5 Pick-up Reagent with specified volume. Total aspirate volume needs to be less than syringe volume
8 Aspi	virate 1 uL	Sample	Speed:	1 Height:	3 Pick-up Sample with specified volume. Total aspirate volume needs to be less than syringe volume.
9 vVait	t 00:00:05				Pause for specified time
10 Aspi	oirate 0 uL	Sample	Speed:	1 Height:	3 Pick-up Sample with specified volume. Total aspirate volume needs to be less than syringe volume.
11 Aspi	nirate 5 uL	Reagent-1	Speed:	1 Height:	5 Pick-up Reagent with specified volume. Total aspirate volume needs to be less than syringe volume
12 vVait	t 00:00:05				Pause for specified time
13 Aspi	oirate 0 uL	Reagent-1	Speed:	1 Height:	5 Pick-up Reagent with specified volume. Total aspirate volume needs to be less than syringe volume
14 Exter	ernal Events	Start Gradient 1			Start LC Gradient 1
15 Valv	ve	Injector Inject			Switch AS injector valve to Inject position (1-2)
16 Exter	ernal Events	Wait for Gradient 1 Inject			Wait for Gradient 1 injection complete
17 Valv	ve	Injector Load			Valve Position Control
18 Exter	ernal Events	Start Gradient 2			Start Gradient 2
19 Dispe	bense 25 uL	rVaste	Speed:	5 Height:	0 Dispense specified volume from syringe to Waste
20 Need	dle Wash 200 uL	Port 1			Perform needle wash
21 END					

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

🖡 Autosampler Settings								
Autosam	pler Procedure						System Configuration	
Name	AS2 10uLloop 1uLin	ij Ch2∀alve Trap				Save	Eksigent AS-2 edit	
X »	Valve		Injector Load	ľ		Valve Position Control		
	2 External Events		Wait for Gradient 1 Ready	L.		Wait for Gradient 1 ready to start		
	External Events		Wait for Gradient 2 Ready			Wait for Gradient 2 ready to start		
	Needle Wash	50 uL	Port 1			Perform needle wash		
	Aspirate	19 uL	Reagent-1	Speed:	1 Height:	5 Pick-up Reagent with specified volume. Total	aspirate volume needs to be less than syringe volume.	
	ò vVait	00:00:05				Pause for specified time		
3	Aspirate	0 uL	Reagent-1	Speed:	1 Height:	5 Pick-up Reagent with specified volume. Total	aspirate volume needs to be less than syringe volume.	
1	Aspirate	1 uL	Sample	Speed:	1 Height:	3 Pick-up Sample with specified volume. Total a	spirate volume needs to be less than syringe volume.	
	9 v/vait	00:00:05				Pause for specified time		
1	Aspirate	0 uL	Sample	Speed:	1 Height:	3 Pick-up Sample with specified volume. Total a	spirate volume needs to be less than syringe volume.	
1	Aspirate	5 uL	Reagent-1	Speed:	1 Height:	5 Pick-up Reagent with specified volume. Total	aspirate volume needs to be less than syringe volume.	
1:	2 vVait	00:00:05				Pause for specified time		
1:	B Aspirate	0 uL	Reagent-1	Speed:	1 Height:	5 Pick-up Reagent with specified volume. Total	aspirate volume needs to be less than syringe volume.	
1	External Events		Start Gradient 1			Start LC Gradient 1		
1	5 Valve		Injector Inject			Switch AS injector valve to Inject position (1-2	0	
1	External Events		Wait for Gradient 1 Inject			Wait for Gradient 1 injection complete		
13	Valve		Injector Load			Valve Position Control		
1	B External Events		Start Gradient 2			Start Gradient 2		
1	Dispense	25 uL	Waste	Speed:	5 Height:	0 Dispense specified volume from syringe to Wa	iste	
2	Needle Wash	200 uL	Port 1			Perform needle wash		
2	END							
Test on A	1 Stop!	Print ToPDI	F Audit				OK Cancel	

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.

	LC Meth	od Settings				X
	Selected M	lethod				
	Name [_oad Pump 10 mir	n Trap Wash		~	Save Print
	Summary	Run Conditions	Gradient Profile	Gradient Table		
						Elow Mode
		Time (min)	Qa (µL/min)	Event		
	1	0	2			
	2	10	2			• Isocratic
	х » з					
	4					
	5					
	6					
	7					
	8					
	9					
	10					
	11					
	12					
	13					•
0	Delete	View Audit Trail				OK Cancel

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.

