Supplementary Methods for "High sensitivity quantitative proteomics using accumulated ion monitoring and automated multidimensional nano-flow chromatography"

Here we describe a chromatographic system that enables automated pre-fractionation of tryptic peptides by high-resolution SCX chromatography prior to capillary reverse phase separation on line with electrospray emitter (Figure 1). This chromatographic mode was chosen because of its efficiency in resolving phosphorylated peptides, and because the mobile phases used are compatible with subsequent reverse phase.

The protocol described was optimized to overcome several technical issues. However, the protocol might require further refining to meet specific experimental requirements. In particular, we found that salt precipitation in the HPLC lines may be a major cause of clogs, as efficient elution requires mobile phases containing relatively high (up to 1M) salt concentration. Traditionally SCX fractionation used a gradient of potassium chloride in phosphate buffer to provide eluting cations, but this salt is not desirable for capillary chromatography because it may form precipitates, thus clogging capillary lines and fittings, and is not volatile, thus interfering with electrospray ion sources. Ammonia cations, with either acetate or formate as counter-ions, alleviated these issues. In addition, the risk of precipitation was further reduced by flushing the injection system and relevant HPLC components with 0.1% formic acid (Supplementary figures 1f). A second issue considered was column geometry, as this parameter is critical to achieve adequate resolution. While short and wide-bore (>150 μm) trap-like columns are desirable because of their low resistance and robustness, we found that this geometry does not enable sufficient resolution for SCX fractionation. We implemented instead a 25 cm x 100 μm inner diameter column. In a step gradient, sub-optimal elution volumes may significantly reduce chromatographic separation. In particular, if the salt plug volume is excessive, mass elution becomes the dominant regime, deteriorating chromatographic resolution. Likewise, the volumes of mobile phase necessary to drive sample and SCX-plugs may also contribute to gradient-independent mass elution. To alleviate this, the chromatography method was optimized to minimize the volume of mobile phases driven through the SCX column (Supplementary figures 1b, 1c, and 1d). On the other hand, since insufficient plug volume results in incomplete elution, we suggest to empirically verify complete recovery using the specified plug volume. We observed that loading salt plugs into the injection loop was a major source of technical variability, because the volume loaded might be incorrect (this can happen if air bubbles form in the syringe pump), or because the element of liquid containing the elution plug was diluted by the flanking element of LC buffer. To alleviate this issue, a high-efficiency SCX elution method was designed to

first fill the injection loop entirely with an excess of elution buffer, then drive the content of the loop using the isocratic pump to equilibrate the lines before and after the SCX column, and finally, by including the SCX column in the flow-path for the exact time required to deliver the plug volume. Once the required volume of salt solution has been delivered, the loop is excluded from the flow path to prevent mass elution.

MATERIALS

Reagents

- Formic Acid 99+% (Thermo Scientific, part nr. 28905)
- Acetonitrile Optima LC/MS grade (Fisher Scientific, par nr. A955-212)
- Water Optima LC/MS grade (Fisher Scientific, par nr. W64)
- Methanol (Honeywell, part nr. LC230)
- Formamide (Sigma Life Science, part nr. F9037)
- Ammonium hydroxide (Sigma-Aldrich, part nr. 44273)
- Ammonium formate (Sigma-Aldrich, part nr. 55674)
- Ammonium bicarbonate (Sigma-Aldrich, part nr. 09830)
- Dithiothreitol (Sigma Life Science, part nr. D9163)
- Iodoacetamide (Sigma Life Science, part nr. I6125)
- Guanidine Hydrochloride (Sigma Life Science, part nr. G3272)
- LysC protease (Wako Pure Chemical Industries, part nr. 125-05061)
- Porcine trypsin protease, sequencing grade (Promega, part nr. V5111)
- Bovine Serum Albumin (Sigma Life Science, part nr. A3059)
- Casein (Sigma Life Science, part nr. C6780)
- KaSil potassium silicate solution (PQ Corporation, part nr. COS.301341)
- SCX stationary phase: Polysulfoethyl A 5μm, 200Å particles (PolyLC, Part nr. 410.992.5400)
- Trap column stationary phase: POROS10 R2 reversed phase 10μm, 2000Å particles (Applied Biosystems, part nr. 1-1118-02)
- Analytical column stationary phase: ReproSil-Pur 120 C18-AQ, 1.9 μm particles (Dr. Maisch GmbH, part nr. r119.aq.)
- Compressed High purity helium, with pressure regulator manifold
- PhoStop phosphatase inhibitor tablets (Roche Applied Science, part nr. 04906845001)

