STANDARD OPERATING PROCEDURE

Title: nano-Liquid Chromatography for Experiment 1 and Experiment 2

SOP#: WU-SOP-LC1-01

Version #: 2

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1. PURPOSE

This document describes the configuration and methods for *nano*-liquid chromatography (*nano*-LC) with parallel reaction monitoring (PRM) mass spectrometry of synthetic peptides in a complex sample matrix (i.e. tryptic digest of tumor lysate).

2. SCOPE

The procedures encompass the setup of a dual column *nano*-LC for generating the MS data for Experiments 1 and 2 as, described in the CPTAC document, "Assay development guidelines". The configuration, optimization and benchmarking of the mass spectrometer are described in WU-SOP-MS1-01.

3. RESPONSIBILITIES

It is the responsibility of person(s) performing this procedure to be familiar with laboratory safety procedures. The interpretation of results must be done by a person trained in the procedure and familiar with such interpretation.

4. EQUIPMENT

nano-LC consisting of a 2DPlus nano-LC (950-0061), cHiPLC system® (950-00070) and AS1 autosampler (Eksigent, Dublin,CA)

5. MATERIALS

Column: 200 µm x 15 cm ChromXP C_{18}-CL, 3 µm, 120 Å (Eksigent, 804-00001)
Injection loop: 20 µL PEEKsil™, 100 µm (Eksigent,)

Mobile Phase A. Water containing 0.1% formic Acid (Honeywell Burdick & Jackson, cat# LC452-2.5, 2.5L)

Mobile Phase B. Acetonitrile containing 0.1% formic Acid (Honeywell Burdick & Jackson, cat# LC441-2.5, 2.5L)

6. REAGENTS

A. Pour the vendor mixed solvents from the 2.5 L glass bottle to 250 mL glass media bottles.

B. Fill the 20 mL reservoir on the LC pump from the 250 mL glass media bottles.

Gradient 1, mobile phase A: 0.1% FA in H_2O
Gradient 1, mobile phase B: 0.1% FA in AcN

Gradient 2, mobile phase A: 0.1% FA in H_2O
Gradient 2, mobile phase B: 0.1% FA in AcN

7. PROCEDURE

A. **Instrument Configuration**
The flow path for the dual column system that is used to execute Experiments 1 and 2 is shown in Diagrams I and II. The Eksigent autosampler (AS1), LC pump1 (Eksigent 1) and LC pump 2 (Eksigent 2) are configured in the hardware profile in the Analyst® software. Methods for AS1, LC1 and LC2 are associated with the software by clicking each icon under Companion Software and selecting the appropriate .ini file. For further details, see user manual. The two channels, Analytical (Diagram I) or Calibrant (Diagram II) that are used to analyze samples (e.g calibrants or standard peptide H/L admixtures) is controlled by two event sequences and gradients as shown in the following Tables.

Table I. Autosampler method for instrument calibration run:

Step #	Operation	Value	Parameter	Speed	Height	Description
1	Valve		Injector Load			Valve Position Control
2	Output		1-OFF			Initialize LC channel1
3	Output		2-OFF			Initialize LC channel2
4	Output		2-ON			Start LC run on channel 2
5	Output		1-ON			Start LC run on channel 1
6	Wait	00:55:00				Pause for specified time
7	Aspirate	12.5	Reagent-1	1	6	Pick-up Reagent with specified volume.

8	Wait	00:00:05				Pause for specified time
9	Aspirate	0	Reagent-1	1	6	Aspirate specified volume
10	Aspirate	10	Sample	1	6	Aspirate specified volume
11	Wait	00:00:05				Pause for specified time
12	Aspirate	0	Sample	1	6	Aspirate specified volume
13	Aspirate	2.5	Reagent-1	1	6	Aspirate specified volume
14	Wait	00:00:05				Pause for specified time
15	Aspirate	0	Reagent-1	1	6	Aspirate specified volume
16	Valve		Injector Inject			Switch AS injector valve to Inject position (1-2)
17	Dispense	25	Waste	5	0	Dispense specified volume
18	Needle Wash	200	Port 1			Perform needle wash
19	END					

B. Eksigent 1 method for calibration column:

1) Flow rate (nL/min): 800
2) Temperature (ºC): 35
3) Run Conditions:
    i. Pre-run
    ii. Flush column for 2 min using 100% initial flow conditions.

Table II. Software "timetable" for Calibration sample

Time (min)	% Mobile phase B composition
0	2
1	2
14	90
	90
	2
	2

C. Eksigent 2 method for calibration column:

1) Flow rate (nL/min): 800
2) Temperature (ºC): 35
3) Run Conditions:
    i. Pre-run
    ii. Flush column for 2 min using 100% initial flow conditions.