🖹 LC Method Settings 🛛 🔀
Selected Method
Name Load Pump 10 min Trap Wash Save Print
Summary Run Conditions Gradient Profile Gradient Table
~ Pre-Run
✓ Flush column for 0.1 minutes using 100 % initial flowrate conditions.
First, establish a column pressure of 3000 psi.
Sample Injection
None.
Standard: Sample valve opens prior to beginning Flow Profile and remains open.
Metered: Inject nL of sample at 100 % initial flowrate conditions.
Rapia: injectinc of sample at maximum flow/rate, maintaining initial mixture conditions.
Post-Run
Flush column for 1 minutes using 100 % ending flowrate conditions.
View Audit Trail

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.

elected M	ethod :H2 45min 300nL_	min nanoflex trap		~		iave Print
Summary	Run Conditions	Gradient Profile	Gradient Table			
						- Flow Mode
	Time (min)	% A	%В	Event	-	Conserved flow
x » 1	0	95	5	AUX3 TTL Low		
2	0	95	5	AUX4 TTL High		O Independent now
3	0.1	95	5	AUX3 TTL High		Profile Editor
4	16	75	25			Total flowrate:
5	18	20	80			300 nL/min
6	24	20	80			
7	27	95	5			
8	45	95	5	AUX4 TTL Low		
9						
10						
11						
12						
13					-	
- 1	1	1			-	

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.

Name	CH2 45MIN 300NLPERMIN TRAP Save Print					ave Print
ummary	Run Conditions	Gradient Profile	Gradient Table			
0.000.000	Time (min)	% A	% B	Event		Flow Mode
(» ·	1 0	95	5	Valve Inject		Conserved flow
:	2 1	95	5			O Independent flow
;	3 16	75	25			Profile Editor
	4 18	20	80			Total flowrate: 300 nL/min
4	5 24	20	80			
(5 27	95	5			
7	7 45	95	5	Valve Load		
(в					
9	9					
10	D					
1	1					
12	2					
13	3				-	

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

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	1
Declustering Potential (DP)	70

Table 2-5 Key Parameters (Continued)

** 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 T	ransitions 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

 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.

Analyst - [Acquisition Method: D:\Analyst Data\Projects\xw sop	test\Acquisition Methods\SOP test bgal MRM-041311-short MRM list]					
Eile Edit View Acquire Tools Explore Window Script Help						
📔 🗃 🖬 🎒 🔃 🏦 😫 🛍 🕰 🕰 🗜 Acquire Mode 💿 🔢 🖆 🔂 Example 💿 🗐 💷 💽						
╡┪╠┺┺┺●┺╙┺ॾछ┺╯╴⋈╭┰ヾ						
Configure Acquisition method Acquisition A	Software Application Properties Path: C:\Program Files\Eksigent NanoLC\settings\method Filename: CH2 45min 300nLmin Nanoflex trap.ini					

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.

a Analyst - [Acquisition Method: D:\Analyst Data\Projects\xw sop t	test\Acquisition Methods\SOP test bgal MRM-041311-short MRM list]						
Eile Edit View Acquire Tools Explore Window Script Help							
🖹 🖆 🖬 🎒 🖪 👗 🐘 🛍 🕰 🕰 生 🛛 Acquire Mode	🔽 📄 🔁 Example 💽 🚽 🛤 🗖 🗖						
<u> </u> ** ** to & & & & ● & U & ¥ 🖼 & → > ★ ▼ T 🛠							
Configure Configure Configuration Configuration	Software Application Properties Path: C:\Program Files\Eksigent NanoLC\settings\method Filename: 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 $pmol/\mu L$.

- 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/ μ L concentration) into 50 μ L 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

- 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 μ L 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	150°C		
Compound			
Declustering Potential	70		









- 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.

Add Sample			
- Sample name- P <u>r</u> efix:	Sample	<u>S</u> ample number: N <u>u</u> mber of digits:	▼ 3
Data file Prefi <u>x</u> : Sub Fol <u>d</u> er:	System Functional Test	Set n <u>a</u> me: Auto <u>I</u> ncrement:	F F Browse
New samples- <u>N</u> umber:	1		
	OK	Cancel	Help

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.