Consumables

- 1.7 ml maximum recovery tubes (Axygen, par nr. MCT-175-L-C)
- 2 ml glass ampoules, flat bottom (Kimble Chase, part nr. 60940A 2)
- Fused silica with 75µm inner diameter (ID) and 360-µm outer diameter (OD) (Polymicro, cat. no. TSP075375)
- Fused silica with 150-µm ID and 360-µm OD. (Polymicro, cat. no. TSP150375)
- Fused silica with 100-µm ID and 360-µm OD. (Polymicro, cat. no. TSP100375)
- Fused silica with 50-µm ID and 360-µm OD. (Polymicro, cat. no. TSP050375)
- Integrated ferrule/nut (Valco, part nr. C360NFPK)
- 0 dead volume metal unions, 50-µm ID (Valco, part nr. C360UFS2)

- Fitting ferrules 1/32OD (SCIEX, part nr. 910-00087)
- Nuts 1/32OD (SCIEX, part nr. 910-00085)
- Sleeves, PEEK, 1/32OD, .015"ID, (SCIEX, part nr. 910-00088)
- Quick mount tee, 0 dead-volume 50-μm ID (Valco, part nr. C360QTPK2)
- Glass filters for solvent inlet, 2 μm (Agilent, part nr. 5041-2168)
- Autosampler vials
- Autosampler caps
- Magnetic stirrer 2 mm (Sigma Aldrich)
- ESI uncoated silica emitters, tip 2 μm ID. Alternatively, 10 μm ID emitters can be used from New Objective (part nr. FS360-20-10-D-20)
- C18 solid phase extraction macro spin column (Nest Group, part nr. SMM SS18V)

Equipment

- Silica capillary cutter
- Optional: silica capillary end polishing station
- Thermostated heating block
- Vortex mixer
- Pressure vessel for capillary packing, equipped with gas manifold
- Magnetic stirrer
- Silica capillary laser puller (Sutton instruments)
- Nano HPLC system (Eksigent nanoLC400)
- Thermo Fusion mass spectrometer (Thermo Scientific)
- Picoview ESI ion source (New Objective)
- Computer station, with MaxQuant 1.5 and related software components.

EQUIPMENT CONFIGURATION

The chromatographic system (Figure 1) was designed for a chromatographic system including two 10-port rotary valves and one 6-port valve, along with one autosampler module, one binary nano pump (flow-rate range 100-500 nl/min) and one isocratic micro-pump (flow-rate range 0.5-5 μl/min). In our configuration, the final reverse phase column is in line with a nano- electro-spray ion source, and thus with a high resolution mass spectrometer capable of recording both precursor and fragmentation ions.

In this system, solution in autosampler vials are initially aspirated in an element of peek tubing (10 µl volume), and then driven by the isocratic pump either to the SCX column or directly to the RP trap column. The flow-through from the SCX column may be routed either to the trap column, thus loading for the subsequent reverse phase, or to waste, as in the case of SCX column regeneration (Supplementary figures 1b-e, and 1f).

For final reverse phase chromatography, we adopted a vented configuration in which peptides are loaded at high flow-rate on a trap column and coincidentally desalted, while the flow-through is directed to waste. Once the trap column is put on line with the analytical column, peptide resolution is obtained by an acetonitrile gradient (Supplementary figures 1f).