Table III Timetable for elution for calibration column

Time (min)	% Mobile phase A composition	% Mobile phase B composition	Event
0	98	2	Valve Load
3	98	2	
	20	80	
	98		
			Valve Inject

Table IV. Autosampler method for analytical column						
Step #	**Operation**	**Value**	**Parameter**	**Speed**	**Height**	**Description**
1	Valve		Injector Load			Valve Position Control
2	Output		1-OFF			Initialize LC channel1
3	Output		2-OFF			Initialize LC channel2
4	Output		2-ON			Start LC run on channel 2
5	Output		1-ON			Start LC run on channel 1
6	Wait	3:30:00				Pause for specified time
7	Aspirate	12.5	Reagent-1	1	6	Pick-up Reagent with specified volume.
8	Wait	00:00:05				Pause for specified time
9	Aspirate	0	Reagent-1	1	6	Aspirate specified volume
10	Aspirate	10	Sample	1	6	Aspirate specified volume
11	Wait	00:00:05				Pause for specified time
12	Aspirate	0	Sample	1	6	Aspirate specified volume
13	Aspirate	2.5	Reagent-1	1	6	Aspirate specified volume
14	Wait	00:00:05				Pause for specified time
15	Aspirate	0	Reagent-1	1	6	Aspirate specified volume
16	Valve		Injector Inject			Switch AS injector valve to Inject position (1-2)
17	Dispense	25	Waste	5	0	Dispense specified volume
18	Needle Wash	200	Port 1			Perform needle wash
19	END					

D. Eksigent 1 method for analytical column:

1) Flow rate (nL/min): 800
2) Temperature (ºC): 35
3) Run Conditions:
    i. Pre-run
    ii. Flush column for 2 min using 100% initial flow conditions.

Table V. Gradient times	
Time (min)	**% Mobile phase B composition**
0	2
1	2
14	90
189	90
190	2
240	2

E. Eksigent 2 method for analytical column:

1) Flow rate (nL/min): 800
2) Temperature (ºC): 35
3) Run Conditions:

i. Pre-run
ii. Flush column for 2 min using 100% initial flow conditions.

Table VI Timetable for elution from column1			
Time (min)	**% Mobile phase A composition**	**% Mobile phase B composition**	**Event**
0	98	2	Valve Inject
3	98	2	
205	65	35	
215	20	80	
220	98	2	
240	98	2	Valve Load

F. Cycle Time for calibration and sample run

Calibration column	
steps for calibration run	**duration (min)**
gradient (2%B to 50%B)	73
high Acetonitrile "bump off"	14
reequilibrate to initial conditions	15
Total time (hours)	1.7
wash column/ blank run	190
reequilibrate to initial conditions	20
Cal solution Load on chip column	30
Total time (hours)	4

Analytical column	
steps for calibration run	**duration (min)**
wash column/ blank run	36
reequilibrate to initial conditions	19
Sample Load on chip column	47
Total time (hours)	1.7
gradient (2%B to 50%B)	205
high Acetonitrile "bump off"	15
reequilibrate to initial conditions	20
Total time (hours)	4

Purple = Eksigent 2, data acquisition
White = Eksigent 1, flow diversion

Diagram I Analytical Column Run

10-port position "INJECT"