Add Sample			×
Sample name-	Sample	<u>S</u> ample number: Number of digits:	3
Data file Prefi <u>x</u> : Sub Fol <u>d</u> er:	System Performance Te	Set n <u>a</u> me: Auto <u>I</u> ncrement:	Browse
New samples <u>N</u> umber:	1		
	OK	Cancel	Help

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 lons**.

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

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

Table 2-8	MRM	Transitions	for	Beta-Galact	osidase

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)

- 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.





- 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:

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	
		1		Specif	ication Passed?	

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

Notes



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 μ L 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.

Name	AS2 10uLloop 1uLinj tra	pelute			✓ [S	ave	Eksigent AS-2
1	External Events 🗸		Wait for Loading Pump Ready			vVai	t for Loading Pump ready to start
2	External Events		Wait for Gradient 2 Ready			Wai	it for Gradient 2 ready to start
3	Needle Wash	150 uL	Port 1			Per	form needle wash
4	Valve		Injector Load			Sw	itch AS injector valve to Load position (1-6)
5	Aspirate	16 uL	Reagent-1	Speed:	2 Height:	5 Pick	-up Reagent with specified volume. Total aspirate v
6	rVait	00:00:10				Pau	ise for specified time
7	Aspirate	0 uL	Reagent-1	Speed:	2 Height:	5 Pick	-up Reagent with specified volume. Total aspirate v
8	Aspirate	1 uL	Sample	Speed:	1 Height:	2 Asp	pirate specified volume
9	rVait	00:00:10				Pau	ise for specified time
10	Aspirate	0 uL	Sample	Speed:	1 Height:	2 Asp	pirate specified volume
11	Aspirate	3 uL	Reagent-1	Speed:	1 Height:	5 Pick	-up Reagent with specified volume. Total aspirate v
12	rVait	00:00:10				Pau	ise for specified time
13	Aspirate	0 uL	Reagent-1	Speed:	1 Height:	5 Pick	-up Reagent with specified volume. Total aspirate v
14	External Events		Start Loading Pump			Sta	rt Loading Pump
15	Valve		Injector Inject			Sw	itch AS injector valve to Inject position (1-2)
16	External Events		Wait for Loading Pump Inject			Wai	it for Loading Pump injection complete
17	Valve		Injector Load			Sw	itch AS injector valve to Load position (1-6)
18	External Events		Start Gradient 2			Sta	rt Gradient 2
19	Dispense	20 uL	Waste	Speed:	5 Height:	0 Disp	pense specified volume from syringe to Waste
20	Needle Wash	250 uL	Port 1			Per	form needle wash
21	END						

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

Autosam	npler Settings Ier Procedure					System Configuration	
Name	AS2 10uLloop 1uLinj (Ch2Valve Trap				Save Eksigent AS-2 edit	
X » 1	Valve		Injector Load	ľ		Valve Position Control	
2	External Events		Wait for Gradient 1 Ready			Wait for Gradient 1 ready to start	
3	External Events		Wait for Gradient 2 Ready			Wait for Gradient 2 ready to start	
4	Needle Wash	50 uL	Port 1			Perform needle wash	
5	Aspirate	19 uL	Reagent-1	Speed:	1 Height:	5 Pick-up Reagent with specified volume. Total aspirate volume needs to be less than syringe volum	ne.
6	vVait	00:00:05				Pause for specified time	
7	Aspirate	0 uL	Reagent-1	Speed:	1 Height:	5 Pick-up Reagent with specified volume. Total aspirate volume needs to be less than syringe volum	ne.
8	Aspirate	1 uL	Sample	Speed:	1 Height:	3 Pick-up Sample with specified volume. Total aspirate volume needs to be less than syringe volume	ie.
9	vNait	00:00:05				Pause for specified time	
10	Aspirate	0 uL	Sample	Speed:	1 Height:	3 Pick-up Sample with specified volume. Total aspirate volume needs to be less than syringe volume	ie.
11	Aspirate	5 uL	Reagent-1	Speed:	1 Height:	5 Pick-up Reagent with specified volume. Total aspirate volume needs to be less than syringe volum	ne.
12	vVait	00:00:05				Pause for specified time	
13	Aspirate	0 uL	Reagent-1	Speed:	1 Height:	5 Pick-up Reagent with specified volume. Total aspirate volume needs to be less than syringe volum	ne.
14	External Events		Start Gradient 1			Start LC Gradient 1	
15	Valve		Injector Inject			Switch AS injector valve to Inject position (1-2)	
16	External Events		Wait for Gradient 1 Inject			Wait for Gradient 1 injection complete	
17	Valve		Injector Load			Valve Position Control	
18	External Events		Start Gradient 2			Start Gradient 2	
19	Dispense	25 uL	v/vaste	Speed:	5 Height:	0 Dispense specified volume from syringe to Waste	
20	Needle Wash	200 uL	Port 1			Perform needle wash	
21	END						
Test on A1	Stop!	Print ToPDI	F Audit			OK Cance	;el