PROCEDURE

Columns preparation

1. Frit empty silica capillary, as described in Dhabaria et al. (2015, doi:10.1038/protex.2015.049), to obtain 60 cm (75 µm and 100 µm ID) or 30 cm (150 µm ID) empty columns.
2. Using the silica capillary cutter, cut the fritted end to obtain a 2 mm frit.
3. Optional: inspect the fritted end to verify optimal cut and/or polish the end using the silica end polishing station.
4. Dissolve stationary phases in methanol, in clean glass ampoules.
5. Wash particles 3 times in methanol (let silica particles deposit on the bottom of the vial, carefully aspire the methanol before resuspending the particles in fresh methanol).
6. Fill fritted capillary using the pressure vessel. Regulate helium pressure tot <2000 psi (140 bar) and <500 psi (35 bar) for 75µm ID and 100-150 µm ID columns respectively.
- Analytical RP column: 75 µm ID
- SCX column: 100 µm ID
- Trap RP column: 150 µm ID.
7. Pack columns. Mount the columns onto the HPLC outlet drive 10 column volumes (CV) of 0.1% formic acid (FA) in 5% acetonitrile (ACN) in water, followed by 10 CV 0.1% FA in 85% ACN in water. Repeat ten times, and verify that pressure remains stable for at least one hour under continuous operation at fixed composition and flow-rate.
8. Visually inspect the columns to ensure homogenous packing.

9. Slide the open side of each column into an integrated ferrule, and cut the column to the final length, immediately before mounting them:
- SCX column: 250 mm
- RP trap column: 40 mm
- RP analytical column: 400 mm.

Assemble chromatographic setup

10. Assemble the chromatographic setup as described in Figure 1. All silica capillaries used for connections are 50 µm ID.
11. Prepare LC mobile phases:
- Buffer A: 0.1% FA in water
- Buffer B: 0.1% FA in acetonitrile
- Needle wash: 50% methanol in water.

  Mobile phases should be prepared in light-protected clean glass bottles, and mobile phase line should be provided with glass filters.
12. FACULTATIVE: Set column heater to 50 °C, and wait for temperature to stabilize. CAUTION: Prolonged exposure to temperatures >65 °C may accelerate degradation of silica-based stationary phases.
13. Monitoring backpressure, verify correctness of flow-path and absence of leaks. Expected measured pressures are as follow (note that pack pressure may vary by up to 15% because of frits features and environmental temperature):

Columns tested	Flowrate (buffer A)	Driving pump	Injector Valve position	Valve A position	Valve B position	Expected pressure (psi)
SCX	2 µl/min	Isocratic	1-2	1-2	1-2	4500
Trap RP	2 µl/min	Isocratic	1-2	1-10	1-10	500
Analytical + trap RP	200 nl/min	Binary	NA	1-10	1-2	5000 (50°C) 6000 (25°C)
SCX + trap RP	1 µl/min	Isocratic	1-2	1-10	1-2	5000
SCX + trap RP + loop	1 µl/min	Isocratic	1-6	1-10	1-2	5500

14. Prepare salt plugs, mixing 0.1% formic acid (pH=2.8) and 1M ammonium formate, 0.1% formic acid (pH 2.8). Since ammonium formate is volatile, salt solution in autosampler vials should be replaced at least weakly. Stock solution

should be kept in clean glass bottles and refrigerated. Ammonium formate powder should be stored in a desiccator cabinet. Plug concentration for 5X and 10X SCX fractionation are suggested.

Salt concentration (mM)	0.1% formic acid (µl)	1M amm. formate/0.1% formic acid (µl)	5X SCX	10X SCX
0	1000	0		X
50	950	50	X	X
75	925	75		X
100	900	100	X	X
125	875	125		X
150	850	150	X	X
200	800	200		X
300	700	300		X
500	500	500		X
1000	0	1000	X	X

15. Prepare SCX reconditioning buffer: 1M ammonium formate, 25% ACN. Adjust pH to 9 using NH_3OH.

Program LC operations

16. The following HPLC programs are optimized for an Eksigent nanoLC400 instrument, with the setup described.

- Program 1: SCX sample load and low-resolution SCX elution

#	Command	Description
1	Initialize	Autosampler Device
2	Valve	Switch injection valve to load (6-1)
3	Valve	Switch valve A to load (1-10)
4	Valve	Switch valve B to SCX (1-10)
5	Wash	Pre-wash needle using washing buffer
6	Wait	For binary pump ready to start
7	Wait	For isocratic pump ready to start
8	Get sample	5 µl pick up, 13.6 µl: 2 mm from bottom*
9	Valve	Switch injection valve to Inject (1-2)
10	Start	Start isocratic pump (1 µl/min buffer A, 20 minutes)
11	Wait	For isocratic pump injection complete
12	Start	Start binary pump (see below)
13	Valve	Switch valve A to inject (1-2)

14	Wash	Pre-wash needle using washing buffer
15	Wait	For binary injection complete

*: sample volume may vary. The syringe pump draws first a volume of buffer A equal to 13.6 μl minus sample volume + 2 μl, then the sample, then 2 μl buffer A.

