

# STANDARD OPERATING PROCEDURE

**Title:** Liquid Chromatography using an Eksigent NanoLC-2D, an Eksigent AS1 autosampler, two Agilent 10-port valves, a C18 trap column, and a ReproSil C18 analytical column for immuno-MRM experiments

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**Author:** Paulovich Lab

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## Purpose

The purpose of this document is to describe the liquid chromatography setup and methods upstream of a mass spectrometer capable of MRM for quantitative analysis of immunoaffinity-enriched peptide samples.

## Scope

This procedure includes the setup of the autosampler and LC and the corresponding methods. It is specific to the operation of an Eksigent NanoLC-2D, an Eksigent AS1 autosampler, and the use of two Agilent 10-port valves to enable back-flushing of the C18 trap column for the last 2 minutes of a 38 minute method.

## 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

- Eksigent NanoLC-2D
- Eksigent autosampler (Spark, 920)
- Two Agilent 1100 Series 2/10 Micro Valves (G1163A)
- Column heater (PST-CHC and PST-CH-10 or PST-CH-15, Phoenix S&T)

## Materials

- PEEKsil™ sample loop, 10 µL, 1/16" OD (620-00039, Eksigent)
- 50 µm ID, 360 µm OD fused silica capillary (TSP050375, Polymicro Technologies)

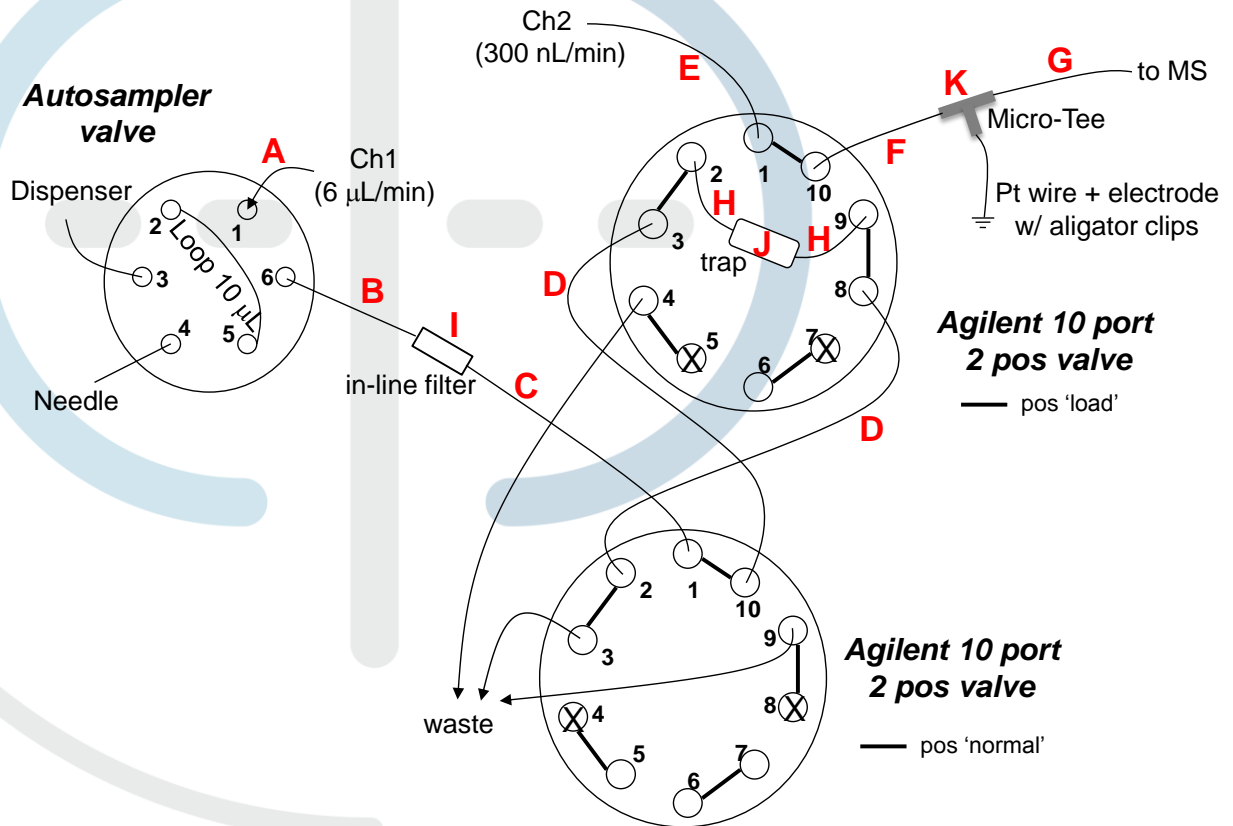
- 25  $\mu\text{m}$  ID, 360  $\mu\text{m}$  OD fused silica capillary (TSP025375, Polymicro Technologies)
- 50  $\mu\text{m}$  ID, 1/32" OD, 20 cm PEEKsil™ tubing (35020, Western Analytical)
- 25  $\mu\text{m}$  ID, 1/32" OD, 15 cm PEEKsil™ tubing (32515, Western Analytical)
- 0.5  $\mu\text{m}$  in-line solvent MicroFilter (M-520 and M-120X, Western Analytical)
- 300  $\mu\text{m}$  ID x 5 mm LC Packings trap column (Acclaim PepMap 100 C18, 5  $\mu\text{m}$ , 100 Å, 160454, Dionex) in a precolumn holder (160431, Dionex)
- Micro-Tee (P-875, Western Analytical)
- 75  $\mu\text{m}$  ID, 360  $\mu\text{m}$  OD IntegraFrit column (IF360-75-50-N-5, New Objective), packed in-house to 10.0 cm with ReproSil-Pur C18-AQ, 3  $\mu\text{m}$  (Dr. Maisch GmbH, Ammerbuch, Germany), and connected to an Advance CaptiveSpray ion source (Bruker)
- 20  $\mu\text{m}$  ID CaptiveSpray tapered tip (SS9/25000/20, Bruker), for use with an Advance CaptiveSpray ion source
- Water, Optima® LC/MS, suitable for UHPLC-UV (W6-4, Fisher Scientific)
- Acetonitrile (ACN), Optima® LC/MS, suitable for UHPLC-UV (A955-4, Fisher Scientific)
- Formic acid (FA) (1.11670.1000, EMD Millipore)
- 2-Propanol, Optima® LC/MS, suitable for UHPLC-UV (A461-4, Fisher Scientific)