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.

E L	C Me	eth d M	od Settings ethod				_	
	Name		.oad Pump 2 min	I rap Wash		*		Save Print
s	umma	ry	Run Conditions	Gradient Profile	Gradient Table	1		
Г			Time (min)	Qa (µL/min)		Event		Flow Mode
0	x »	1	0	3.5				
		2	2	3.5			_	• Isocratic
		3					_	
		4					_	
		5					_	
		6					_	
_		7					_	
-		8					- 1	
-		9					- 1	
-		10					-	
-		11					-	
-		13					-	
L		13						
De	elete		/iew Audit Trail					OK Cancel

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.

E LC Method Settings
Selected Method
Name Load Pump 2 min Trap Wash Save Print
Summary Run Conditions Gradient Profile Gradient Table
Pre-Run
Flush column for 0.1 minutes using 100 % initial flowrate conditions.
First, establish a column pressure of 3000 psi.
Sample injection
None. Standard: Sample valve opens prior to beginning Flow Profile and remains open.
Metered: Inject nL of sample at 100 % initial flowrate conditions.
ORapid: InjectnL of sample at maximum flowrate, maintaining initial mixture conditions.
Post-Kun
Delete View Audit Trail OK Cancel

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.

				_	
un Conditions	Gradient Profile	Gradient Tak	ile		
Time (min)	%A	%B	Event		Flow Mode
0	85	15	AUX3 TTL Low		Conserved flow
0	85	15	AUX4 TTL High		O Independent flow
0.1	85	15	AUX3 TTL High		Profile Editor
5	60	40			Total flowrate:
6	20	80			400 nL/min
8	20	80			
9	95	5			
15	95	5	AUX4 TTL Low		
				-	
	Time (min) Image: Constraint of the second sec	Conditions Gradient Profile Time (min) % A 0 85 0 85 0 85 0.1 85 5 60 6 20 9 95 15 95 15 95 20 20	Image: Constructions Gradient Profile Gradient Take Time (min) % A % B 0 85 15 0 85 15 0 85 15 0 85 15 0.1 85 15 5 60 40 6 20 80 9 95 5 15 95 5 15 95 5 15 95 5	Image: Construction of the second s	Image Gradient Profile Gradient Table Time (min) % A % B Event 0 85 15 AUX3 TTL Low 0 85 15 AUX3 TTL High 0.1 85 15 AUX3 TTL High 5 60 40 40 6 20 80 40 9 95 5 4000 15 95 5 4000 9 95 5 4000 15 95 5 4000 9 95 5 4000 9 95 5 4000 9 95 5 4000 9 95 5 4000 9 95 5 4000 9 9 9 9 9 9 9 9 9 9 9 9 9 9 9 9 9 9

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.

Name	CH2 15MIN 400NL	PERMIN TRAP		~		Save Print
ummary]	Run Conditions	Gradient Profile	Gradient Table			
	Time (min)	% A	% B	Event		Flow Mode
x » 1	0	85	15	Valve Inject		Conserved flow
2	5	60	40			O Independent flow
3	6	20	80			Profile Editor
4	8	20	80			Total flowrate:
5	9	95	5			400 nL/min
6	15	95	5	Valve Load		
7						
8						
9						
10						
11						
12						
13					-	

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.