Binary pump gradient

Time (min)	Flow-rate (nl/min)	Buffer A (%)	Buffer B (%)
0	250	95	5
5	250	95	5
65	250	62	38
70	250	20	80
100	250	20	80
102	250	95	5
120	250	95	5

- Program 2: high-resolution SCX elution (this program minimizes mass elution from mobile phase used to drive salt plugs through the SCX column).

#	Command	Description
1	Initialize	Autosampler Device
2	Valve	Switch injection valve to load (6-1)
3	Valve	Switch valve A to load (1-10)
4	Valve	Switch valve B to 1D (Inject, 1-10)
5	Wash	Pre-wash needle using washing buffer
6	Wait	For binary pump ready to start
7	Wait	For isocratic pump ready to start
8	Get sample	Fill loop with sample/plug (35μl): 2 mm from bottom*
9	Valve	Switch injection valve to Inject (1-2)
10	Start	Start isocratic pump (1 μl/min buffer A, 20 minutes)
11	Valve	Switch injection valve to inject (1-2)
12	Wait	1:30 minutes
13	Valve	Switch valve B to SCX (load, 1-10)
14	Wait	4 minutes
15	Valve	Switch injection valve to load (1-2)
16	Wait	4 minutes
17	Start	Start binary pump (see below)
18	Valve	Switch valve A to inject (1-2)
19	Valve	Switch valve B to 1D (inject, 1-2)
20	Wait	15 minutes
21	Wash	Pre-wash needle using washing buffer
22	Wait	For binary injection complete

Binary pump gradient

Time (min)	Flow-rate (nl/min)	Buffer A (%)	Buffer B (%)
0	250	95	5
5	250	95	5
65	250	62	38
70	250	20	80
100	250	20	80
102	250	95	5
120	250	95	5

- Program 3: Single dimension RP analysis

#	Command	Description
1	Initialize	Autosampler Device
2	Valve	Switch injection valve to load (6-1)
3	Valve	Switch valve A to load (1-10)
4	Valve	Switch valve B to 1DRP (1-2)
5	Wash	Pre-wash needle using washing buffer
6	Wait	For binary pump ready to start
7	Wait	For isocratic pump ready to start
8	Get sample	5 μl pick up, 13.6 μl: 2 mm from bottom*
9	Valve	Switch injection valve to Inject (1-2)
10	Start	Start isocratic pump (1 μl/min buffer A, 20 minutes)
11	Wait	For isocratic pump injection complete
12	Start	Start binary pump (see below)
13	Valve	Switch valve A to inject (1-2)
14	Wash	Pre-wash needle using washing buffer
15	Wait	For binary injection complete

*: sample volume may vary. The syringe pump draws first a volume of buffer A equal to 13.6 μl minus sample volume + 2 μl, then the sample, then 2 μl buffer A.

Binary pump gradient

Time (min)	Flow-rate (nl/min)	Buffer A (%)	Buffer B (%)
0	250	95	5
5	250	95	5
65	250	62	38
70	250	20	80
100	250	20	80
102	250	95	5
120	250	95	5

- Program 4: columns reconditioning and equilibration. SCX column is reconditioned injecting 10 μl SCX reconditioning buffer (1M ammonium formate, 25% ACN, pH 9), with flow-through directed to waste. Reverse phase columns are reconditioned and equilibrated in parallel.

