NanoLC™ Systems

For QTRAP® Systems

System Integration Test and Data Log

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Introduction 1

This guide describes the steps for preparing for and performing an LC-MS/MS system integration test for a NanoLC System combined with the SCIEX 4000, 4500, 5500/5500+, or 6500 QTRAP® mass spectrometer. The system might include the cHiPLC® System or an external column and one of the following NanoLC Systems:

- NanoLC™ 400 System
- NanoLC Ultra® System
- NanoLC™ System
- Tempo™ System

Note: Read the safety instructions in the *Operator Guide* prior to running the system integration test.

Note: For information about assembly and operation of the NanoSpray® or OptiFlow™ Turbo V Ion Source, refer to the *Ion Source Operator Guide*. For information about assembly and operation of the Digital PicoView® Nanospray Ion Source, refer to the *Digital PicoView Nanospray Source Hardware Manual*.

Note: Optimize the ion source before beginning this test.

About the Test

The test in this document determines the performance level of the LC-MS system. This test can be used as a measure of the system performance. The results from this test can provide a baseline measurement of system performance. The test can be performed regularly and used as a system quality control test in the future. Record the test results in the System Integration Test Data Log and Signoff.

Perform this test when the mass spectrometer is known to be operating well and meeting performance specifications. Refer to the *Ion Source Tests, Specifications, and Data Log* document.

If the LC system has been idle for two weeks or more, re-initialize transducers, then verify the flow rate and, if necessary, calibrate.

Before starting the test, make sure that the system is plumbed in a trap-and-elute configuration (with either the cHiPLC® System or an external trap and column). Refer to Plumbing Diagrams.

Note: The test in this document is written with Gradient 1 as the low-flow channel. If this is not true for the system being tested (for example, a 2D system), then make the appropriate changes throughout the test. Refer to Table 1-1.

Table 1-1 Channel Flow Rate

Pump	Channel	Flow Rate
Gradient 1	Low flow	300 nL/min
Loading pump	High flow	5 µL/min

Time Required

Approximate time required:

1. Create the methods and batch: 45 minutes
2. Prepare the system for testing: 60 minutes (or 3 to 4 hours if the column needs conditioning)
3. Perform the test: 3 hours

Required Materials

Note: If you are not using pre-mixed solvents, make sure that you use LC-MS-grade water, acetonitrile, and formic acid.

Note: We do not recommend using Milli-Q water because it is not of suitable quality for use in LC-MS systems.

Table 1-2 Required Materials for All Configurations

	Vendor	Part Number
LC-MS Peptide Calibration kit	SCIEX	4465867
Beta-galactosidase digest	SCIEX	4465938
Burdick and Jackson acetonitrile with 0.1% formic acid (or equivalent)	VWR	BJLC441-1.0
Burdick and Jackson water with 0.1% formic acid (or equivalent)	VWR	BJLC452-1

Table 1-3 Required Materials for a NanoLC System with a cHiPLC® System

Description	Part Number
Reversed phase cHiPLC® column (75 µm × 15 cm ChromXP™ C18-CL 3 µm 120 Å)	804-00001
cHiPLC® trap (200 µm × 0.5 mm ChromXP™ C18-CL 3 µm 120 Å)	804-00006
Trap-and-elute jumper chip	800-00389

Table 1-4 Required Materials for a NanoLC System with an External Column

Ion Source	Description	Part Number
NanoSpray® or Digital PicoView® Nanospray Ion Source	Reversed phase ChromXP™ nanoLC column (75 µm ID × 15 cm, ChromXP™ C18 3 µm 120 Å)	805-00120
	ChromXP™ nanoLC Trap column (350 µm ID × 0.5 mm, ChromXP™ C18 3 µm 120 Å)	5016752
	NanoSpray emitter tip	910-00051
OptiFlow™ Turbo V Ion Source	bioZen Peptide Polar-C18 column (75 µm ID × 15 cm, Polar-C18 3 µm 100 Å)[1]	SCIEX 5073903 Phenomenex 00F-4782-AW-SX
	Nano Trap RP-1 trap column (75 µm ID × 1.0 cm, RP-1)[1]	SCIEX 5073904 Phenomenex 05N-4252-AW
	SteadySpray NANO probe with a NANO electrode	5068771

[1] This column only available for purchase by users from Phenomenex. The equivalent SCIEX part is only available to Field Service Employees (FSEs).

LC-MS System Integration Test 2

Create the Methods

Create the autosampler and LC methods in the Eksigent Control Software and the acquisition method in the Analyst® Software.

Create the Autosampler Method

1. Plumb the autosampler valve with a 10 µL sample loop.
2. Open the Analyst® Software.
3. In the **Autosampler** control window, click **Method Editor**.
4. Create the autosampler method for a trap-elute configuration. Refer to the appropriate appendix for the system.
   - Methods for NanoLC™ 400 Systems with a cHiPLC® System
   - Methods for NanoLC™ 400 Systems with an External Trap and Column
   - Methods for NanoLC Ultra® Systems with a cHiPLC® System
   - Methods for NanoLC Ultra® Systems with an External Trap and Column
   - Methods for NanoLC™ and Tempo™ Systems with a cHiPLC® System
   - Methods for NanoLC™ and Tempo™ Systems with an External Trap and Column
5. Type the name of the method as specified in the appendix, and then click **Save**.
6. Click **OK**.

Create the LC Methods

Fill the aqueous channel for each pump (side A) with buffer A. Fill the organic channel (side B) with buffer B. For the loading pump, buffer A is always used. Required buffers are shown in Table 2-1.

Table 2-1 Mobile Phase Mixtures

	Buffer A	Buffer B	Modifier
Gradient 1	100% water	100% acetonitrile	0.1% formic acid
Gradient 2	100% water	100% acetonitrile	0.1% formic acid
Loading pump	100% water	N/A	0.1% formic acid

Create the Loading Pump Method

In this method, the loading pump is the channel with the microflow module.

1. Open the Analyst® Software.
2. In the **Acquisition** window that opens (from the Eksigent Control Software), click the arrow in the top, right corner of the window to select the high-flow or loading pump.
3. Click **LC Methods**.
4. Create the loading pump method. Refer to the appropriate appendix for the system:
   - Methods for NanoLC™ 400 Systems with a cHiPLC® System
   - Methods for NanoLC™ 400 Systems with an External Trap and Column
   - Methods for NanoLC Ultra® Systems with a cHiPLC® System
   - Methods for NanoLC Ultra® Systems with an External Trap and Column
   - Methods for NanoLC™ and Tempo™ Systems with a cHiPLC® System
   - Methods for NanoLC™ and Tempo™ Systems with an External Trap and Column
5. Type the method name as specified in the appropriate appendix.
6. Click **Save**.
7. Click **OK**.

Create the Gradient Method

For the analytical gradient (typically on the gradient 1 channel with the nanoflow module), create the gradient method.

1. In the **Acquisition** window that opens (from the Eksigent Control Software), click the arrow in the top, right corner of the window to select the nanoflow or gradient pump.
2. Click **LC Methods**.
3. Create the gradient method. Refer to the appropriate appendix for the system:

- Methods for NanoLC™ 400 Systems with a cHiPLC® System
- Methods for NanoLC™ 400 Systems with an External Trap and Column
- Methods for NanoLC Ultra® Systems with a cHiPLC® System
- Methods for NanoLC Ultra® Systems with an External Trap and Column
- Methods for NanoLC™ and Tempo™ Systems with a cHiPLC® System
- Methods for NanoLC™ and Tempo™ Systems with an External Trap and Column

4. Type the method name as specified in the appropriate appendix.
5. Click **Save**.
6. Click **OK**.

Create the Acquisition Method

1. Close the Eksigent Control Software, if it is open.
2. Verify that the Analyst® Software drivers are installed. Refer to the appropriate installation document for details.
3. Open the Analyst® Software.
4. Create a hardware profile.
5. Create the acquisition method.

   Use the values in Table 2-2 as a starting point, updating the method with optimized values.

Note: The acquisition time should be more than 1 minute shorter than the LC run time. In the methods created below, the acquisition time is 5 minutes shorter than the LC run time.

Table 2-2 Acquisition Method Parameters—Analyst® Software

Parameter	Value
MS	
Scan Type	MRM Scan
Dwell time (msec)	50
Polarity	Positive
Q1/Q3 Masses and CE	Refer to Table 2-3.
Acquisition time (min)	40
Advanced MS	

Table 2-2 Acquisition Method Parameters—Analyst® Software (continued)

Parameter	Value
Q1 Resolution	Unit
Q3 Resolution	Unit
Source/Gas**	
Curtain Gas (CUR)	20 to 25 (or as optimized)
CAD Gas	HIGH Feedback should read between 2.1×10^5 and 2.4×10^5
IonSpray Voltage (IS) (V)	2100 to 2400 (or as optimized)
Ion Source Gas 1 (GS1)	2 to 15 (or as optimized)
Interface Heater (°C) (IHT)	150 (or as optimized)
Compound	
Declustering Potential (DP)	70
Collision Exit Potential (CXP)	30

** Source/Gas parameters may vary between systems and emitter tips. Determine the best value for the system. If applicable, make sure that the emitter tip position is optimized before creating the acquisition method.

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

Note: In the Analyst® Software 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 2-3 MRM Transitions for Beta-galactosidase

Q1	Q3	Dwell	ID	CE
433.9	723.4	50	BG_ELNYGPHQWR	30
450.7	524.3	50	BG_FNDDFSR	28
503.2	760.3	50	BG_YSQQQLMETSHR	27
528.9	855.4	50	BG_RDWENPGVTQLNR	25
542.3	636.4	50	BG_GDFQFNISR	26
550.3	871.4	50	BG_IDPNAWVER	27

Table 2-3 MRM Transitions for Beta-galactosidase (continued)

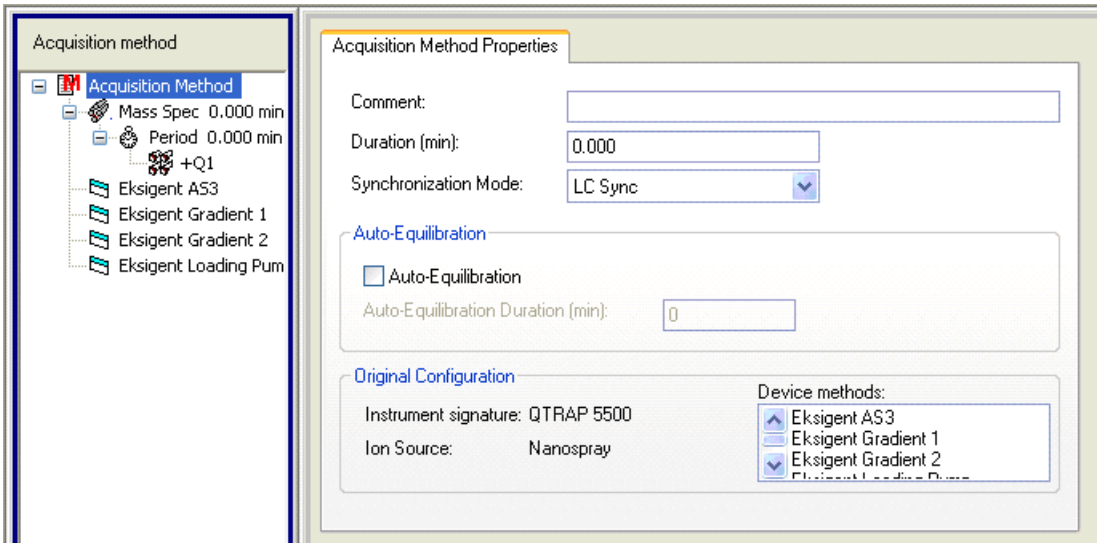
Q1	Q3	Dwell	ID	CE
567.1	932.5	50	BG_DVSLLHKPTTQISDFHVATR	30
607.9	685.4	50	BG_ITDSLAVVLQR	39
671.3	755.5	50	BG_VDEDQPFPAVPK	33
697.9	821.5	50	BG_LPSEFDLSAFLR	35
714.9	884.5	50	BG_DWENPGVTQLNR	32
729.4	832.5	50	BG_APLDNDIGVSEATR	48
871.9	915.5	50	BG_LSGQTIEVTSEYLFR	40
879.4	664.3	50	BG_VNWLGLGPQENYPDR	40

7. Save the Analyst® software method (for example, System Integration Test).

Add LC Information to the Acquisition Method

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

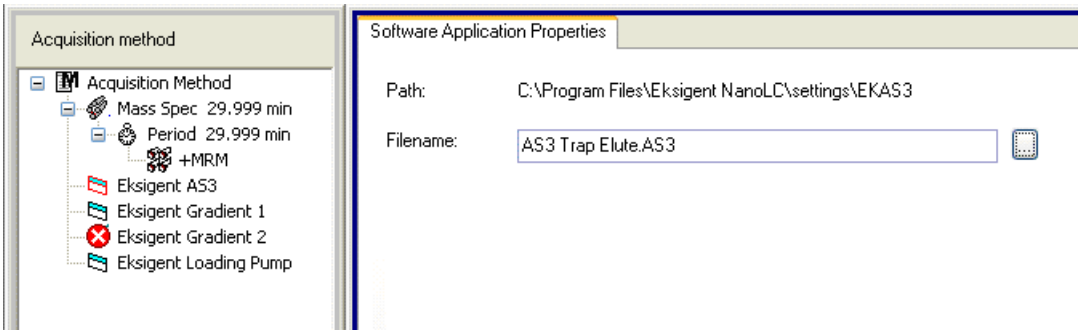
Figure 2-1 Acquisition Method Properties Tab—Synchronization Mode



2. Click the autosampler (for example, Eksigent AS2 or AS3) and then select the appropriate autosampler method. Refer to Create the Autosampler Method.

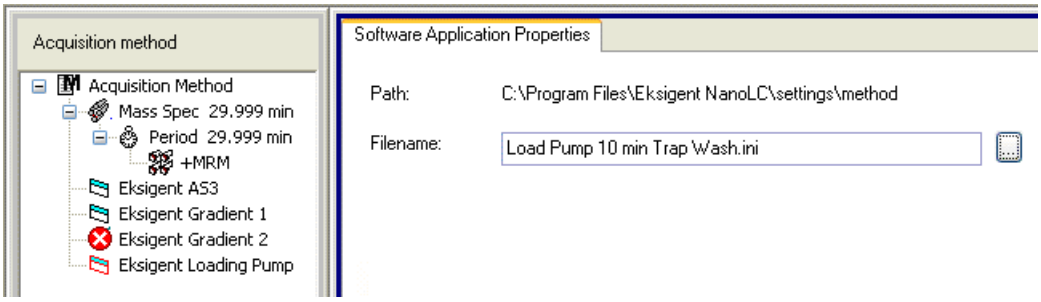
3. If Eksigent Gradient 2 appears in the device list, right-click **Eksigent Gradient 2** and then clear the "Use" selection as it is not being used.

Figure 2-2 Software Application Properties Tab—Autosampler Method



4. Click **Eksigent Gradient 1** and then select the appropriate gradient pump method. Refer to Create the Gradient Method.
5. Click **Eksigent Loading Pump** and then select the appropriate loading pump method. Refer to Create the Loading Pump Method.

Figure 2-3 Software Application Properties Tab—Loading Pump Method



6. Save the acquisition method.

Prepare the System for Testing

Plumb the system in trap-elute configuration to perform the pre-column desalting workflow. Refer to Plumbing Diagrams. For wiring information for a NanoLC™ or Tempo™ System with the cHiPLC®. Refer to Wire Connections.

Prepare the 1 pmol/µL Stock Solution of Beta-Galactosidase Digest Solution

WARNING! Toxic Chemical Hazard. Refer to the chemical product *Safety Data Sheets* and follow all of the recommended safety procedures when handling, storing, and disposing of chemicals. For health and safety precautions, refer to the *Operator Guide*.