🗟 LC Method Settings 🛛 🛛 🔀
Selected Method
Name CH2 15min 400nl-min nanoflex trap Save Print
Summary Run Conditions Gradient Profile Gradient Table
Pre-Run Pre-Run Prush column for 0.1 minutes using 100 % initial flowrate conditions.
Sample Injection None. Standard: Sample valve opens prior to beginning Flow Profile and remains open. Metered: Inject Standard: Inject Standard: Sample at 100 % initial flowrate conditions. Rapid: Inject Standard: Inject
Post-Run Flush column for 1 minutes using 100 % ending flowrate conditions.
Delete View Audit Trail OK Cancel

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

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

Table 3-2 Key Parameters (Continued)

** 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.

a Analyst - [Acquisition Method: D:\Ana	alyst Data\Projects\xw sop 1	test\Acquisition Methods\SOP test bgal MRM-041311-short MRM list]						
Eile Edit View Acquire Tools Explore Wi	⊻indow <u>S</u> cript <u>H</u> elp							
🖹 😂 🖶 🎒 🔃 🗘 ڬ 🗄 🛍 🕰 🕰 🛓 Acquire Mode 💽 📄 🔂 Example 💽 🚽 💷 🐨 🗔 🗔 🗔 🗔 👘								
≝ ≝ ta & & & L L L E E E t + ≍ ☆ T ℃								
Configure Configuration Hardware Configuration Hardware Configuration Report Template Editor Acquire (1) Compound Optimization A Instrument Optimization Build Acquisition Method Build Acquisition Method Report Explore Acquire (1) Compound	cquisition method Acquisition Method Mass Spec 14.003 min Period 14.003 min Heisgent A52 Eksigent Gradient 1 Eksigent Gradient 2 Eksigent Loading Pump	Acquisition Method Properties Quration (min): Quration (min): 14.003 Synchronization Mode: LC Sync Auto-Equilibration Auto-Equilibration Auto-Equilibration Auto-Equilibration Auto-Equilibration Original Configuration Driginal Configuration Instrument signature: 4000 Q TRAP Instrument signature: 4000 Q TRAP Ion Source: Nanospray Plusient Landon Pure						

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.

analyst - [Acquisition Method: D:\Analyst Data\Projects\xw sop test\Acquisition Methods\SOP test bgal MRM-041311-short MRM list]								
🛃 Eile Edit View Acquire Iools Explore Window Script Help								
🎽 😂 🖬 🚳 💫 🕰 🖢 🛍 🕰 🕰 🖌 Acquire Mode 💿 📄 🔂 Example 💿 🚽 🔤 🗮 🛛 🗃 🖿 🖿 🖿								
₩₩1033401012201401000000000000000000000000								
Configure Configure Configuration Configuration Configuration Compound Optimization Compound Optimization	nethod Software Application Properties sition Method Path: c:\Program Files\Eksigent NanoLC\settings\EKAS1 period 14.003 min Filename: AS2 10uLloop 1uLinj trap elute.ini							

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.

a Analyst - [Acquisition Method: D:	\Analyst Data\Projects\xw sop t	testMcquisition Methods\SOP test bgal MRM-041311-short MRM list]							
🛃 Eile Edit View Acquire Iools Explore Window Script Help									
🖹 🖆 🖶 🎒 🖪 🕺 🖏 🛍 🕰 🕰 🛓 Acquire Mode 💽 📑 🔁 Example 💽 🚽 💷 🐨 🖿 🖽 🔐									
∬≒≒toXLA●LU2	╡┪╔┰┱┎┎┰┺ѽ┰╴╡┆╷								
Configure Security Configuration Hardware Configuration Report Template Editor (III) Tune and Calibrate Compound Optimization AV Instrument Optimization	Acquisition method	Software Application Properties Path: C:\Program Files\Eksigent NanoLC\settings\method Filename: CH2 15min 400nL-min nanoflex trap.ini							
السريخ Manual Tuning المحصينة (1)									

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 μ L 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.