#	Command	Description
1	Initialize	Autosampler Device
2	Valve	Switch injection valve to load (6-1)
3	Valve	Switch valve A to Inject (1-2)
4	Valve	Switch valve B to SCX (1-10)
5	Wash	Pre-wash needle using washing buffer
6	Wait	For binary pump ready to start
7	Wait	For isocratic pump ready to start
8	Get sample	Fill loop with SCX recondition buffer (15 μl): 2 mm from bottom*
9	Valve	Switch injection valve to Inject (1-2)
10	Start	Start isocratic pump (1 μl/min buffer A, 65 minutes)
14	Start	Start binary pump (see below)
17	Wait	For binary injection complete

Binary pump gradient

Time (min)	Flow-rate (nl/min)	Buffer A (%)	Buffer B (%)
0	250	80	20
10	250	20	80
40	250	20	80
45	250	95	5
70	250	95	5

17. Prepare silica nanoESI emitters, as described in Cifani et al., (2015 doi:10.1038/protex.2015.053). Alternative, commercial 10 μm ID emitters can be used.
    FACULTATIVE: Setting the emitters to be washed in 50% MetOH in water during sample loading and SCX fractionation highly improves maintaining ionization efficiency constant over time, by reducing depositing of salt and organic matter on the tip ID. Optional: narrow bore emitters require careful voltage optimization for optimal performance. Progressively decreasing voltage as a function of water content in the buffer highly improves spray efficiency and stability. For the suggested elution profile and flow-path, the following voltage gradient may be used:

Time (min)	Voltage (V)
0	1750
20	1700
30	1650
40	1600
50	1550
60	1500
70	1450
119	0*

*: emitter moved to washing station

18. Set the mass spectrometer to perform precursor scan every 2 seconds, followed by data-dependent precursor selection and fragmentation by HCD.

Preparation of SCX peptide standard

19. Prepare a 2 mg/ml solution of BSA and casein in 1M guanidine hydrochloride/100 mM ABC pH 8.5. Mix equal volumes of the two solutions to obtain a 1:1 (v/v) BSA/casein standard solution, and freeze in 500 μl aliquots.
20. To 500 μl standard, add 55 μl 100 mM DTT, 100mM ABC (freshly prepared). Incubate at 56°C for 30 minutes.
21. Let the solution cool to room temperature (25°C).
22. Add 60 μl 550 mM iodoacetamide/100mM ABC (freshly prepared). Incubate at room temperature for 20 minutes, in the dark.
23. Add 65 μl 100 mM DTT, 100mM ABC (freshly prepared). Incubate at 56°C for 20 minutes.
24. Add 335 μl 100 mM ABC.
25. Add 50 μl of 0.2 μg/μl LysC protease in 100 mM ABC. Incubate 8 hours at 37°C.
26. Transfer half the LyC digest (525 μl), in a clean tube. Acidify with 1% (5 μl) formic acid, and freeze.
27. To the remaining 525 μl LysC digest, add 975 μl of 50 mM ABC.
28. Add 50 μl of 0.2 μg/μl Trypsin protease in 100 mM ABC. Incubate 16 hours at 37°C.
29. Acidify with 1% (15 μl) formic acid.
30. Mix 3 volumes tryptic digest with 1 volume LysC digest and mix by vortexing.
31. Desalt by solid phase extraction, using 4 macro spin columns.

Step	Buffer	Volume (μl/column)	Centrifugation
Activation	99.9% ACN/0.1% FA	300	100 rcf/ 60 s
Equilibration	99.9% water/0.1% FA	2 x 300	100 rcf/ 60 s
Loading	sample	500	100 rcf/ 120 s
Wash#1	99.9% water/0.1% FA	300	100 rcf/ 120 s
Wash#2	99.9% water/0.1% FA	300	200 rcf/ 120 s
Elution	60% ACN in water/0.1% FA	400	200 rcf/ 120 s

32. Divide eluate in ten 100 μl aliquots (100 μg/aliquot) and vacuum centrifuge to dryness.
33. Just before injection, resuspend one aliquot in 200 μl 0.1% FA/3% ACN (0.5 μg/μl).

SCX standard quality control

34. Using LC Program 3 (Single dimension RP analysis), analyze 1 μg (2 μl) SCX standard.
35. Submit the MS raw file to MaxQuant for peptide spectral matching against a fasta file containing the protein sequences of BSA and casein. Set C-carbamidomethylation as fixed modification. Set M, oxidation, S/T/Y-phosphorylation and N/Q deamidation as variable modifications. Allow for up to 5 missed cleavages.