Required Materials
• Beta-galactosidase digest (PN 4465938) • Buffer A

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 to step 3 to confirm dissolution.
5. Aliquot the stock solution into 50 µL volumes and then store them at –35 °C to –15 °C for up to one year.

Note: Aliquots of the beta-galactosidase stock solution can be stored at 4 °C for up to three days after thawing.

Condition the System

A trap and column typically require two to three runs with 200 fmol/µL of protein digest for conditioning. Prepare a 200 fmol/µL dilution and use it in two or three 1 µL injections at the beginning of the batch.

If the column is already conditioned, this procedure is not required. Proceed to Prepare the Sample for the System Integration Test.

Prepare a 200 fmol/µL Dilution of the Beta-Galactosidase to Condition the System

Required Materials
• Stock solution, prepared in Prepare the 1 pmol/µL Stock Solution of Beta-Galactosidase Digest Solution

1. Combine 40 µL of buffer A (100% water:0.1% formic acid) with 10 µL of the stock solution in a clean vial.
2. Mix the solution for at least 30 seconds using a vortex mixer.

This step produces a 1:5 dilution, with a concentration of 200 fmol/µL.

3. Transfer the solution to the autosampler vial, making sure there is no bubble on the bottom of the vial.

Prepare the Sample for the System Integration Test

Required Materials
• Beta-galactosidase stock solution, prepared in Prepare the 1 pmol/µL Stock Solution of Beta-Galactosidase Digest Solution • Water with 0.1% formic acid (Buffer A)

1. Combine the specified amount of the 1 pmol/µL beta-galactosidase stock solution with Buffer A in clean vial. Refer to Table 2-4.

Table 2-4 Sample Dilutions by Mass Spectrometer

System	Buffer A (µL)	Stock Solution (µL)	Final Concentration
4000 QTRAP® System QTRAP® 4500 System	190	10	50 fmol/µL
QTRAP® 5500/5500+ System QTRAP® 6500 System	495	5	10 fmol/µL

2. Mix the solution for at least 30 seconds using a vortex mixer.
3. Transfer the solution to the autosampler vial, making sure that there is no bubble on the bottom of the vial.

Verify System Readiness

Make sure that the LC system meets performance specifications.

Note: For information about assembly and operation of the NanoSpray® or OptiFlow™ Turbo V Ion Source, refer to the *Ion Source Operator Guide*. For information about assembly and operation of the Digital PicoView® Nanospray Ion Source, refer to the *Digital PicoView Nanospray Source Hardware Manual*.

Note: Optimize the ion source before beginning this test.

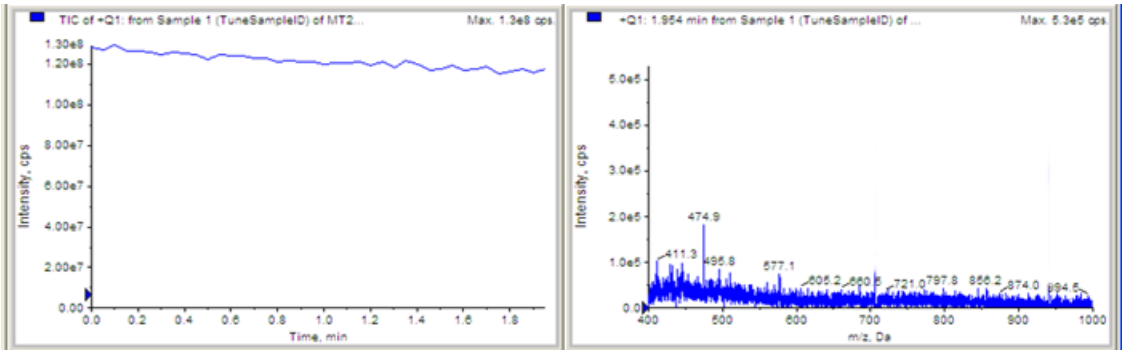
1. Connect the flow from the LC system to the ion source and verify that the spray is stable by monitoring the background signal in the Analyst® Software.

2. Equilibrate the LC-MS system with the starting conditions of the method outlined above.
3. Type the key parameters and then click **Start** to begin acquisition.

Table 2-5 Key Parameters

Parameter	Value
MS	
Scan type	Q1 Scan
Scan speed (Da/sec)	1000
Polarity	Positive
MCA	Off
Start Mass	400
Stop Mass	1000
Run Time (min)	2
Source/Gas	
Curtain Gas (CUR)	20 to 25 (or as optimized) Readback should read between 2.1 x 10^5 and 2.4 x 10^5
IonSpray Voltage (IS) (V)	2100 to 2400 (or as optimized)
Ion Source Gas 1 (GS1)	2 to 15 (or as optimized)
Interface Heater (ºC)	150 (or as optimized)
Compound	
Declustering Potential (DP)	70
Collision Exit Potential (CXP)	30

Figure 2-4 Q1 MS Scan for Background Noise



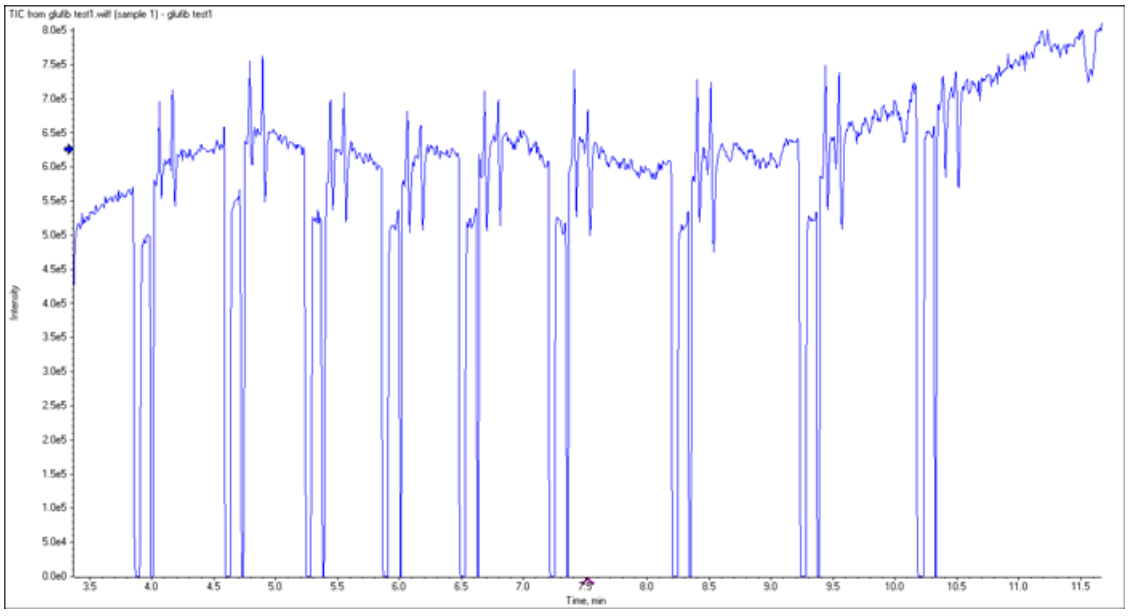
4. Make sure that the spray is still stable by monitoring the background signal with a Q1 MS scan.

   An example of stable spray is shown on the left side of the previous figure (the spectrum on the right will vary system to system). Refer to Figure 2-4.

   Unstable spray can be caused by air bubbles in the spray and appears as shown in the following figure. Bubbles in the spray are almost always caused by a poor post-column fitting.

   - If air bubbles are present in the spray, verify that all post-column fittings and connections have been made properly.
   - If the spray is not stable, tune the ion source again by infusion. Refer to the *Operator Guide*.

Figure 2-5 Unstable Spray Induced by Air Bubbles



Perform the System Integration Test

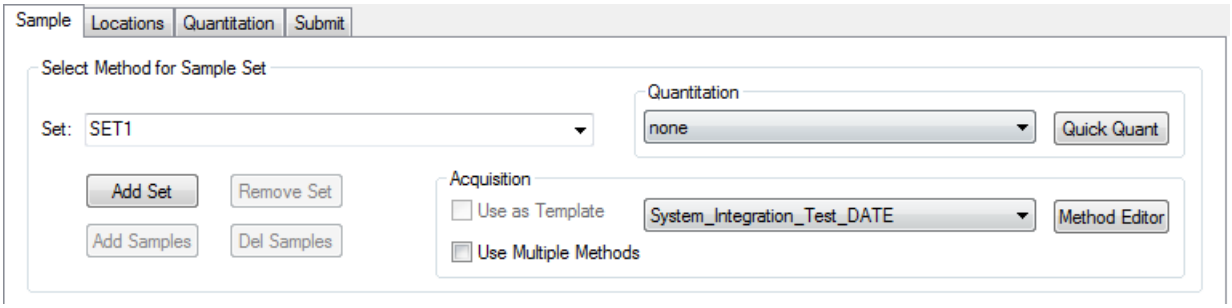
Create the acquisition batch, run the batch and then verify the results. Type the test results in the System Integration Test Data Log and Signoff.

Note: If the system includes a cHiPLC® System, make sure that the cHiPLC® System is in the Load position before beginning this test.

Create the LC-MS Acquisition Batch and Quantitation Method

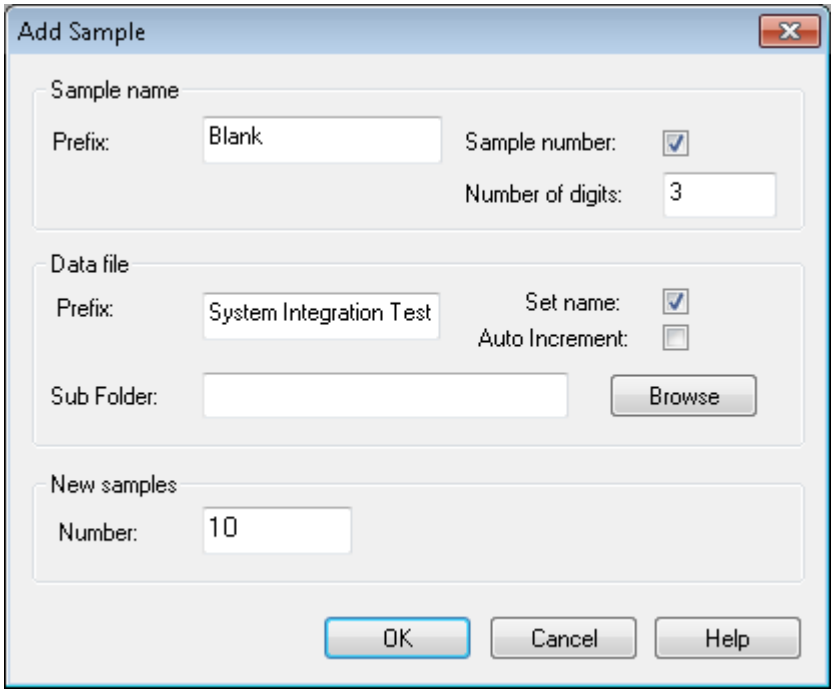
1. Double-click **Build Acquisition Batch** on the **Navigation** bar.
2. On the **Sample** tab, in the **Acquisition** group, select the acquisition method created in Create the Acquisition Method from the list.

Figure 2-6 Sample Tab—Acquisition Group



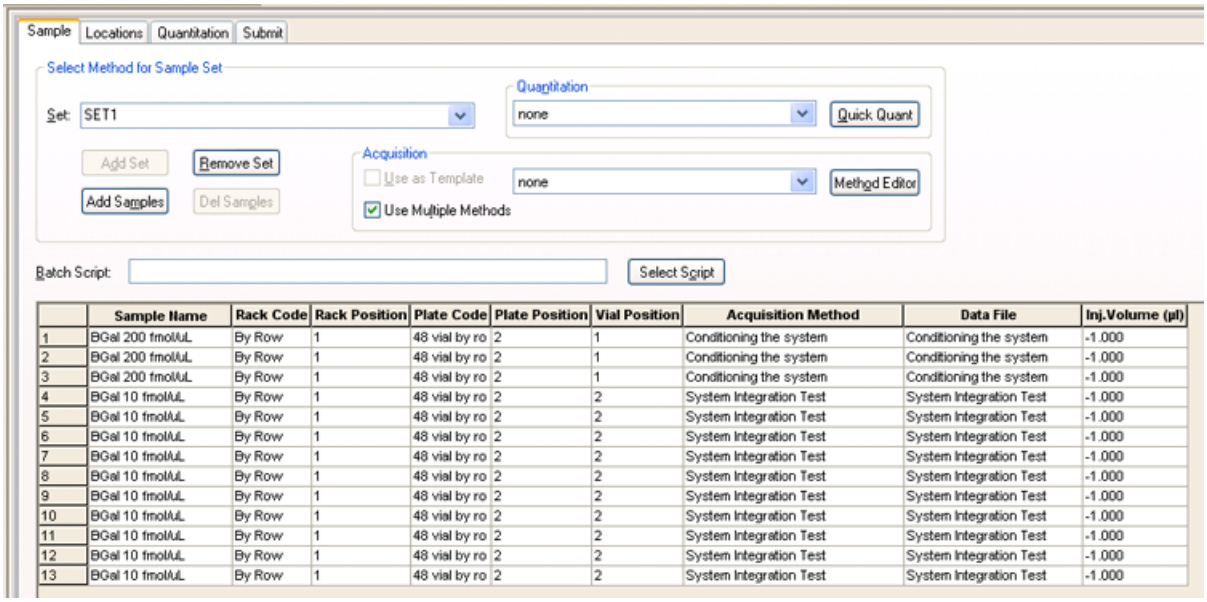
3. Click **Add Set**.
4. Click **Add Samples**.

Figure 2-7 Add Sample Dialog



5. Specify the sample information as shown in the previous figure. Refer to Figure 2-7.
   a. In the **Number** field, type **10**.
   b. Click **OK**.
6. Specify the rack and plate position for the samples as shown in the following figure. Refer to Figure 2-8.

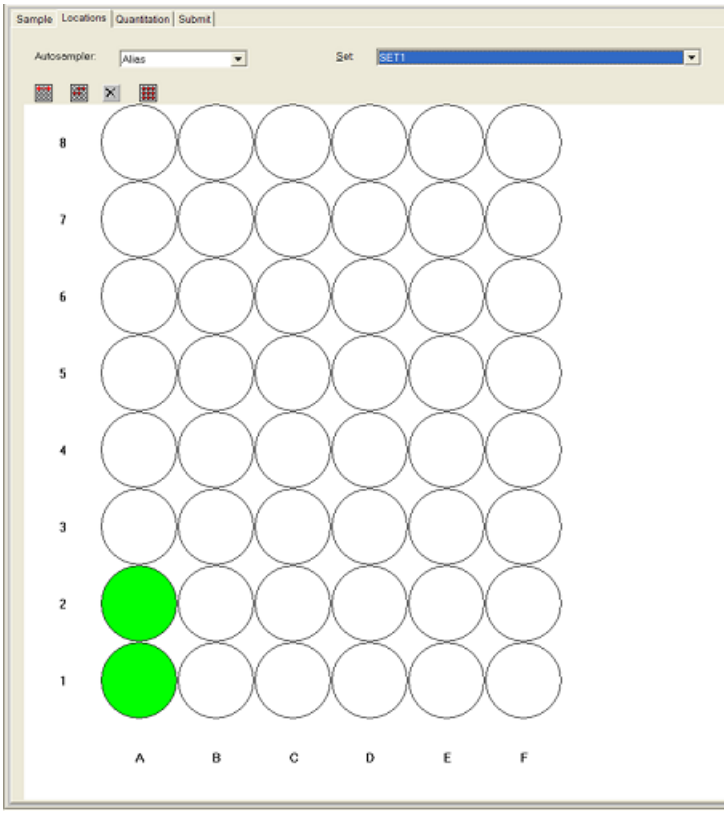
Figure 2-8 Sample Tab—Method Creation



	Sample Name	Rack Code	Rack Position	Plate Code	Plate Position	Vial Position	Acquisition Method	Data File	Inj.Volume (µl)
1	BGal 200 fmol/uL	By Row	1	48 vial by ro	2	1	Conditioning the system	Conditioning the system	-1.000
2	BGal 200 fmol/uL	By Row	1	48 vial by ro	2	1	Conditioning the system	Conditioning the system	-1.000
3	BGal 200 fmol/uL	By Row	1	48 vial by ro	2	1	Conditioning the system	Conditioning the system	-1.000
4	BGal 10 fmol/uL	By Row	1	48 vial by ro	2	2	System Integration Test	System Integration Test	-1.000
5	BGal 10 fmol/uL	By Row	1	48 vial by ro	2	2	System Integration Test	System Integration Test	-1.000
6	BGal 10 fmol/uL	By Row	1	48 vial by ro	2	2	System Integration Test	System Integration Test	-1.000
7	BGal 10 fmol/uL	By Row	1	48 vial by ro	2	2	System Integration Test	System Integration Test	-1.000
8	BGal 10 fmol/uL	By Row	1	48 vial by ro	2	2	System Integration Test	System Integration Test	-1.000
9	BGal 10 fmol/uL	By Row	1	48 vial by ro	2	2	System Integration Test	System Integration Test	-1.000
10	BGal 10 fmol/uL	By Row	1	48 vial by ro	2	2	System Integration Test	System Integration Test	-1.000
11	BGal 10 fmol/uL	By Row	1	48 vial by ro	2	2	System Integration Test	System Integration Test	-1.000
12	BGal 10 fmol/uL	By Row	1	48 vial by ro	2	2	System Integration Test	System Integration Test	-1.000
13	BGal 10 fmol/uL	By Row	1	48 vial by ro	2	2	System Integration Test	System Integration Test	-1.000

7. Create a batch. Refer to Figure 2-8.
8. Save the batch file as **YYYYMMDD LC MRM BGal Integration Test**.
9. On the **Location** tab, identify the position of the beta-galactosidase sample vial.