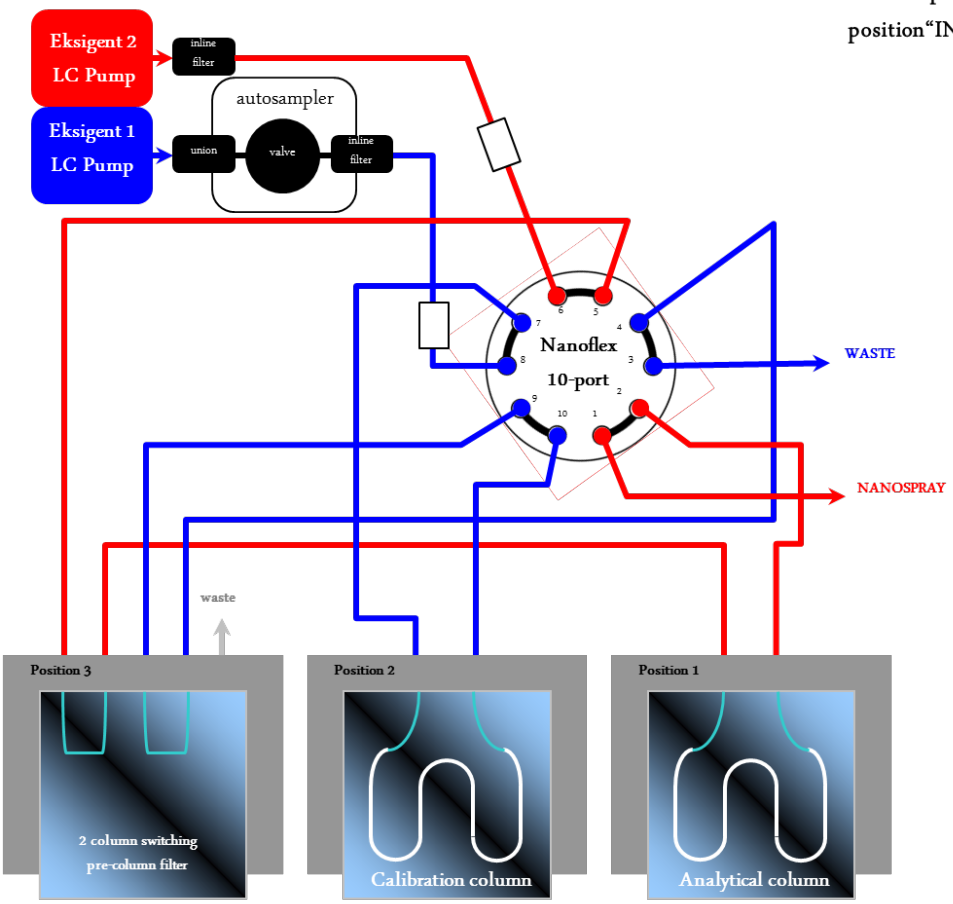
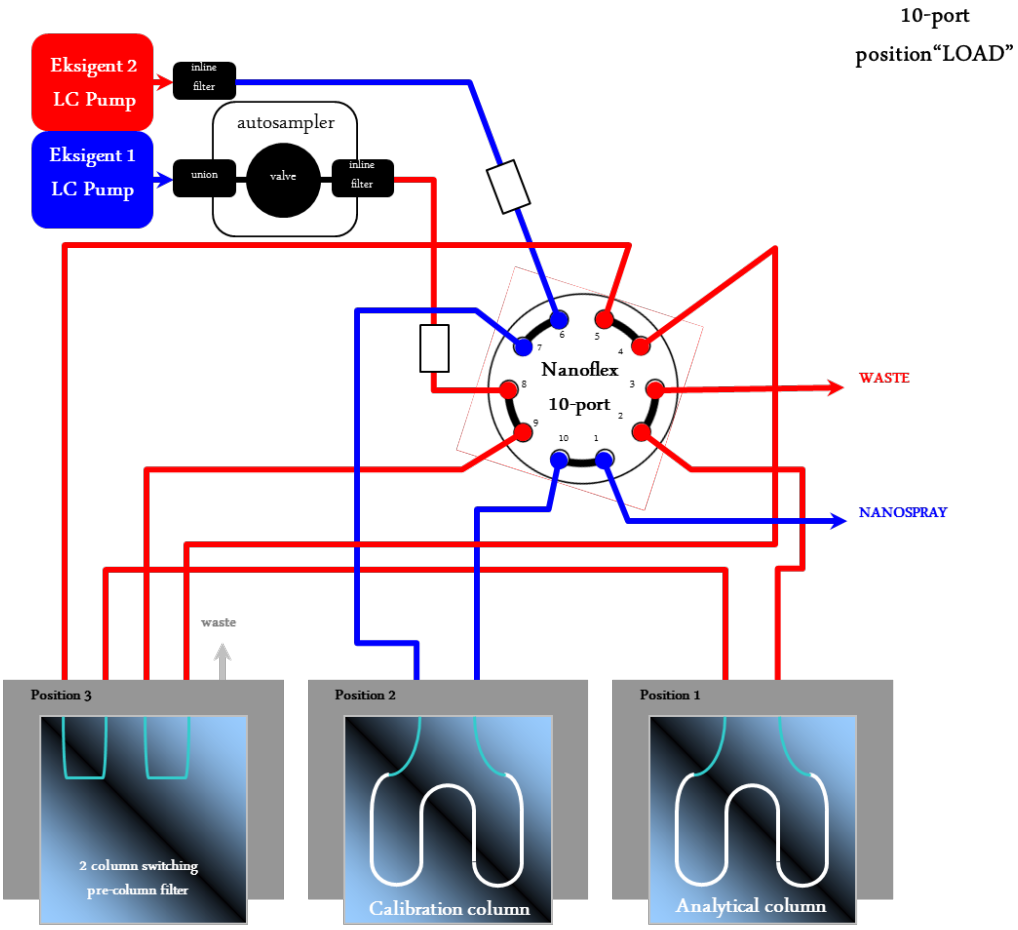


Diagram II Calibration Column Run



8. REFERENCED DOCUMENTS

A. WU-SOP-MS1-01- Optimizing Mass Spectrometer Performance for Experiments 1 and 2