🖡 Autosampler Settings								
Autosampl	ler Procedure						System Configuration	
Name	AS2 10uLloop 1uL	inj trap elute			v [5	ave	Eksigent AS-2	edit
1	External Events	~	Wait for Loading Pump Ready			Wa	it for Loading Pump ready to start	
2	External Events		Wait for Gradient 2 Ready			٧Və	it for Gradient 2 ready to start	
3	Needle Wash	150 uL	Port 1			Per	form needle wash	
4	Valve		Injector Load			Sw	ritch AS injector valve to Load posit	ion (1-6)
5	Aspirate	16 uL	Reagent-1	Speed:	2 Height:	5 Pic	k-up Reagent with specified volume	e. Total aspirate volu
6	v∿ait	00:00:10				Pau	use for specified time	
7	Aspirate	0 uL	Reagent-1	Speed:	2 Height:	5 Pic	k-up Reagent with specified volume	e. Total aspirate volu
8	Aspirate	1 uL	Sample	Speed:	1 Height:	2 As	pirate specified volume	
9	v∕vait	00:00:10				Pau	use for specified time	
10	Aspirate	0 uL	Sample	Speed:	1 Height:	2 As	pirate specified volume	
11	Aspirate	3 uL	Reagent-1	Speed:	1 Height:	5 Pic	k-up Reagent with specified volume	e. Total aspirate volu
12	r∕Vait	00:00:10				Pau	use for specified time	
13	Aspirate	0 uL	Reagent-1	Speed:	1 Height:	5 Pic	k-up Reagent with specified volume	e. Total aspirate volu
14	External Events		Start Loading Pump			Sta	rt Loading Pump	
15	Valve		Injector Inject			SW	ritch AS injector valve to Inject posit	ion (1-2)
16	External Events		Wait for Loading Pump Inject			vVə	it for Loading Pump injection comple	te
17	Valve		Injector Load			SW	ritch AS injector valve to Load posit	ion (1-6)
18	External Events		Start Gradient 2			Sta	rt Gradient 2	
19	Dispense	20 uL	Waste	Speed:	5 Height:	0 Dis	pense specified volume from syring	ge to Waste
20	Needle Wash	250 uL	Port 1			Per	form needle wash	
21	END							
Test on A1	Stop!	Print ToPDF	Audit				ОК	Cancel

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

Autosa	mpler Settings						
Autosamp	ler Procedure						System Configuration
Name	AS2 10uLloop 1uLinj (Ch2Valve Trap				Save	Eksigent AS-2 edit
X » 1	Valve		Injector Load	ſ		Valve Position Control	
2	External Events		Wait for Gradient 1 Ready	ι.		Wait for Gradient 1 ready to start	
3	External Events		Wait for Gradient 2 Ready			Wait for Gradient 2 ready to start	
4	Needle Wash	50 uL	Port 1			Perform needle wash	
5	Aspirate	19 uL	Reagent-1	Speed:	1 Height:	5 Pick-up Reagent with specified volume. Total	aspirate volume needs to be less than syringe volume.
6	vVait	00:00:05				Pause for specified time	
7	Aspirate	0 uL	Reagent-1	Speed:	1 Height:	5 Pick-up Reagent with specified volume. Total	aspirate volume needs to be less than syringe volume.
8	Aspirate	1 uL	Sample	Speed:	1 Height:	3 Pick-up Sample with specified volume. Total a	aspirate volume needs to be less than syringe volume.
9	v∕Vait	00:00:05				Pause for specified time	
10	Aspirate	0 uL	Sample	Speed:	1 Height:	3 Pick-up Sample with specified volume. Total a	aspirate volume needs to be less than syringe volume.
11	Aspirate	5 uL	Reagent-1	Speed:	1 Height:	5 Pick-up Reagent with specified volume. Total	aspirate volume needs to be less than syringe volume.
12	vVait	00:00:05				Pause for specified time	
13	Aspirate	0 uL	Reagent-1	Speed:	1 Height:	5 Pick-up Reagent with specified volume. Total	aspirate volume needs to be less than syringe volume.
14	External Events		Start Gradient 1			Start LC Gradient 1	
15	Valve		Injector Inject			Switch AS injector valve to Inject position (1-2	2)
16	External Events		Wait for Gradient 1 Inject			Wait for Gradient 1 injection complete	
17	Valve		Injector Load			Valve Position Control	
18	External Events		Start Gradient 2			Start Gradient 2	
19	Dispense	25 uL	Waste	Speed:	5 Height:	0 Dispense specified volume from syringe to We	aste
20	Needle Wash	200 uL	Port 1			Perform needle wash	
21	END						
Test on A1	Stop!	Print ToPDI	F Audit				OK Cancel

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.