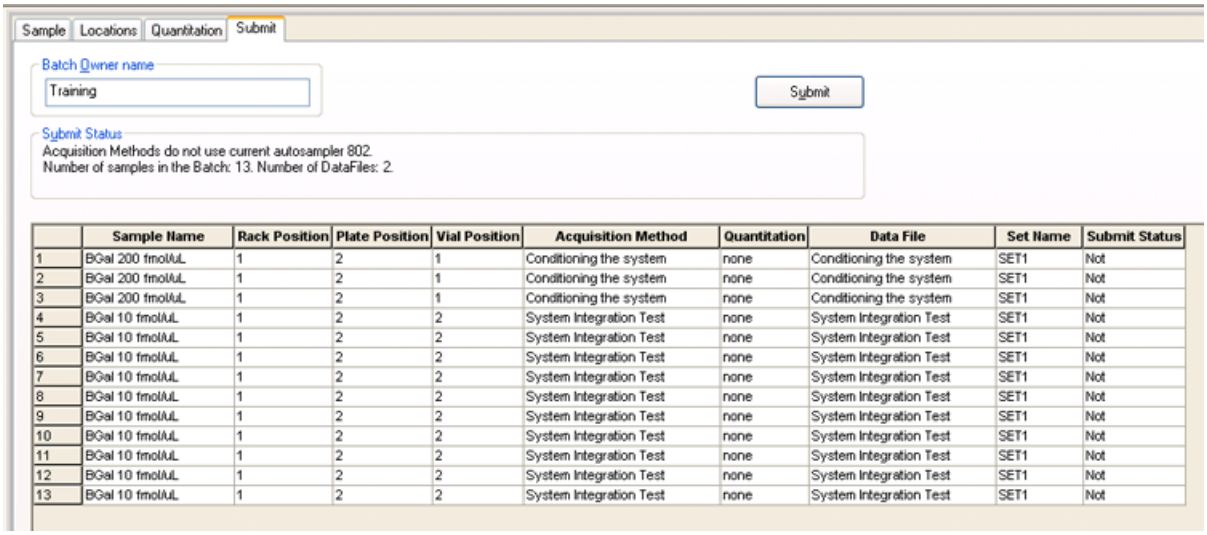
Figure 2-9 Locations Tab



Run the Batch

1. Click **Submit** on the **Submit** tab.

Figure 2-10 Submit Tab



	Sample Name	Rack Position	Plate Position	Vial Position	Acquisition Method	Quantitation	Data File	Set Name	Submit Status
1	BGal 200 fmol/uL	1	2	1	Conditioning the system	none	Conditioning the system	SET1	Not
2	BGal 200 fmol/uL	1	2	1	Conditioning the system	none	Conditioning the system	SET1	Not
3	BGal 200 fmol/uL	1	2	1	Conditioning the system	none	Conditioning the system	SET1	Not
4	BGal 10 fmol/uL	1	2	2	System Integration Test	none	System Integration Test	SET1	Not
5	BGal 10 fmol/uL	1	2	2	System Integration Test	none	System Integration Test	SET1	Not
6	BGal 10 fmol/uL	1	2	2	System Integration Test	none	System Integration Test	SET1	Not
7	BGal 10 fmol/uL	1	2	2	System Integration Test	none	System Integration Test	SET1	Not
8	BGal 10 fmol/uL	1	2	2	System Integration Test	none	System Integration Test	SET1	Not
9	BGal 10 fmol/uL	1	2	2	System Integration Test	none	System Integration Test	SET1	Not
10	BGal 10 fmol/uL	1	2	2	System Integration Test	none	System Integration Test	SET1	Not
11	BGal 10 fmol/uL	1	2	2	System Integration Test	none	System Integration Test	SET1	Not
12	BGal 10 fmol/uL	1	2	2	System Integration Test	none	System Integration Test	SET1	Not
13	BGal 10 fmol/uL	1	2	2	System Integration Test	none	System Integration Test	SET1	Not

2. Click **View > Sample Queue.**
3. Click **Acquire > Start Sample.**

Assess the Results

1. On the Navigation toolbar, click **Open Data File**.
2. Right-click the TIC for the MRM experiment and then click **Extract Ions**.
3. In the **Extract Ions** dialog, select the four MRMs from Table 2-6 and then click **OK**.

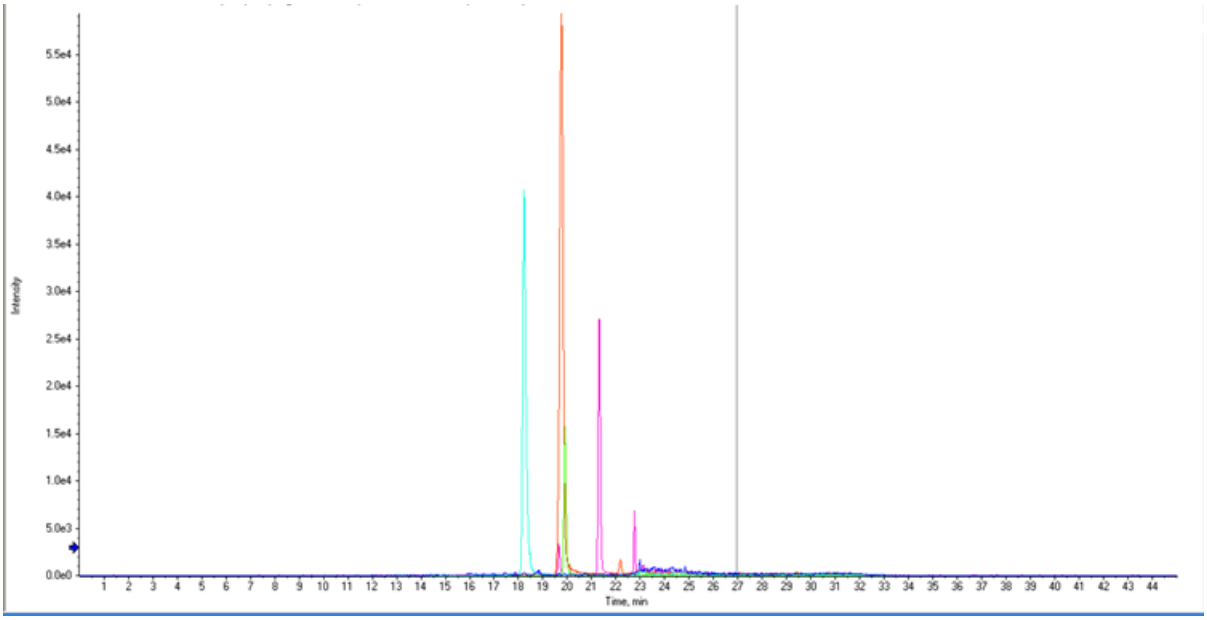
Note: These four peaks are the most stable and most reproducible from batch-to-batch and from lot-to-lot.

Table 2-6 MRM Transitions for Beta-Galactosidase

Q1	Q3	ID
542.3	636.4	BG_GDFQFNISR
671.3	755.5	BG_VDEDQPFPAVPK
714.9	884.5	BG_DWENPGVTQLNR
729.4	832.5	BG_APLDNDIGVSEATR

The following figure shows typical data. Refer to Figure 2-11.

Figure 2-11 MRM XICs of Peptides from Beta-Galactosidase NanoLC™ Pre-column Desalting Run



4. Record the peak areas of the specified MRM transitions in the System Integration Test Data Log and Signoff.

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

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5. Record the retention times of the chosen peaks. Retention time will vary with each system. Time of elution of the first peak indicates delay volume of the system. Minimize delay volume where possible.

Tip! Use a column oven to increase the retention time reproducibility. The temperature effect on retention time differs from peptide to peptide. In order to get the retention time reproducibility within specification, the temperature of the column should be stable within 1 °C. Factors to consider include not only the column temperature itself, but the room temperature, especially during overnight runs as the room temperature can shift dramatically.

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6. Repeat the acquisition until peak shape, retention time, and peak intensity are consistent (a minimum of three times for columns that are already conditioned).

   For new columns, acquisition might need to be repeated 10 or more times to produce consistent peak shape, retention time, and peak intensity. If required, refer to Troubleshoot Peak Issues.

7. Record the average peak area of the last three injections. Refer to System Integration Test Data Log and Signoff.

8. Compare the average peak area for four QC peaks in the last three injections to make sure that they meet the minimum requirements. Refer to System Integration Test Data Log and Signoff.
9. In the System Integration Test Data Log and Signoff, record the retention time for the last three samples analyzed for the following three peptides:
    - 729.4 *m/z*
    - 671.3 *m/z*
    - 542.3 *m/z*
10. Calculate the difference between the latest and earliest elution of each peptide and record the difference. Refer to System Integration Test Data Log and Signoff.
11. Make sure that the difference meets the minimum requirements specified in the System Integration Test Data Log and Signoff.
12. Verify that the Pc traces of the system integration test runs are of the correct shape. Refer to Figure 2-13.

Troubleshoot Peak Issues

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 the trap column. A small increase in peak width is often seen when a trap column is used.
- Broad peaks may also result from overloading the column. If this occurs when conditioning the column, ignore it. If this occurs during a sample run, either decrease the injection volume or dilute the sample and then inject the sample again.

CAUTION: Potential System Damage: If peak with problems persist and a cHiPLC® System is in use, 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 drop upon injection is not too severe in the high-flow channel (change in pressure less than 300 psi).

- A large pressure drop 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 with trap and elute will often be less than with direct injection. Components that elute comparably on the trap and analytical column will not resolve on the analytical column and, as a result, spread out or bunch together.

Peak intensity or peak area is too low

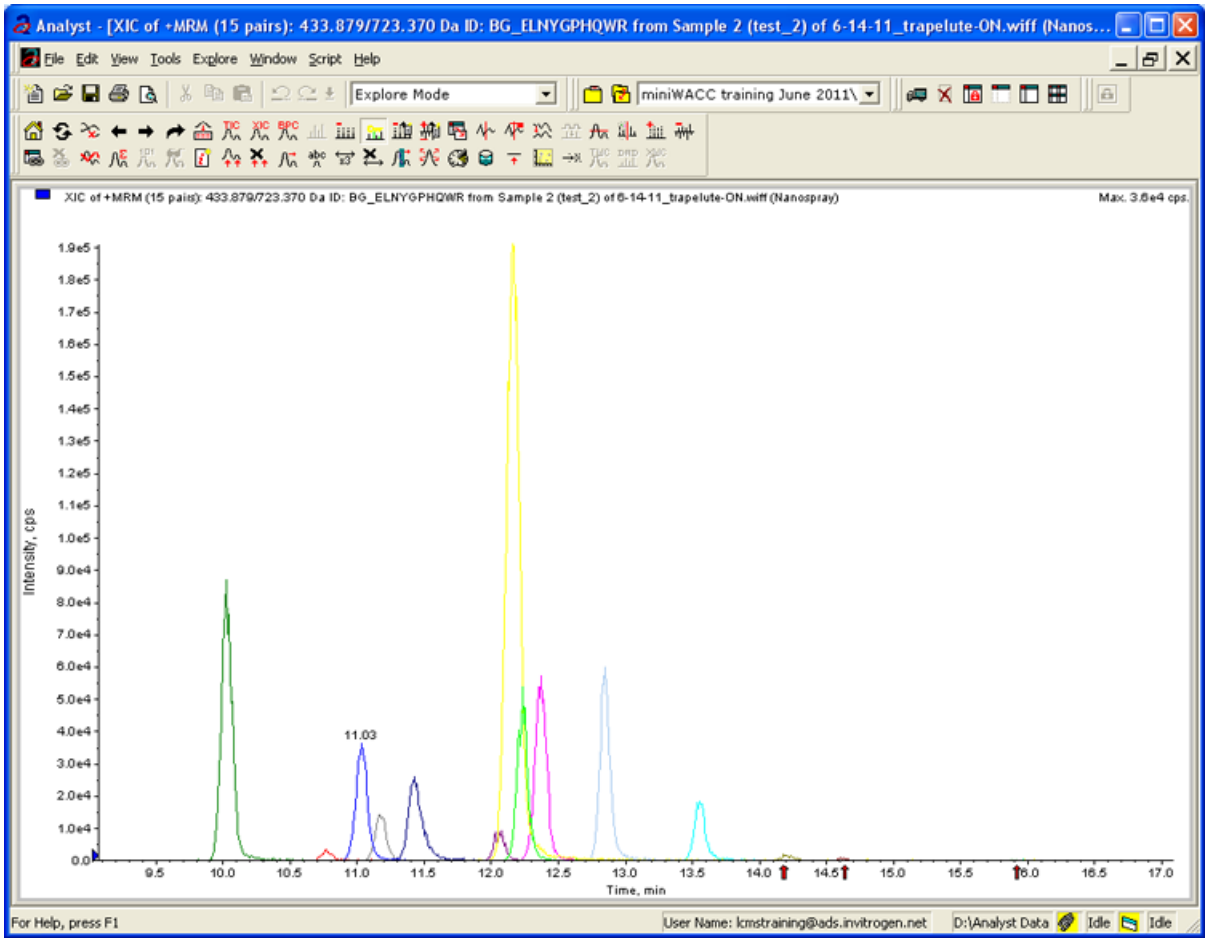
- Verify that the correct amount of sample has been withdrawn from the autosampler vial by performing an aspiration test.
- Verify the performance of the mass spectrometer and the ion source spray.
  - For the NanoSpray® or OptiFlow™ Turbo V Ion Sources, refer to the infusion tests in the *Ion Source Tests, Specifications, and Data Log* document.
  - For the Digital PicoView® Nanospray Ion Source, refer to the *NanoLC Systems Installation Checklist and Data Log.*
- If there is a large pressure drop upon injection, inspect for air bubbles in the sample loop or trap column plumbing.
- Verify that the trap and analytical column are well conditioned with protein digest injections before performing this test. A trap typically requires two to three runs with 200 fmol of protein digest on the column for conditioning.
- Perform a direct injection with a protein digest on the analytical column to determine whether the problem is related to the trap, or 10-port switching valve, or the cHiPLC® System.
- If the first LC peak does not elute for a long time, inspect the system for dead volume.