## Solutions

- Autosampler Reagent Reservoirs 1 and 3: 0.1% FA in water
- Autosampler Needle wash solution: 20% 2-propanol in water
- Channel 1, mobile phase A: 0.1% FA in water
- Channel 1, mobile phase B: 0.1% FA in 90% ACN in water
- Channel 2, mobile phase A: 0.1% FA in water
- Channel 2, mobile phase B: 0.1% FA in 90% ACN in water

## Procedure

### 1. Autosampler and LC plumbing



**A, B, C:** 50 µm ID, 360 µm OD fused silica capillary

**D:** 20 cm, 50 µm ID, 1/32" OD PEEKsil™ tubing

**E, F:** 25 µm ID, 360 µm OD fused silica capillary

**G:** ~25 cm x 75 µm ID, 360 µm OD IntegraFrit column, packed to 10.0 cm w/ ReproSil-Pur AQ, C18, 3 µm (Dr. Maisch GmbH); heated to 45 °C using a Phoenix column heater

**H:** 15 cm, 25 µm ID, 1/32" OD PEEKsil™ tubing

**I:** 0.5 µm in-line solvent MicroFilter

**J:** 300 µm ID x 5 mm LC Packings trap column

**K:** Micro-Tee

## 2. Autosampler method

Autosampler temperature: 8 °C

Step	Operation	Value	Unit	Parameter	Speed	Height	Description
1	Output			1-OFF			TTL Output control
2	Output			2-OFF			Initialize LC channel 2
3	Valve			Injector Load			Switch AS injector valve to Load position (1-6)
4	Aspirate	9	uL	Reagent-1	1	5	Pick-up Reagent with specified volume
5	Aspirate	0	uL	Reagent-1	1	5	Pick-up Reagent with specified volume
6	Aspirate	11	uL	Sample	1	9	Pick-up Sample with specified volume
7	Aspirate	0	uL	Sample	1	9	Pick-up Sample with specified volume
8	Aspirate	0	uL	Reagent-3	1	5	Pick-up Reagent with specified volume
9	Aspirate	5	uL	Reagent-1	1	5	Pick-up Reagent with specified volume
10	Output			1-ON			Start LC run on channel 1
11	Output			2-ON			Start LC run on channel 2
12	Valve			Injector Inject			Switch AS injector valve to Inject position (1-2)
13	Marker			Inject			
14	Wait	00:05:00	hr:min:sec				Pause for specified time
15	Valve			Injector Load			Switch AS injector valve to Load position (1-6)
16	Dispense	25	uL	Waste	3	0	Dispense specified volume from syringe to Waste
17	Needle Wash	100	uL	Port 1			Perform needle wash
18	END						

## 3. LC Channel 1 method

- a. Flow rate: Conserved flow, 6.0  $\mu$ L/min
- b. Run conditions:
  - i. Pre-Run: Flush column for 0.1 minutes using 100% initial flow rate conditions.
  - ii. Sample injection: None.
- c. Timetable:

Time (min)	Mobile phase A composition (%)	Mobile phase B composition (%)
0	97	3
38	97	3

#### 4. LC Channel 2 method

- a. Flow rate: Independent flow (see Timetable below)
- b. Run conditions:
  - i. Pre-Run: Flush column for 0 minutes using 100% initial flow rate conditions.
  - ii. Sample injection: None.
- c. Timetable:

Time (min)	Qa (nL/min)	Qb (nL/min)
0	285	15
4	285	15
22	150	150
23	30	270
24	30	270
24	285	15
29	285	15
29	332	17
38	332	17

These flow rates yield the following effective mobile phase compositions:

Time (min)	Effective mobile phase A composition (%)	Effective mobile phase B composition (%)
0	95	5
4	95	5
22	50	50
23	10	90
24	10	90
24	95	5
29	95	5
29	95	5
38	95	5

5. Agilent 10 port valve methods for back-flushing the trap column for 2 minutes at the end of a 38 min run

1. Timetable for the 10 port valve that includes the trap column and the capillary from LC channel 2 (see the Autosampler and LC plumbing diagram above):

Line	Time (min)	Position	Position Description
1	0.00	Position 1	load
2	4.00	Position 2	elute
3	35.50	Position 1	load

2. Timetable for the 10 port valve that includes the capillary from LC channel 1 (see the Autosampler and LC plumbing diagram above):

Line	Time (min)	Position	Position Description
1	0.00	Position 1	normal
2	35.50	Position 2	back-flush
3	37.90	Position 1	normal