STANDARD OPERATING PROCEDURE

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

SOP#: WU-SOP-LC2-02

Version #: 1

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Purpose

This document describes the configuration, benchmarks and gradient methods for nano-liquid chromatography (nano-LC) using an EASY nanoLC™ 1000 (https://tools.thermofisher.com/content/sfs/manuals/Man-60053-97227-EASY-nLC-1000-User-Man6005397227-C-EN.pdf) coupled to an Active Background Ion Reduction Device (ABIRD, ESI Source Solutions) that is interfaced to a triple quadrupole-Orbitrap (ThermoFisher, Q-Exactive™). The system is used to acquire scheduled full scan MS2 spectra (PRM) for the high-purity synthetic H/L peptide admixture given in WU-SOP-EXP1-02 (V3) in a complex matrix (tryptic digest of a pooled tumor lysate).

Scope

The procedures encompass the setup of a single 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-MS3-01 and WU-SOP-MS4-01.

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.

Equipment

- EASY-nLC™ 1000 (Thermo Scientific, LC120).
- Active Background Ion Reduction Device-ABIRD (ESI Source Solutions, Woburn Ma)

Materials

- EASY-Spray Column: 75 µm x 50 cm PepMap™ RSLC C18, 2 µm, 100 Å (Thermo Scientific, ES803)
- Injection loop: 20 µL PEEKsil™, 100 µm (Thermo Scientific, LC472)

Reagents

- 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, 2.5 L).
- Pierce Retention Time Calibration Mixture, Thermo Scientific (88321)
- Pierce HeLa Protein Digest Standard, Thermo Scientific (88328)

Solutions

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

- Fill the 25 mL reservoir on the LC pump from the 250 mL glass media bottles.
    - Pump-A, mobile phase A: 0.1% FA in water
    - Pump-B, mobile phase B: 0.1% FA in AcN

- Loading pump-S, mobile phase A: 0.1% FA in H_2O
- All solvents that go on the instrument are sonicated for 5 minutes to degas with the cap loosened. All reservoirs are rinsed 3 times before refilling with the appropriate mobile phase.

Procedure

1. Instrument Configuration
    a. The flow path for the EASY-nLC system that is used to execute Experiments 1 and 2 is shown in Diagram I. The Thermo Scientific EASY-nLC system is configured in the configuration file in the Xcalibur® software. Methods for the autosampler and LC are written in each method file. For further details, see user manual. The method used to analyze samples (e.g. calibrants or standard peptide H/L admixtures) is controlled by event sequence and gradient as shown in the following Tables.

Table I. Autosampler Method for PRM Sample Run:						
Step	**Operation**	**Value**	**Parameter**	**Speed**	**Pressure**	**Description**
1	Sample Pickup	2.5 µL	Injector Load	1 µL/min		Pull up sample into loop
2	Wait					Until column pre-equilibration is finished
3	Sample Load	7 µL	Injector Inject		800 bar	Loading sample onto column
4	Wait					Wait until gradient starts
5	Autosampler wash	100 µL				
6	END					

2. EASY-nLC method for PRM sample run:
    a. Flow rate (nL/min): 300
    b. Temperature (ºC): 50
    c. Run Conditions:
        i. Column pre-equilibration to initial conditions (20µL at 800bar)
        ii. Load sample for 7µL at 700bar
        iii. Prepare gradient

Table II. Timetable for Column Flow for PRM Sample Run Exp1 and 2		
Time (min)	**% Mobile phase A composition**	**% Mobile phase B composition**
0	100	0
5	100	0
112	70	30
113	5	95
120	5	95

Table III. Autosampler Method for System Performance Run						
Step	**Operation**	**Value**	**Parameter**	**Speed**	**Pressure**	**Description**
1	Sample Pickup	2 µL	Injector Load	1uL/min		Pull up sample into loop
2	Wait					Until column pre-equilibration is finished
3	Sample Load	6 µL	Injector Inject		800 bar	Loading sample onto column
4	Wait					Wait until gradient starts
5	Autosampler wash	100 µL				
6	END					

3. EASY-nLC method for PRTC and HeLa digest runs:
    a. Flow rate (nL/min): 300
    b. Temperature (ºC): 50
    c. Run Conditions:
        i. Column pre-equilibration to initial conditions at 800bar.
        ii. Load sample for 6µL at 800bar
        iii. Prepare gradient

Table IV. Timetable for Column Flow for System Performance PRTC's		
Time (Min)	**% Mobile Phase A Composition**	**% Mobile Phase B Composition**
0	100	0
2	100	0
32	70	30
33	5	95
36	5	95

Table V. Timetable for Column Flow for System Performance Hela Digest		
Time (min)	**% Mobile phase A composition**	**% Mobile phase B composition**
0	98	2
5	98	2
105	80	20
125	68	32
126	5	95
133	5	95