  Tip! Minimize tubing length wherever possible and make sure that all the tubing for the flow path for the gradient channel has an inner diameter of 18 µm to 25 µm.

- If the early eluting peaks are not visible or are very low in intensity, replace the trap. Invisible or low-intensity peaks can result from trapping efficiency.

- If the late eluting peaks are not visible or are very low in intensity, the column might be getting old. In rare cases, the beta-galactosidase standard is degraded. The following figure is an example of a scan with an older column. Refer to Figure 2-12.

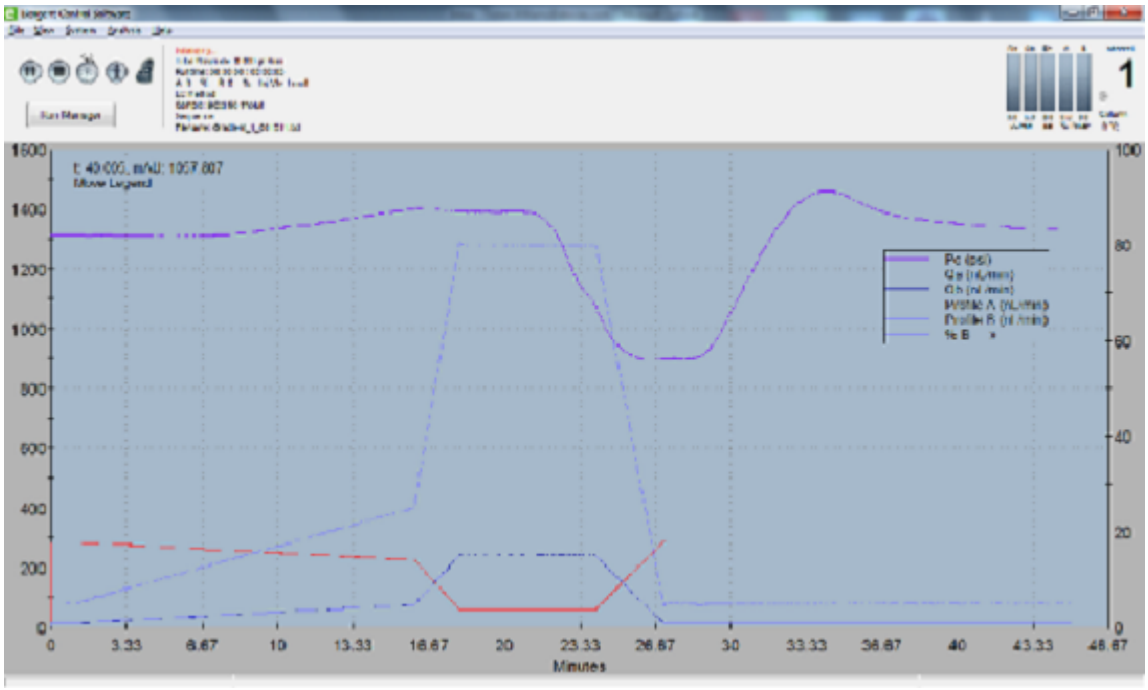
Figure 2-12 Extraction of All Peaks, Late Eluting Peaks Not Present



- Always monitor the column and trap pressure over time. Increasing pressure often indicates increasing blockage, probably at the ion source emitter tip. If the pressure drops quickly when the connection between the column and the ion source is unfastened, the tip is getting clogged and should be changed.
- For better long-term column lifetime, verify that the pressure drops at least 30% 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.

- The following figure shows a minimal pressure change upon injection (at time 0) and a 30% pressure decrease (at 23 minutes to 30 minutes) during the high organic flush. Refer to Figure 2-13.

Figure 2-13 Good Pressure Profile for a Trap-and-Elute Run



System Integration Test Data Log and Signoff 3

Beta-galactosidase lot number:	

System Information

Table 3-1 Pump Information

Model	
Location	
Serial number	

Table 3-2 Autosampler Information

Model	
Serial number	

Table 3-3 (Optional) cHiPLC® System Information

Model	
Serial number	

Table 3-4 (Optional) A/D Converter

Model	
Serial number	

Table 3-5 (Optional) Column Oven

Model	
Serial number	

Table 3-6 Ion Source Information

Model	
Serial number	

Table 3-7 Mass Spectrometer Information

Model	
Serial number	

Peak Area

Most of the XICs obtained should have peak widths of approximately 0.2 min half height on average. Some peaks will be narrower and some will be broader.

Additionally, verify that the peaks elute within five minutes of each other.

Table 3-8 Peak Area

Q1	Q3	Dwell	Peptide ID	CE	Peak Area Spec		Actual
					4000/4500/ 5500/5500+	6500/ 6500+	
542.3	636.4	50	BG_GDFQFNISR	26	5.0×10^5	1.5×10^6	
671.3	755.5	50	BG_VDEDQPFPAVPK	33	5.0×10^5	1.5×10^6	
714.9	884.5	50	BG_DWENPGVTQLNR	32	7.0×10^4	2.0×10^5	
729.4	832.5	50	BG_APLDNDIGVSEATR	48	2.0×10^5	6.0×10^5	
Specification Passed?							

Retention Time

For the three peptides, record the retention time for the last three samples analyzed then calculate the difference between the latest and earliest retention times.

Tip! Use a column oven to increase the retention time reproducibility. The temperature effect on retention time differs from peptide to peptide. In order to get the retention time reproducibility within specification, the temperature of the column should be stable within 1 °C. Factors to consider include not only the column temperature itself, but the room temperature, especially during overnight runs as the room temperature can shift dramatically.

Table 3-9 Retention Time for Beta-Galactosidase Peptides

Peptide m/z	Retention Time			Difference
	Run A	Run B	Run C	
729.4				
671.3				
542.3				
For NanoLC™ 400 Systems, difference should be < 0.3 min. For NanoLC Ultra®, NanoLC™ and Tempo™ Systems, difference should be < 0.5 min. Specification Passed?				

Comments and Exceptions

N/A

Signoff

Organization			
Customer contact name		Date (yyyy-mm-dd)	
Customer contact signature			
FSE name		Date (yyyy-mm-dd)	
FSE signature			

Methods for NanoLC™ 400 Systems with a cHiPLC® System

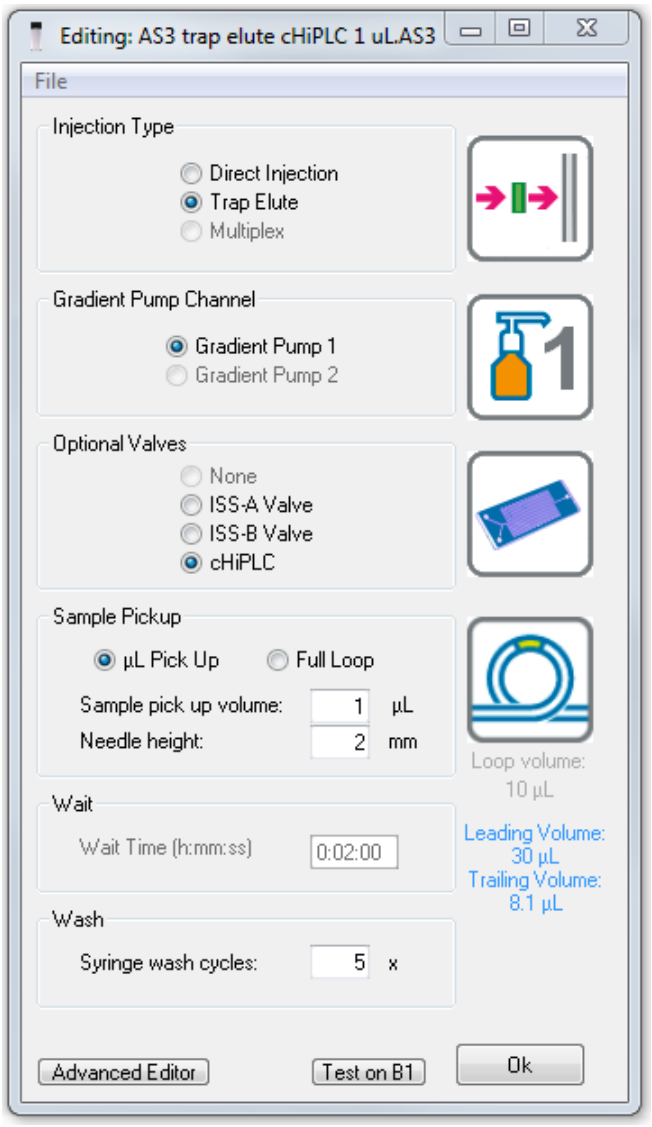
A

This appendix includes method parameters for a NanoLC™ 400 System with a cHiPLC® System.

For methods for a NanoLC™ 400 System with an external trap and column, refer to Methods for NanoLC™ 400 Systems with an External Trap and Column.

Autosampler Method Parameters

Figure A-1 Method Editor Dialog



The following table contains the autosampler method parameters as they are shown in the **Advanced Editor** dialog. Refer to Table A-1.

Table A-1 Advanced Editor Autosampler Method Parameters

Command	Description
Initialize	Autosampler Device
Wait	for 0:00:01
Needle Wash	Pre-wash—1 ×
Wait	for Gradient 1 ready to start
Wait	for Loading pump ready to start
Get Sample	µL Pickup—1 µL—0.5 µL/s—2 mm from bottom
Start	Loading Pump
Valve	Injector Inject
Wait	Wait for loading pump injection complete
Start	Gradient Pump 1
Valve	Injector Load
Needle Wash	Clean Up—5 ×

Loading Pump Method Parameters

Figure A-2 LC Method Settings Dialog—Run Conditions

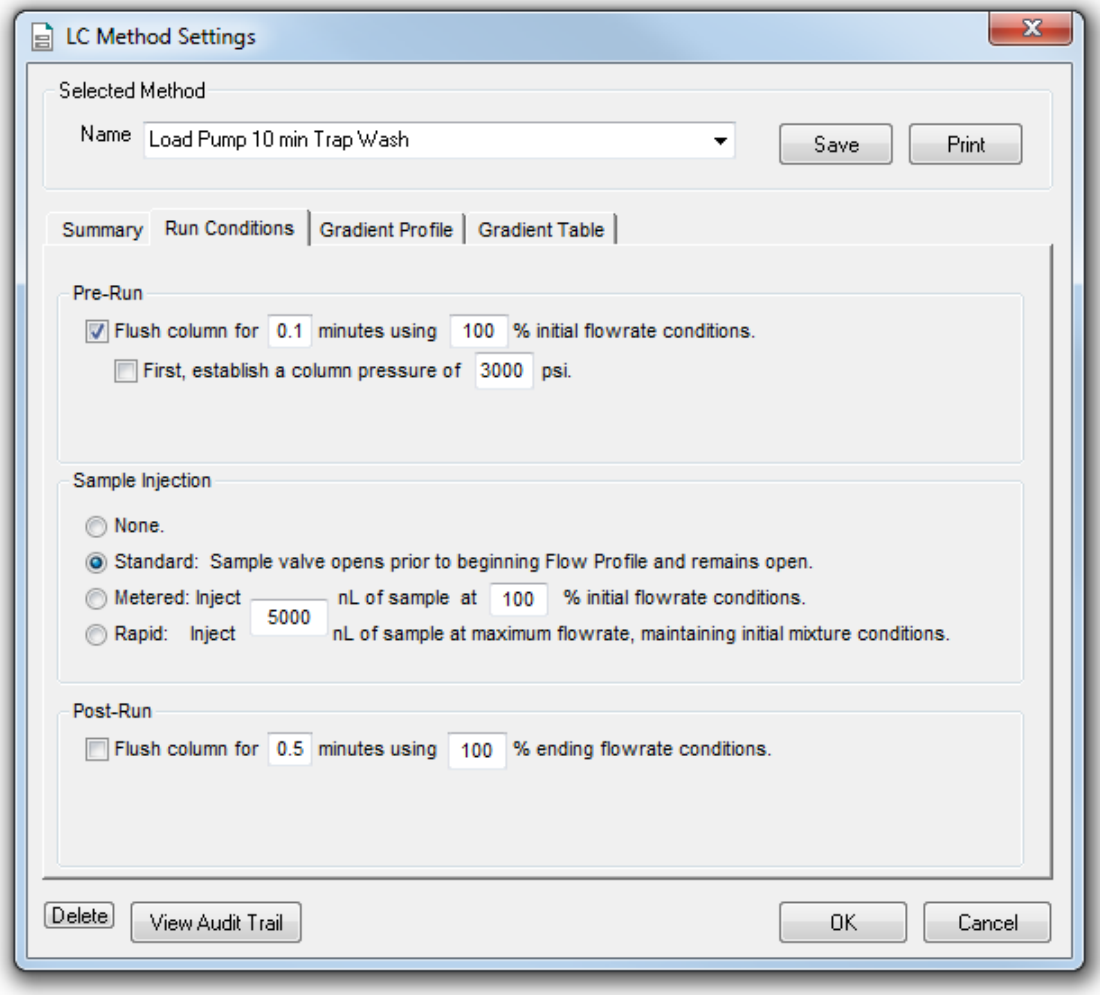


Table A-2 Loading Pump Method Parameters (Gradient Table Tab)

Step	Time	Qa
1	0	2
2	10	2

Gradient Pump Method Parameters

Figure A-3 LC Method Settings Dialog—Run Conditions Tab (cHiPLC® System)

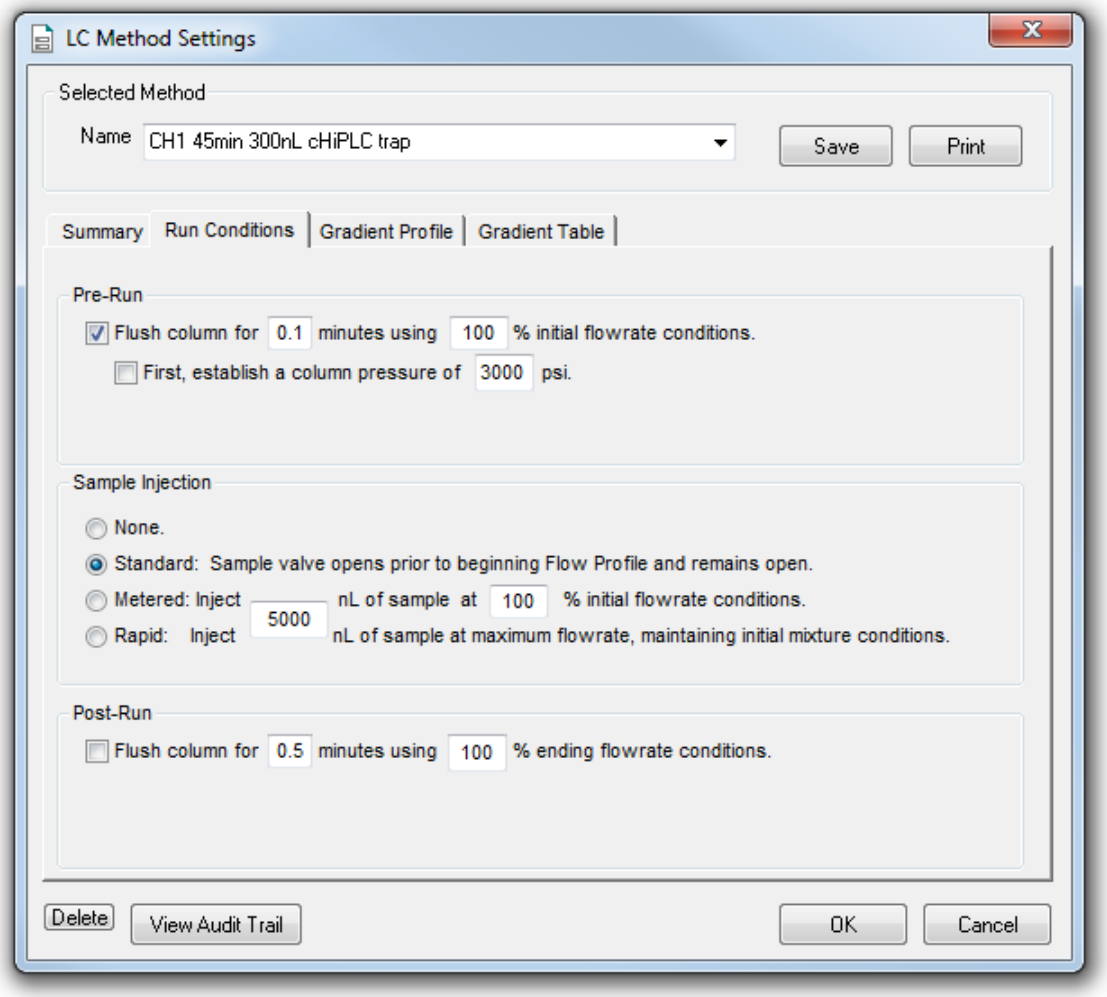


Table A-3 Gradient Method Parameters (Gradient Table Tab)

Step	Time	% A	% B	Event
1	0	95	5	
2	1	95	5	
3	16	75	25	
4	18	20	80	

Table A-3 Gradient Method Parameters (Gradient Table Tab) (continued)

Step	Time	% A	% B	Event
5	24	20	80	
6	27	95	5	
7	45	95	5	

Note: Flow rate is set to 300 nL/min.