LC Meth	od Settings				×
Selected M	lethod				
Name [Load Pump 10 min	i Trap Wash		*	Save Print
Summary	Run Conditions	Gradient Profile	Gradient Table		
					Flow Mode
	Time (min)	Qa (µL/min)	Event		
1	0	2			
2	10	2			
<u>х</u> » з					
4					
5					
6					
7				_	
8					
9					
10					
11					
12					
13					•
	View Audit Trail				OK Cancel

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.

E LC Method Settings
Selected Method
Name Load Pump 10 min Trap Wash Save Print
Summary Run Conditions Gradient Profile Gradient Table
Pre-Run ✓ Flush column for 0.1 minutes using 100 % initial flowrate conditions. First, establish a column pressure of 3000 psi.
Sample Injection
O None.
Standard: Sample valve opens prior to beginning Flow Profile and remains open. Metered: Inject
Rapid: Inject 12000 nL of sample at maximum flowrate, maintaining initial mixture conditions.
Post-Run
Flush column for 1 minutes using 100 % ending flowrate conditions.
Delete View Audit Trail OK Cancel

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.

Selected M	ethod	min nonoflou tran				
	.HZ 40ININ 300NL	inin nanonex (rap		*		Save Print
Summary	Run Conditions	Gradient Profile	Gradient Table]		
	Time (min)	% A	%В	Event		Flow Mode
x » 1	0	95	5	AUX3 TTL Low		Conserved flow
2	0	95	5	AUX4 TTL High		
3	0.1	95	5	AUX3 TTL High		Profile Editor
4	16	75	25			Total flowrate:
5	18	20	80			300 nL/min
6	24	20	80			
7	27	95	5			
8	45	95	5	AUX4 TTL Low		
9						
10						
11						
12						
13					-	

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.

Name	CH2 45MIN 300N	LPERMIN TRAP		~	S	ave Print
ummary	Run Conditions	Gradient Profile	Gradient Table			
0.000.000	Time (min)	% A	% B	Event		Flow Mode
(» ·	1 0	95	5	Valve Inject		Conserved flow
:	2 1	95	5			O Independent flow
;	3 16	75	25			Profile Editor
	4 18	20	80			Total flowrate:
4	5 24	20	80			300 nL/min
(5 27	95	5			
7	7 45	95	5	Valve Load		
(в					
9	9					
10	D					
1	1					
12	2					
13	3				-	

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.



 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**		

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.

a Analyst - [Acquisition Method: D:\Analyst Data\Projects\xw sop test\Acquisition Methods\SOP test bgal MRM-041311-short MRM list]								
Eile Edit View Acquire Iools Explore Window Script Help								
🖹 🖆 🖶 🚭 🖪 🖄 🖺 🛍 🗅 🗠 🛨 🗛 Cquire Mode 💽 📑 🔂 Example 💽 🚽 🚚 🛪 🛅 🗖 🖽 🗃								
▝▝▝▌▖▙▙▟▋▋▆▆▙ᡔ≍᠅▖								
Configure Configuration Hardware Configuration Hardware Configuration Report Template Editor Tune and Calibrate Compound Optimization A' Instrument Optimization If Manual Tuning Acquire (1) Y: IDA Mathed Wireard	Acquisition method	Software Application Properties Path: C:\Program Files\Eksigent NanoLC\settings\EKAS1 Filename: AS2 10uLloop 1uLinj trap elute.ini						

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.

a Analyst - [Acquisition Method: D:\Analyst Data\Projects\xw sop test\Acquisition Methods\SOP test bgal MRM-041311-short MRM list]							
Eile Edit View Acquire Iools Explore Window Script Help							
🖀 🖨 🖶 🚳 🔃 🕰 🛓 🛍 🋍 🕰 🕰 🛓 Acquire Mode 💽 💼 🔂 Example 💽 🖷 🖛 🗶							
║╡╡ ╔╶╧╘ ┻╺╝╝	L 🛛 🖬 🕹 🕂 🛏 🌾 🔳 🛠						
	Acquisition method	Software Applicatic Path: Filename:	ion Properties C:\Program Files\Eksigent NanoLC\settings\method CH2 45min 300nLmin Nanoflex trap.ini				

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 $pmol/\mu L$.