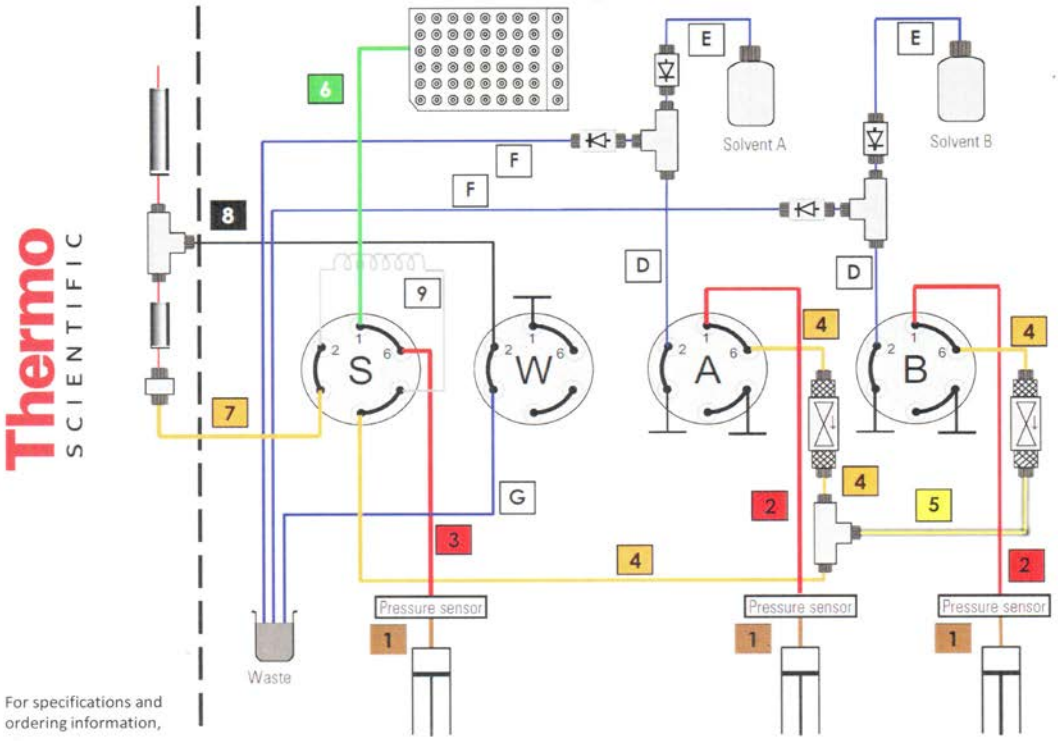
4. Cycle Time for system performance test and PRM sample run

System Performance Run PRTC's	
Steps for PRM Run	**Duration (Min)**
Re-equilibrate to initial conditions	57
Sample Load on chip column	15
gradient (0%B to 30%B)	112
High AcN bump off (95%B)	8
Total time (hours)	3.2

System Performance Run Hela Digest	
Steps for System Performance Run	**Duration (Min)**
Re-equilibrate to initial conditions	57
Sample Load on chip column	15
gradient (2%B to 30%B)	125
High AcN bump off (95%B)	8
Total time (hours)	3.41

PRM Sample Run	Exp1 / Exp2
Steps for PRM Run	**Duration (Min)**
Re-equilibrate to initial conditions	57
Sample Load on chip column	15
gradient (0%B to 30%B)	112
High AcN bump off (95%B)	8
Total time (hours)	3.2

Diagram I: flow path



ID	Connections	Tubing	Part number
1	Pump outlet to pressure sensor inlet	Stainless steel, 250 µm ID, 150 mm length	LC512
2	Pressure sensor outlet to valve A or B	Stainless steel, 250 µm ID, 150 mm length	LC513
3	Pressure sensor outlet to valve S	Stainless steel, 250 µm ID, 150 mm length	LC514
4	Mixing Tee to valve S, Valve A to flow sensor A, Valve B to flow sensor B, Flow sensor A to mixing Tee	nanoViper, 20 um ID, 350 mm length	LC522
5	Flow sensor B to mixing Tee	nanoViper, 10 um ID, 180 mm length	LC543
6	Autosampler needle connected to port 1 of valve S	PEEKsil™, 150 um ID, 550 mm length	LC302
7	Column Out tubing connected to port 3 of valve S	nanoViper, 20 um ID, 550 mm length	LC560
8	Waste In line, venting Tee to port 2 of valve W	nanoViper, 75 um ID, 550 mm length	LC562
9	Sample loop, 20 µL	nanoViper, 250 um ID, 410 mm length	LC472
D	Port 2 of valve A to check valve A Port 2 of valve B to check valve B	Teflon™, 500 um ID, 150 mm length	kit LC230
E	Tubing (2) from check valves to solvent bottles	Teflon, 500 um ID, 390 mm length	kit LC230
F	Tubing (2) from check valves to waste beaker	Teflon, 500 um ID, 390 mm length	kit LC230
G	Tubing from valve W to the waste beaker	Teflon, 500 um ID, 330 mm length	LC263

For a layout diagram, please

Referenced Documents

- WU-SOP-EXP1-02 (V3)-: "Preparation of Standard Peptide Samples for the Generation of Reverse Response Curves-Experiment 1"
- WU-SOP-MS3-01- "Optimizing Mass Spectrometer Performance for Experiments 1 and 2 on the Q-Exactive™ system".
- WU-SOP-MS4-01 – "Mass Spectrometry Using Parallel Reaction Monitoring for
- Experiments 1 and 2"

Abbreviations

- AcN, acetonitrile
- FA, formic acid
- LC-MS, nano-LC interfaced to a high-resolution Quadrupole-Orbitrap mass spectrometer as described in WU-SOP-LC2-02 and WU-SOP-MS4-01
- H or heavy, stable isotopically labeled synthetic peptide
- L or light, natural abundance synthetic peptide
- Q.S., quantum satis
- PDX, patient-derived xenografts
- PRM, parallel reaction monitoring
- PRTC- Pierce Retention Time Calibration Mixture