Methods for NanoLC™ 400 Systems with an External Trap and Column

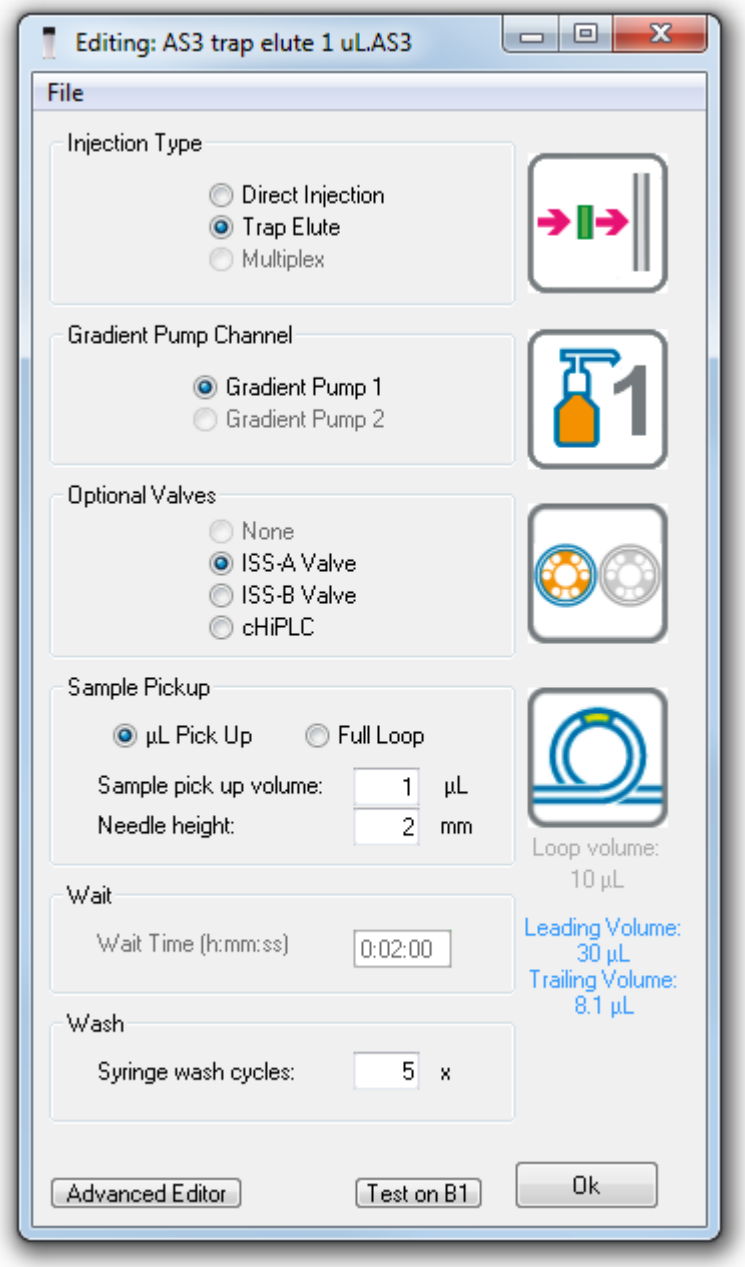
B

This appendix includes method parameters for a NanoLC™ 400 System with an external trap and column.

For methods for a NanoLC™ 400 System with a cHiPLC® System, refer to Methods for NanoLC™ 400 Systems with a cHiPLC® System.

Autosampler Method Parameters

Figure B-1 Method Editor Dialog



The following table contains the autosampler method parameters as they are shown in the **Advanced Editor** dialog. Refer to Table B-1.

Table B-1 Advanced Editor Autosampler Method Parameters

Command	Description
Initialize	Autosampler Device
Valve	ISS-A Load
Needle Wash	Pre-wash—1 × using Wash Solvent 1
Wait	for Gradient 1 ready to start
Wait	for Loading Pump ready to start
Get Sample	µL Pickup—1 µL - 0.5 µL/s - 2 mm from bottom
Start	Loading Pump
Valve	Injector Inject
Wait	for Loading Pump injection complete
Valve	ISS-A Inject
Start	Gradient Pump 1
Valve	Injector Load
Needle Wash	Clean Up—5 × using Wash Solvent 1

Loading Pump Method Parameters

Figure B-2 LC Method Settings Dialog—Run Conditions

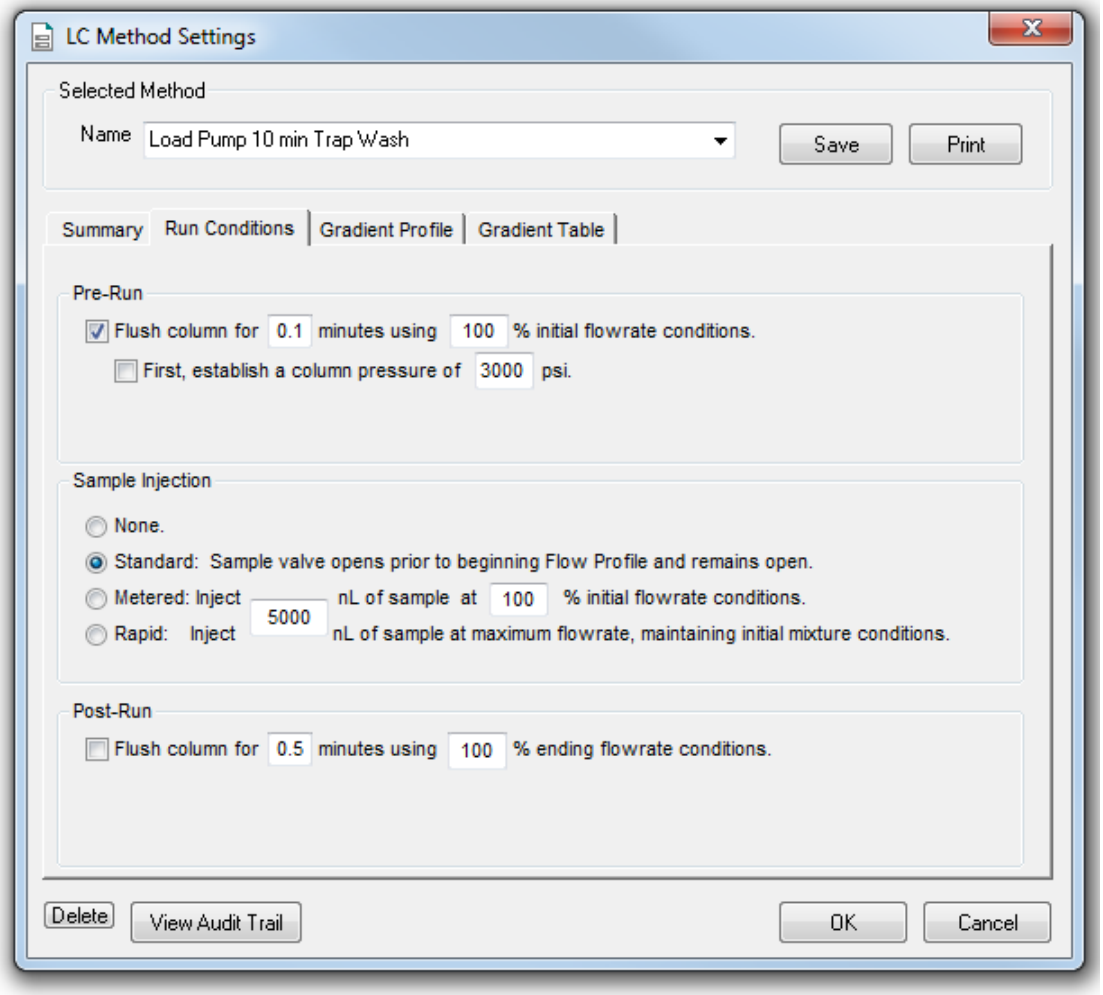


Table B-2 Loading Pump Method Parameters (Gradient Table Tab)

Step	Time	Qa
1	0	2
2	10	2

Gradient Pump Method Parameters

Figure B-3 LC Method Settings Dialog—Run Conditions Tab (External Column)

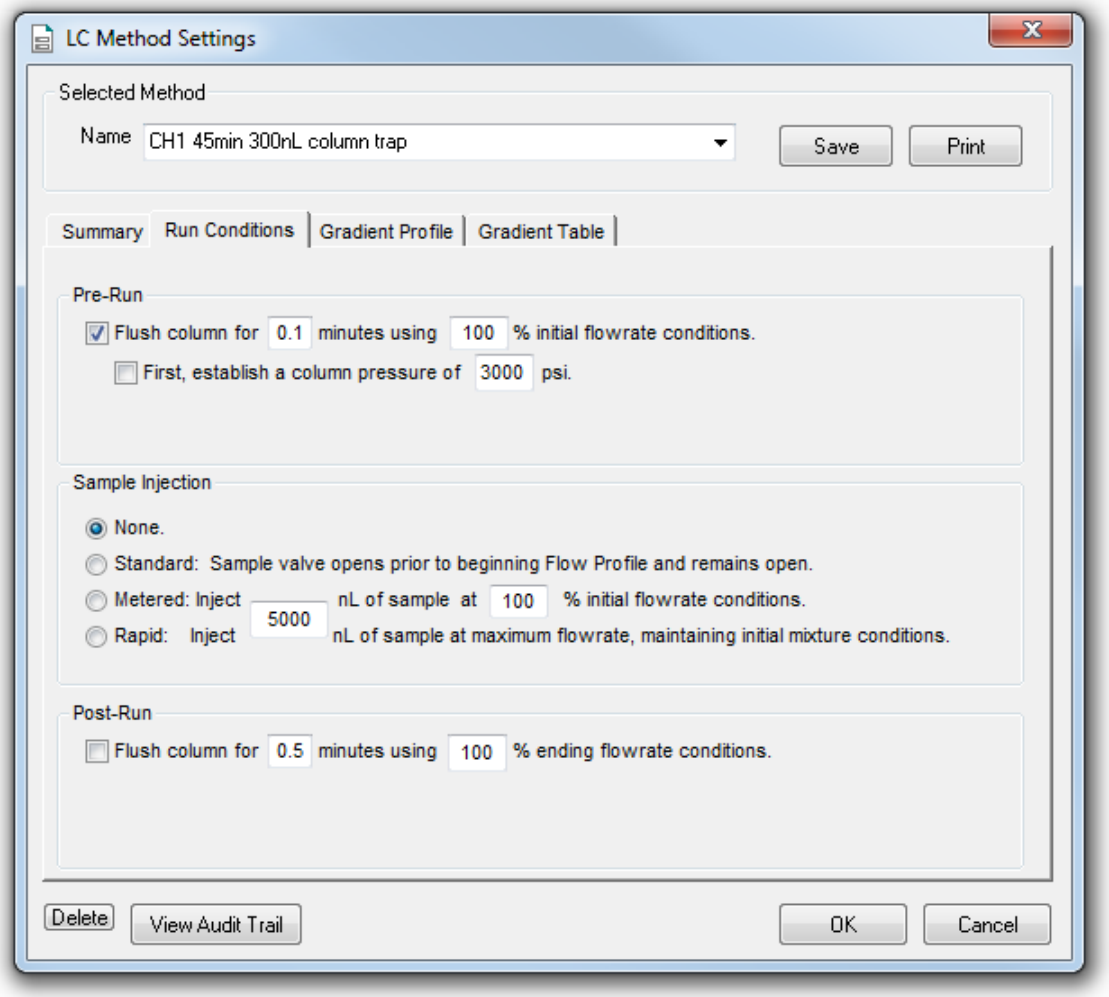


Table B-3 Gradient Method Parameters (Gradient Table Tab)

Step	Time	% A	% B	Event
1	0	95	5	
2	1	95	5	
3	16	75	25	
4	18	20	80	

Table B-3 Gradient Method Parameters (Gradient Table Tab) (continued)

Step	Time	% A	% B	Event
5	24	20	80	
6	27	95	5	
7	45	95	5	

Note: Flow rate is set to 300 nL/min.

Methods for NanoLC Ultra® Systems with a cHiPLC® System C

This appendix includes method parameters for a NanoLC Ultra® System with a cHiPLC® System.

For methods for a NanoLC Ultra® System with an external trap and column, refer to Methods for NanoLC Ultra® Systems with an External Trap and Column.

Autosampler Method Parameters

This table contains the autosampler method parameters as they are shown in the **Method Editor** dialog.