- 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/ μ L concentration) into 50 μ L 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

- 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/ μ L.
- 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 μ L 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/ μ L.
 - 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	150°C				
Compound					
Declustering Potential	70				






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.

Add Sample			
⊢ Sample name - P <u>r</u> efix:	Sample	<u>S</u> ample number: N <u>u</u> mber of digits:	▼ 3
Data file Prefi <u>x</u> : Sub Fol <u>d</u> er:	System Functional Test	Set n <u>a</u> me: Auto <u>I</u> ncrement:	Br <u>o</u> wse
New samples - <u>N</u> umber:	1		
	OK	Cancel	Help

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.

Add Sample			×
⊢ Sample name - P <u>r</u> efix:	Sample	<u>S</u> ample number: N <u>u</u> mber of digits:	3
Data file Prefi <u>x</u> : Sub Fol <u>d</u> er:	System Performance Te	Set n <u>a</u> me: Auto <u>I</u> ncrement:	☐ ☐ Br <u>o</u> wse
New samples- <u>N</u> umber:	1		
	OK	Cancel	Help

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 lons**.

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

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

Table 3-8 MRM Transitions for Beta-Galactosidase

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)

- 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: _____

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	
			· · · · · · · · · · · · · · · · · · ·	Specifi	cation Passed?	

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

Notes





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 [Glu¹]-Fibrinopeptide B Dilution



Note: Prepare the solution just before calibrating the system.

- Add 1200 μL of Standard Diluent (0.1% formic acid, 10% acetonitrile) to the glass amber vial containing 0.1 mg [Glu¹]-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 μ L of a 5 pmol/ μ L 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 μ L of a 500 fmol/ μ L solution.

- 8. Aliquot 50 μ L of the 500 fmol/ μ L solution into a clean 500 μ L 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.

 Store the stock solution aliquots (50 pmol/µL solutions) and the diluted stock solutions (500 fmol/µL solution) of [Glu¹]-Fibrinopeptide B in the freezer at -20°C.

Edit the Calibration Reference Table for [Glu¹]-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 [Glu¹]-Fibrinopeptide B in the **Name** list.
- 5. Add the masses in Figure A-1 to the Reference lons for TOF MS Calibration table (left side of window).
- 6. Click OK.

Name: Glu-fibrinopeptide B New Copy Delete Positive C Negative Calibration Valve Position: A												
Reference Ions for TOF MS Calibration: (Product of 785.84210 Da)												
	Use	Compound Name	Precursor m/z (Da)	Use for MS/MS	CE for MS/MS	DP for MS/MS	Retention Time (min)		Use	Fragment Name	Fragment m/z (Da)	^
1		Diisooctyl phthalate,	391.28429		10.000	100.000	0.00	1			72.08080	
2	N	y4	480.25650	Γ	42.000	100.000	0.00	2			120.08080	
3	<u> </u>	y6	684.34640		42.000	100.000	0.00	3			159.07640	
4		Glu-fibrinopeptide	785.84210	v	42.000	100.000	0.00	4	N		175.11900	
5		Y7	813.38900		42.000	100.000	0.00	5			187.07130	
6	N	Y8	942.43160		42.000	100.000	0.00	6			246.15610	
7	<u> </u>	Y9	1056.47450		42.000	100.000	0.00	7			286.13970	
8	<u> </u>	Y10	1171.50140		42.000	100.000	0.00	8			333.18810	
9	<u> </u>	Y11	1285.54440		42.000	100.000	0.00	9			400.18270	
10								10	<u> </u>		480.25650	
11								11			515.20960	
12								12			627.32490	
13								13			629.25250	
Ital Image: Sector Se												

Figure A-1 [Glu¹]-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 Para	ameters
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 Para	ameters
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 [Glu¹]-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	5		
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		

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		

Table A-2 TOF MS Method Parameters (Continued)

- 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 [Glu¹]-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.

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 Para	ameters
Collision energy (CE) (V)	45
Resolution Parameters	
Q1 resolution	Unit

Table A-3 TOF MS Method Parameters