Table C-1 Autosampler Method Parameters

Command	Value	Parameter	Values
Valve		Injector Load	
External Events		Wait for Gradient 1 Ready	
External Events		Wait for Loading Pump Ready	
Needle Wash	50 µL	Port 1	
Aspirate	19 µL	Reagent-1	Speed: 1 Height: 5
Wait	00:00:05		
Aspirate	0 µL	Reagent-1	Speed: 1 Height: 5
Aspirate	1 µL	Sample	Speed: 1 Height: 3
Wait	00:00:05		
Aspirate	0 µL	Sample	Speed: 1 Height: 3
Aspirate	5 µL	Reagent-1	Speed: 1 Height: 5
Wait	00:00:05		

Table C-1 Autosampler Method Parameters (continued)

Command	Value	Parameter	Values
Aspirate	0 µL	Reagent-1	Speed: 1 Height: 5
External Events		Start Loading Pump	
Valve		Injector Inject	
External Events		Wait for Loading Pump Inject	
Valve		Injector Load	
External Events		Start Gradient 1	
Dispense	25 µL	Waste	Speed: 5 Height: 0
Needle Wash	200 µL	Port 1	
END			

Loading Pump Method Parameters

Figure C-1 LC Method Settings Dialog—Run Conditions

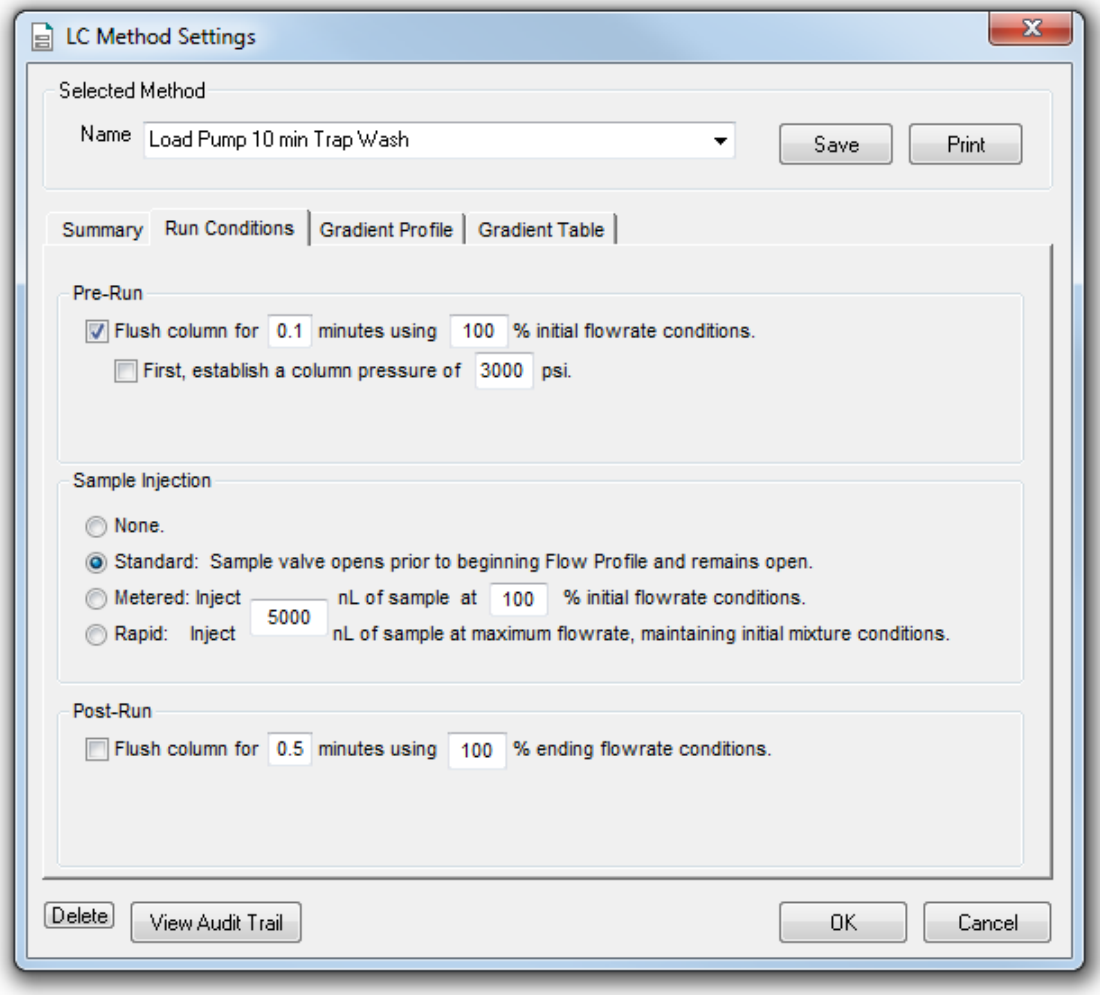


Table C-2 Loading Pump Method Parameters (Gradient Table Tab)

Step	Time	Qa
1	0	2
2	10	2

Gradient Pump Method Parameters

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

Figure C-2 LC Method Settings Dialog—Run Conditions Tab (cHiPLC® System)

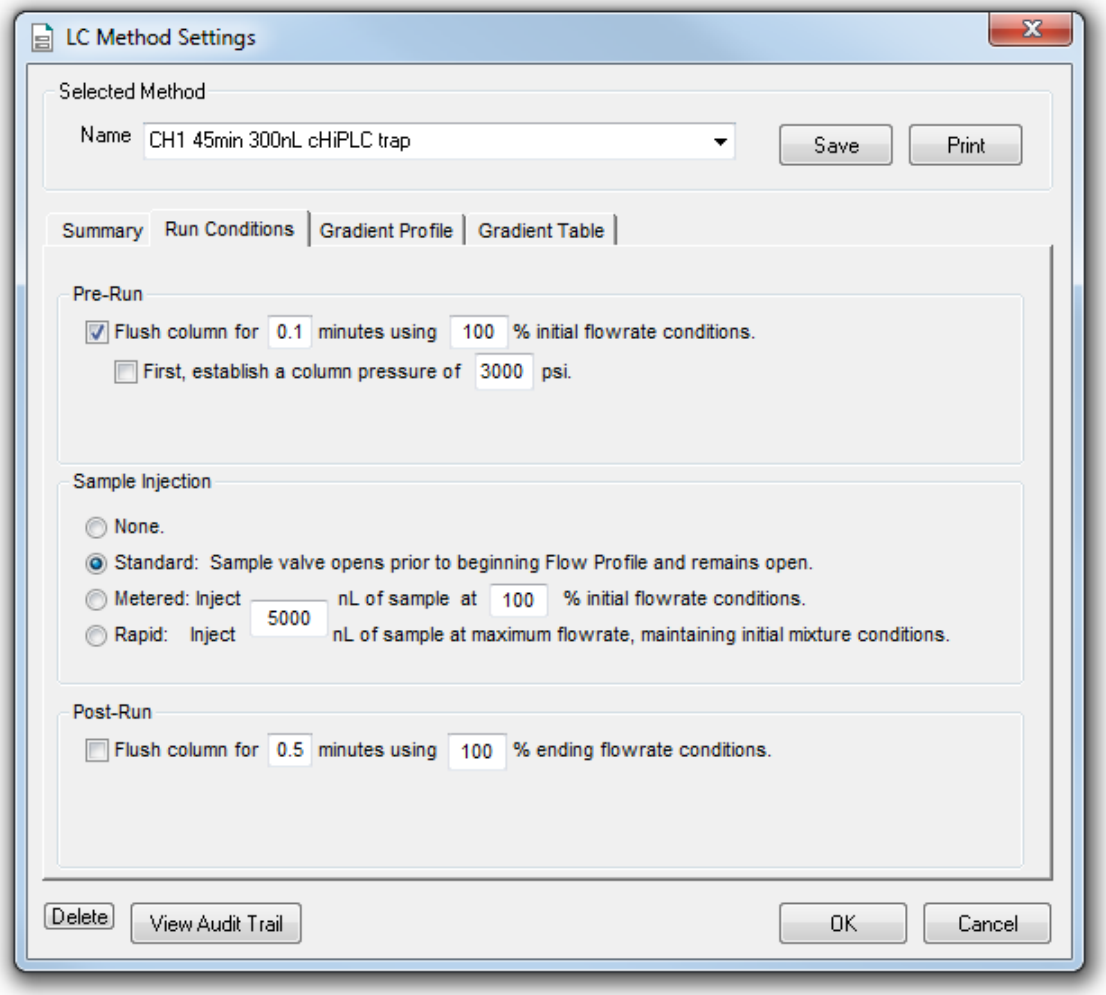


Table C-3 Gradient Method Parameters

Step	Time	% A	% B	Event
1	0	95	5	AUX3 TTL Low
2	0	95	5	AUX4 TTL High
3	0.1	95	5	AUX3 TTL High
4	16	75	25	
5	18	20	80	
6	24	20	80	
7	27	95	5	
8	45	96	5	AUX4 TTL Low

Note: Flow rate is set to 300 nL/min.

Methods for NanoLC Ultra® Systems with an External Trap and Column

D

This appendix includes method parameters for a NanoLC Ultra® System with an external trap and column.

For methods for a NanoLC Ultra® System with a cHiPLC® System, refer to Methods for NanoLC Ultra® Systems with a cHiPLC® System.

Autosampler Method Parameters

This table contains the autosampler method parameters as they are shown in the **Method Editor** dialog.

Table D-1 Autosampler Method Parameters

Command	Value	Parameter	Values
Valve		Injector Load	
External Events		Wait for Gradient 1 Ready	
External Events		Wait for Loading Pump Ready	
Needle Wash	50 µL	Port 1	
Aspirate	19 µL	Reagent-1	Speed: 1 Height: 5
Wait	00:00:05		
Aspirate	0 µL	Reagent-1	Speed: 1 Height: 5
Aspirate	1 µL	Sample	Speed: 1 Height: 3
Wait	00:00:05		
Aspirate	0 µL	Sample	Speed: 1 Height: 3
Aspirate	5 µL	Reagent-1	Speed: 1 Height: 5

Table D-1 Autosampler Method Parameters (continued)

Command	Value	Parameter	Values
Wait	00:00:05		
Aspirate	0 µL	Reagent-1	Speed: 1 Height: 5
External Events		Start Loading Pump	
Valve		Injector Inject	
External Events		Wait for Loading Pump Inject	
Valve		Injector Load	
External Events		Start Gradient 1	
Dispense	25 µL	Waste	Speed: 5 Height: 0
Needle Wash	200 µL	Port 1	
END			

Loading Pump Method Parameters

Figure D-1 LC Method Settings Dialog—Run Conditions

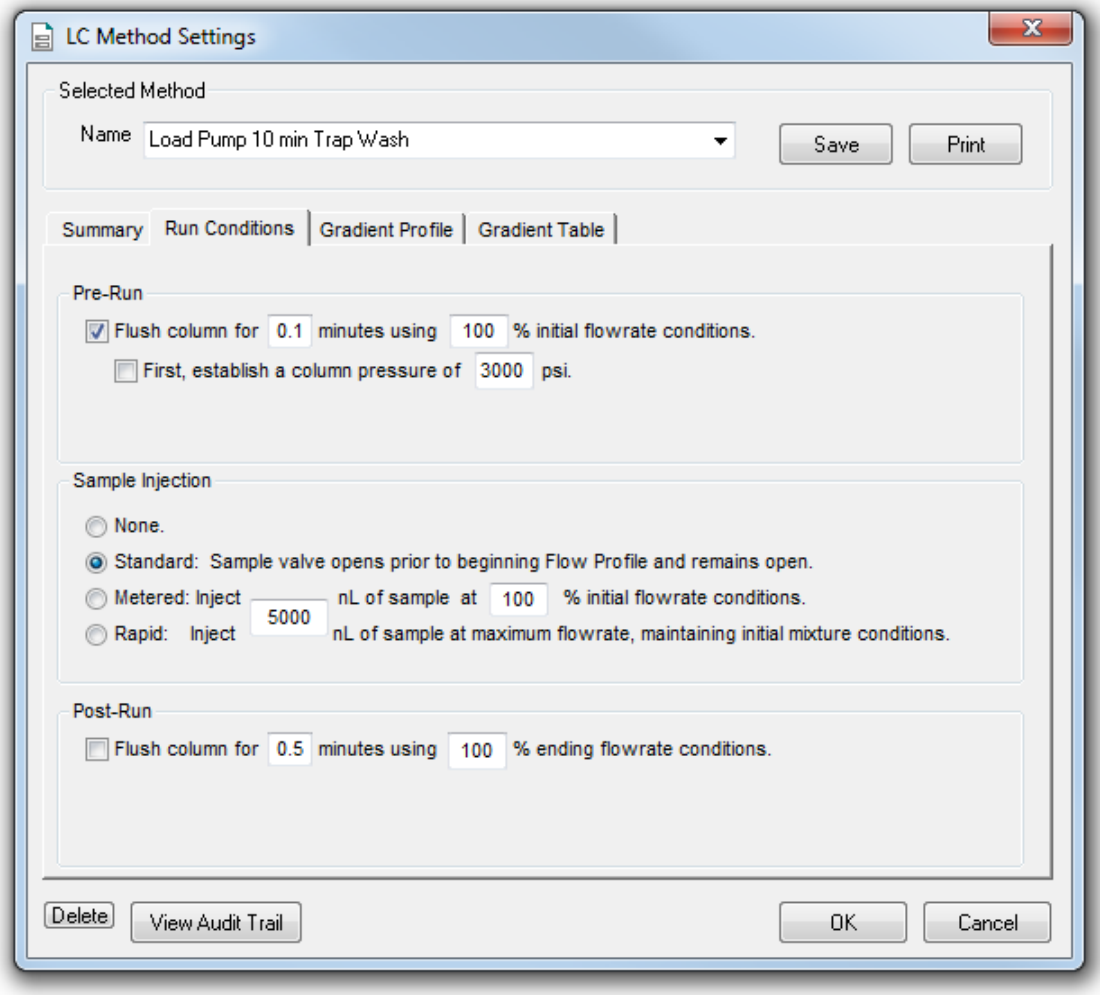


Table D-2 Loading Pump Method Parameters (Gradient Table Tab)

Step	Time	Qa
1	0	2
2	10	2

Gradient Pump Method Parameters

Figure D-2 LC Method Settings Dialog—Run Conditions Tab (External Column)

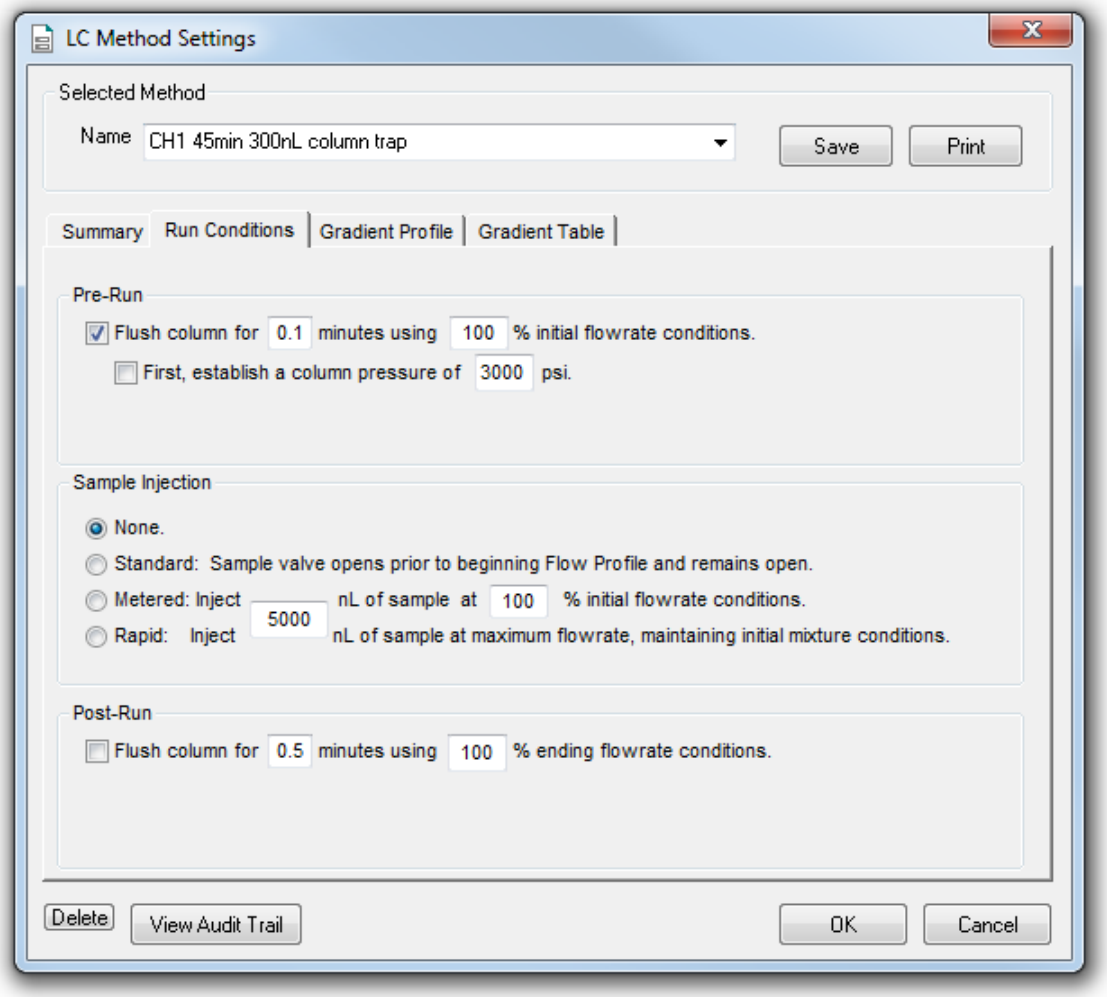


Table D-3 Gradient Method Parameters (Gradient Table Tab)

Step	Time	% A	% B	Event
1	0	95	5	
2	1	95	5	
3	16	75	25	
4	18	20	80	

Table D-3 Gradient Method Parameters (Gradient Table Tab) (continued)

Step	Time	% A	% B	Event
5	24	20	80	
6	27	95	5	
7	45	95	5	

Note: Flow rate is set to 300 nL/min.

Methods for NanoLC™ and Tempo™ Systems with a cHiPLC® System

E

This appendix includes method parameters for a NanoLC™ and Tempo™ Systems with a cHiPLC® System.

Refer to Connecting NanoLC™ and Tempo™ Systems with a cHiPLC® System for plumbing information.

For methods for NanoLC™ and Tempo™ Systems with an external trap and column, refer to Methods for NanoLC™ and Tempo™ Systems with an External Trap and Column.

Autosampler Method Parameters

This table contains the autosampler method parameters as they are shown in the **Method Editor** dialog.

Table E-1 Autosampler Method Parameters

Command	Value	Parameter	Values
Output		1-OFF	
Output		2-OFF	
Valve		Injector Load	
Aspirate	19 µL	Reagent-1	Speed: 1 Height: 5
Wait	00:00:05		
Aspirate	0 µL	Reagent-1	Speed: 1 Height: 5
Aspirate	1 µL	Sample	Speed: 1 Height: 5
Wait	00:00:05		
Aspirate	0 µL	Sample	Speed: 1 Height: 5
Aspirate	5 µL	Reagent-1	Speed: 1 Height: 5
Wait	00:00:05		

Table E-1 Autosampler Method Parameters (continued)

Command	Value	Parameter	Values
Aspirate	0 µL	Reagent-1	Speed: 1 Height: 5
Output		1-ON	
Wait for input		1-LOW	
Valve		Injector Inject	
Wait for input		1-HIGH	
Valve		Injector Load	
Output		2-ON	
Dispense	25 µL	Waste	Speed:5 Height: 0
Needle wash	200 µL	Port 1	
END			

Loading Pump Method Parameters

Figure E-1 LC Method Settings Dialog—Run Conditions

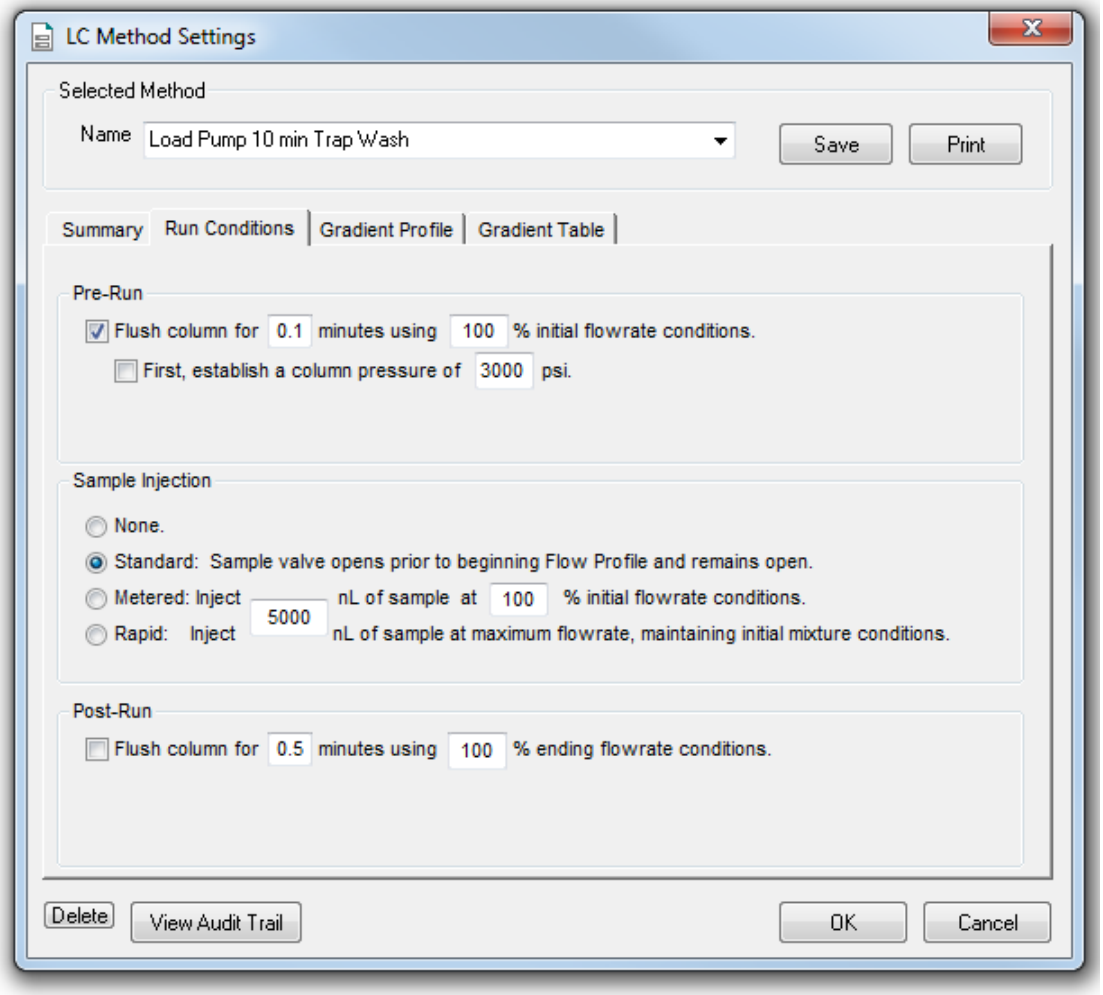


Table E-2 Loading Pump Method Parameters (Gradient Table Tab)

Step	Time	Qa
1	0	2
2	10	2

Gradient Pump Method Parameters

Figure E-2 LC Method Settings Dialog—Run Conditions Tab (cHiPLC® System)

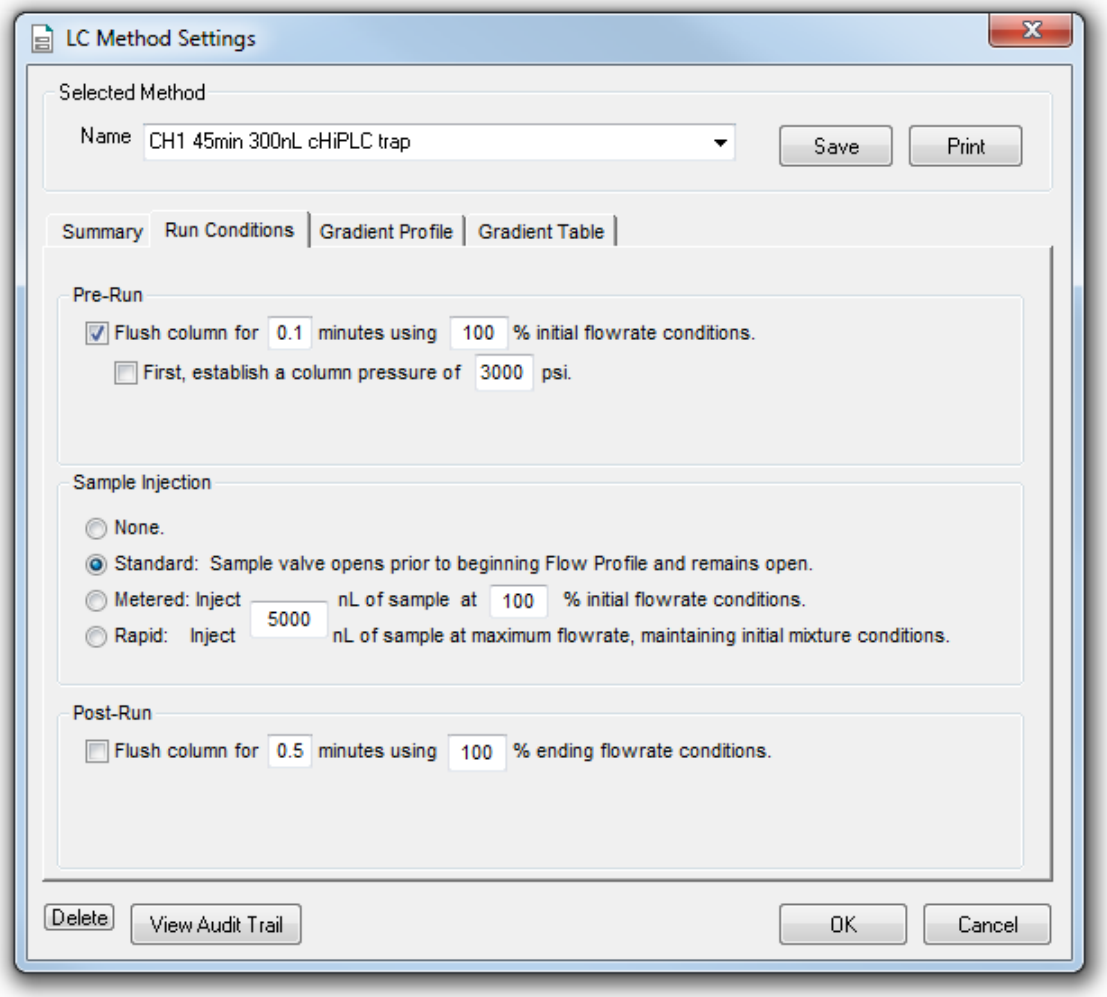


Table E-3 Gradient Method Parameters (Gradient Table Tab)

Step	Time	% A	% B	Event
1	0	95	5	
2	1	95	5	
3	16	75	25	
4	18	20	80	

Table E-3 Gradient Method Parameters (Gradient Table Tab) (continued)

Step	Time	% A	% B	Event
5	24	20	80	
6	27	95	5	
7	45	95	5	

Note: Flow rate is set to 300 nL/min.

Methods for NanoLC™ and Tempo™ Systems with an External Trap and Column

F

This appendix includes method parameters for a NanoLC™ or Tempo™ System with an external trap and column.

For methods for a NanoLC™ or Tempo™ System with a cHiPLC® System, refer to Methods for NanoLC™ and Tempo™ Systems with a cHiPLC® System.

Autosampler Method Parameters

This table contains the autosampler method parameters as they are shown in the **Method Editor** dialog.

Table F-1 Autosampler Method Parameters

Command	Value	Parameter	Values
Output		1-OFF	
Output		2-OFF	
Valve		Injector Load	
Aspirate	19 µL	Reagent-1	Speed: 1 Height: 5
Wait	00:00:05		
Aspirate	0 µL	Reagent-1	Speed: 1 Height: 5
Aspirate	1 µL	Sample	Speed: 1 Height: 5
Wait	00:00:05		
Aspirate	0 µL	Sample	Speed: 1 Height: 5
Aspirate	5 µL	Reagent-1	Speed: 1 Height: 5
Wait	00:00:05		

Table F-1 Autosampler Method Parameters (continued)

Command	Value	Parameter	Values
Aspirate	0 µL	Reagent-1	Speed: 1 Height: 5
Output		1-ON	
Wait for input		1-LOW	
Valve		Injector Inject	
Wait for input		1-HIGH	
Valve		Injector Load	
Output		2-ON	
Dispense	25 µL	Waste	Speed:5 Height: 0
Needle wash	200 µL	Port 1	
END			

Loading Pump Method Parameters

Figure F-1 LC Method Settings Dialog—Run Conditions

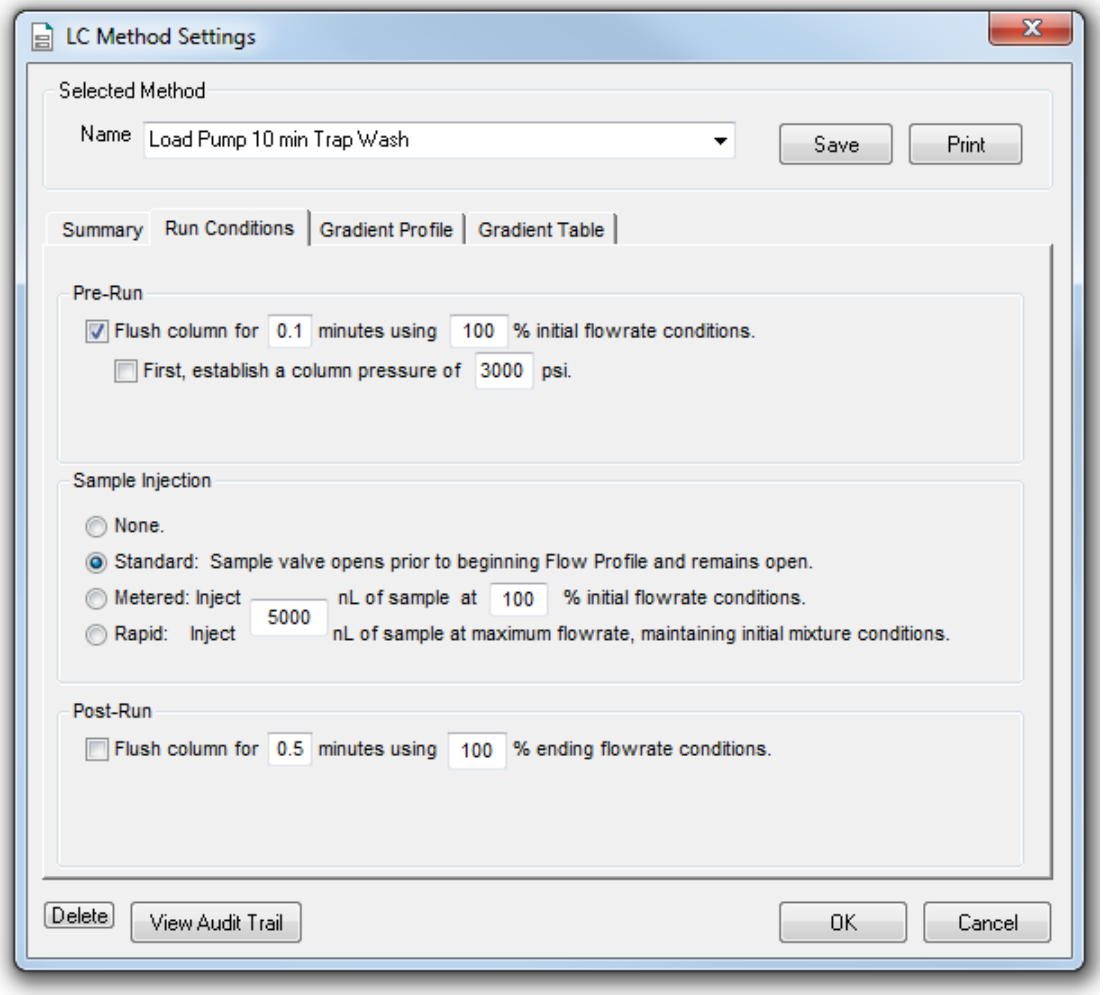


Table F-2 Loading Pump Method Parameters (Gradient Table Tab)

Step	Time	Qa
1	0	2
2	10	2

Gradient Pump Method Parameters

Figure F-2 LC Method Settings Dialog—Run Conditions Tab (External Column)

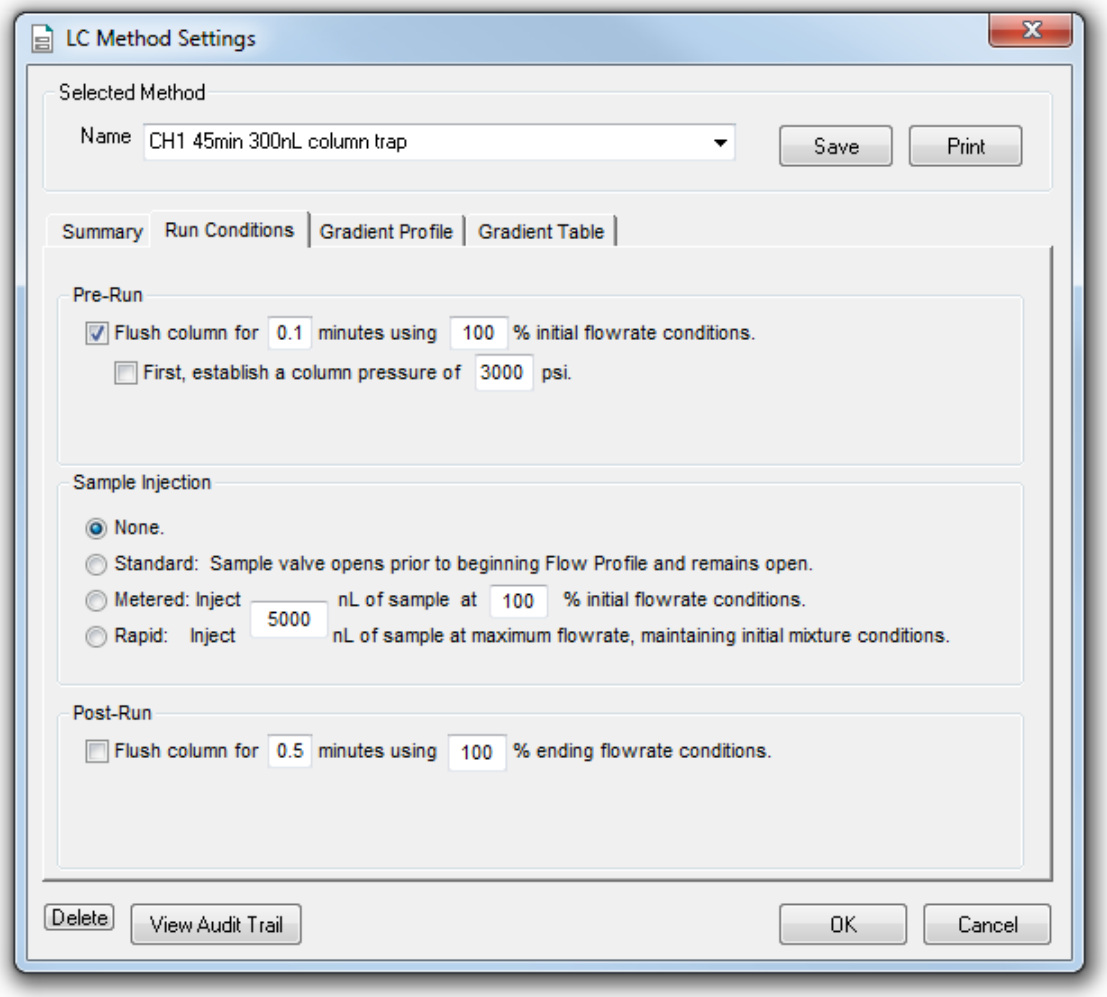


Table F-3 Gradient Method Parameters (Gradient Table Tab)

Step	Time	% A	% B	Event
1	0	95	5	
2	1	95	5	
3	16	75	25	
4	18	20	80	

Table F-3 Gradient Method Parameters (Gradient Table Tab) (continued)

Step	Time	% A	% B	Event
5	24	20	80	
6	27	95	5	
7	45	95	5	

Note: Flow rate is set to 300 nL/min.

Connecting NanoLC™ and Tempo™ Systems with a cHiPLC® System

G

This appendix includes wiring information required when performing the system integration test with a NanoLC™ or Tempo™ System and the cHiPLC® System.

Wire Connections

Table G-1 provides the wiring connections for a NanoLC™ System with the cHiPLC® System. The output on the NanoLC system (4-pin connector) is usually used for switching the external 10-port valve. However, the 10-port valve is not used when using the cHiPLC® System. Use the same communication ports, as shown in Table G-1.

Table G-1 Pin Assignments

Output on NanoLC System (Valve I/O)	Input on cHiPLC® System
GND	Pin 1 (Ground) [1st on Left]
Vlv 1	Pin 3 (Position B)
Vlv 2	Pin 2 (Position A)
Pin 10 (Channel I/O)	Pin 6 (Interlock signal)

Plumbing Diagrams H

This appendix includes two plumbing diagrams for the trap-and-elute configuration, one for external trap-and-elute, the other for the cHiPLC® System.

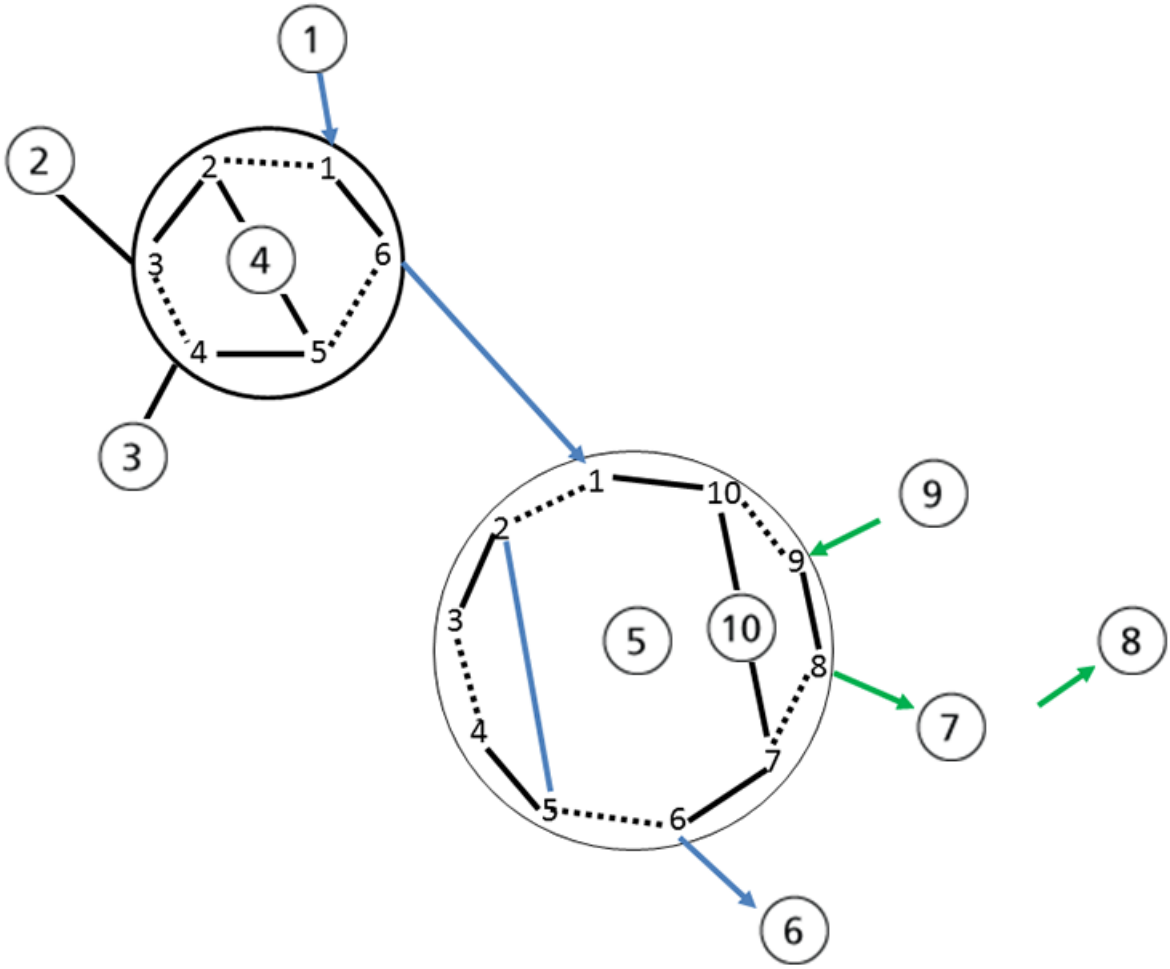
Table H-1 Legend for the Plumbing Diagrams

Item	Description
1	Loading pump
2	Syringe dispenser
3	Sample needle
4	Sample loop
5	10-port valve
6	Waste
7	Column
8	To mass spectrometer
9	Nanoflow pump
10	Trap
11	cHiPLC® valve
12	Filter
13	cHiPLC® column chip
14	cHiPLC® trap column
15	cHiPLC® trap/elute jumper chip
16	Union
———	Load
.....	Inject
———	25 µm i.d., 360 µm o.d.

Table H-1 Legend for the Plumbing Diagrams (continued)

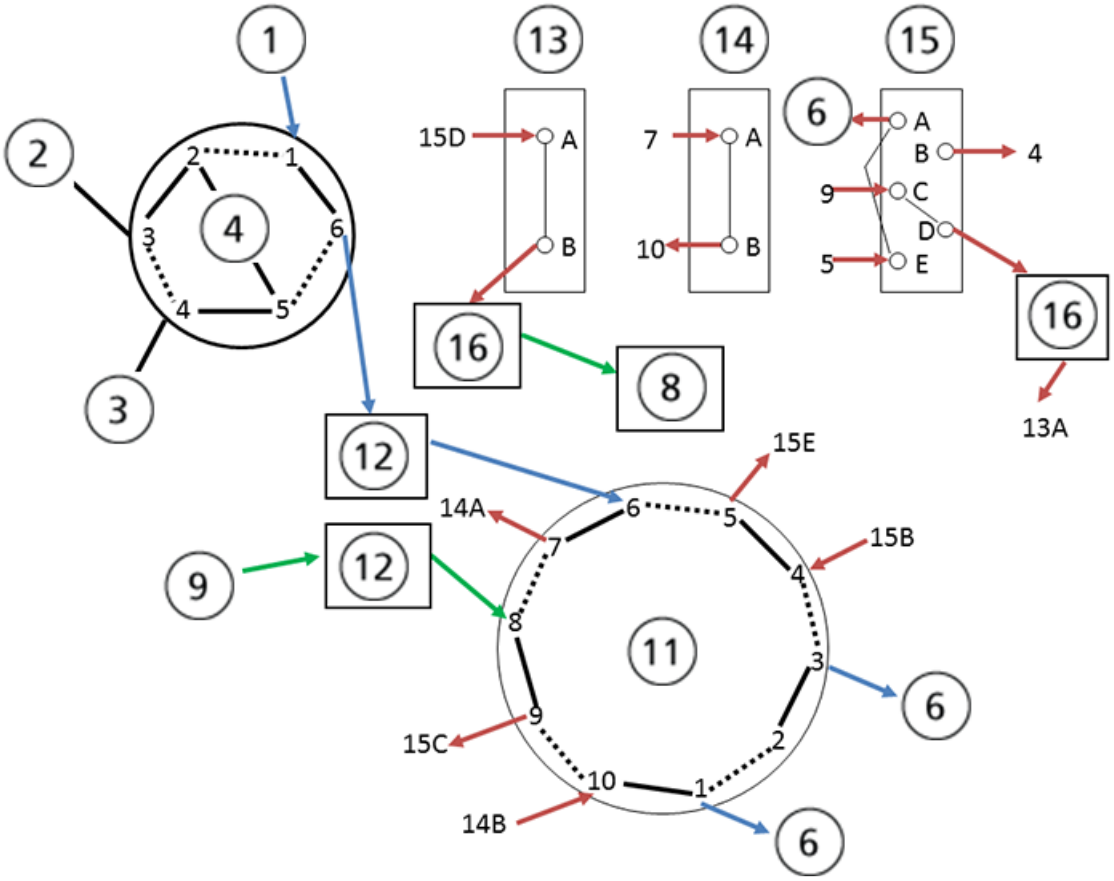
Item	Description
	30 µm i.d., 180 µm o.d.
	50 µm i.d., 360 µm o.d.

Figure H-1 Trap-and-elute Configuration with External Trap and Column



In Figure H-2, the nanoflow channel (usually gradient 2) is plumbed from pump to the filter attached to port 8 of the cHiPLC® System. The microflow channel (usually Channel 1) is plumbed from the pump to port 1 of the autosampler. Port 6 of the autosampler is plumbed to the filter attached to port 6 of the cHiPLC® System.

Figure H-2 Trap-and-elute Experiment with Optional cHiPLC® System



Best Practices

This section describes best practices and sample preparation techniques for a NanoLC System.

- Keep the system turned on and fluid flowing at all times. If the system is shut off for more than a few days, then purge and possibly calibrate the system prior to use. Refer to the appropriate operator guide.
- Use LC-MS-grade pre-made solvents such as those from Burdick-Jackson (that is, HPLC-grade water with 0.1% formic acid and acetonitrile with 0.1% formic acid). These solvents can be ordered from VWR:
  - PN BJLC452-2.5 - Water with 0.1% formic acid
  - PN BJLC441-2.5 - Acetonitrile with 0.1% formic acid

Note: We do not recommend using Milli-Q water because it is not of suitable quality for use in LC-MS systems.

- Verify that the gas flow rate and pressure are consistent. Do not disconnect the gas supply.
- Avoid biological growth.
  - Change solutions frequently.
  - Include 0.1% formic acid in all mobile phase bottles.
- Verify that the mobile phase solutions in the bottles match the mobile phase and composition values in the Eksigent Control Software (**System > Mobile Phases**).

Table I-1 Recommended Mobile Phase Mixtures

	Buffer A	Buffer B	Modifier
Gradient 1	100% water	100% acetonitrile	0.1% formic acid
Gradient 2	100% water	100% acetonitrile	0.1% formic acid
Loading pump	100% water	N/A	0.1% formic acid

- Remove air from the mobile phase bottles weekly by purging the system a minimum of 10 times per channel.
- Keep the pump seal wash bottle filled with 5% methanol and change the solutions quarterly.
- Empty the waste bottle once a week or more often if needed.
- Check the flow rate daily. Re-initialize the pressure transducers weekly.
- Pre-swage all fittings.

- When cutting silica, wash the end with methanol and flow solution through the cut end before connecting.
- Do not cut PEEK-lined fused silica tubing.
- Perform an autosampler Initial Wash or System Wash daily.

Sample Preparation Techniques

WARNING! Toxic Chemical Hazard. Refer to the chemical product *Safety Data Sheets* and follow all of the recommended safety procedures when handling, storing, and disposing of chemicals. For health and safety precautions, refer to the *Operator Guide*.

In general, the standard practices and procedures for reversed phase LC-MS/MS experiments using electrospray mass spectrometry also apply to the use of the NanoLC™ Systems, including the cHiPLC® System.

- Use HPLC- or MS-grade solvents at all times.
- Avoid the use of non-volatile salts and buffers such as CHAPS, phosphate, TRIS, HEPES, and perchlorates. These additives will foul the electrospray source and mass spectrometer orifice.
- Centrifuge all samples at 10 000 rpm for 5 minutes to remove particulates from the sample solution.
- Dilute all samples to a maximum of 5% organic modifier to prevent sample precipitation in the chromatographic system and at the electrospray source. This also ensures binding to the stationary phase.
- Make sure that detergents used during sample preparation are reduced to a concentration less than 0.01% prior to analysis.

Mass Assignments J

Table J-1 Beta-Galactosidase Mass Assignments

Peptide Fragment	Monoisotopic Molecular Weight (Da)	*m/z*	Charge State
ELNYGPHQWR	1298.62	433.88	3
FNDDFSR	899.38	450.7	2
YSQQQLMETSHR	1506.69	503.24	3
RDWENPGVTQLNR	1583.78	528.93	3
GDFQFNISR	1082.51	542.27	2
IDPNAWVER	1098.55	550.28	2
DVSLLHKPTTQISDFHVATR	2264.19	567.06	4
ITDSLAVVLQR	1213.70	607.86	2
VDEDQPFPAVPK	1340.66	671.34	2
LPSEFDLSAFLR	1393.72	697.87	2
DWENPGVTQLNR	1427.68	714.85	2
APLDNDIGVSEATR	1456.72	729.37	2
LSGQTIEVTSEYLFR	1741.89	871.95	2
VNWLGLGPQENYPDR	1756.85	879.43	2

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