SCX recovery

36. Recondition and equilibrate columns using LC Program 4 (columns reconditioning and equilibration).
37. Blank SCX load. Using LC Program 1 (SCX sample load) inject 5 μl 0.1% FA in water.
38. Blank SCX elute. Using LC Program 1 inject 10 μl SCX reconditioning buffer (1M ammonium formate/ 25% ACN, pH=9). Repeat once, and search all MS raw files generated in step 37 and 38 as described in step 35. Verify absence of contaminants.
39. Recondition and equilibrate columns using LC Program 4 (columns reconditioning and equilibration).

40. Standard SCX load. Using LC Program 1 (SCX sample load) inject 2 μl of SCX standard
41. Standard SCX bulk elution. Using LC Program 1 inject 4 μl SCX reconditioning buffer (1M ammonium formate/ 25% ACN, pH=9). Repeat once.
42. Using LC Program 3 (Single dimension RP analysis), analyze 1 μg (2 μl) SCX standard.
43. Search all MS raw files generated in step 40-42 as described in step 35. Verify that >95% peptides elute in a single SCX fraction. Verify that extracted ion chromatograms (XIC) for peptides have similar intensity (±10%) in SCX and 1D RP experiments.

SCX fractionation

44. Recondition and equilibrate columns using LC Program 4 (columns reconditioning and equilibration).
45. Standard SCX load. Using LC Program 1 (SCX sample load) inject 2 μl of SCX standard
46. Standard SCX fractionation. Using LC Program 2 inject 4 μl ammonium formate plugs at 50, 100, 150 mM (pH2.8).
47. Using LC Program 1 inject 10 μl ammonium formate 1M (pH2.8).
48. Using LC Program 1 inject 10 μl SCX reconditioning buffer (1M ammonium formate/ 25% ACN, pH=9).
49. Search all MS raw files generated in step 45-48 as described in step 35.

TROUBLESHOOTING

Clogging of unions and capillary lines. Possible causes include:

- Column frits leaking stationary phase. Suggested solution: replace column. Kasil polymerization to produce frits should be optimized before attempting to pack columns.
- Particulate accumulating in the outer part of capillary being inadvertently introduced in the lines when mounting into frits and sleeves. Suggested solution:

cut the silica capillaries after they are inserted in the fittings and avoid touching the open ends. Avoid storing columns with open ends for extended time.

- Particulate contamination of bulk stationary phase. Make sure spatulas, magnets and glass ampoules are kept clean.
- Degradation of silica stationary phase. Prolonged exposure to pH>9 may induce hydrolysis of unprotected silica particles. Possible solutions: after SCX column reconditioning at high pH, ensure, appropriate column washing with 0.1% Formic Acid in water.
- Salt precipitation in the lines. Salt precipitation may occur if non-volatile salts (such as KCl) are used for elution. Suggested solution: elute peptides using volatile salts such as ammonium formate or ammonium acetate.
- Silica fragments from capillary end cutting. Cutting silica capillary may produce debris and particulate that enter the column or the downstream component producing clogs. Suggested solution: using a phase microscope, inspect all ends to ensure that a clean cut perpendicular to the silica wall is obtained. Small silica splinters may be removed using a silica polishing station. All lines and column should be flushed before being mounted.

Insufficient retention of peptides on SCX columns. Possible causes include:

- Excessive salt concentration in loaded sample. Suggested solution: thoroughly desalt peptide sample by solid phase extraction. Avoid using phosphate buffers.
- Incorrect pH of SCX loading buffer. For best retention all solution must have pH below the pKa of aspartate (3.86). Suggested solution: titrate the pH of all buffers with formic acid to obtain pH=2.8.
- Excessive peptide amount loaded on SCX column. Suggested solution: do not exceed SCX column loading capacity. We observed >90% peptide retention on 100 µm ID x 150 mm SCX column when loading up to 15 µg tryptic peptides in 0.1% formic acid in water.

Insufficient resolution of SCX column. Possible causes include:

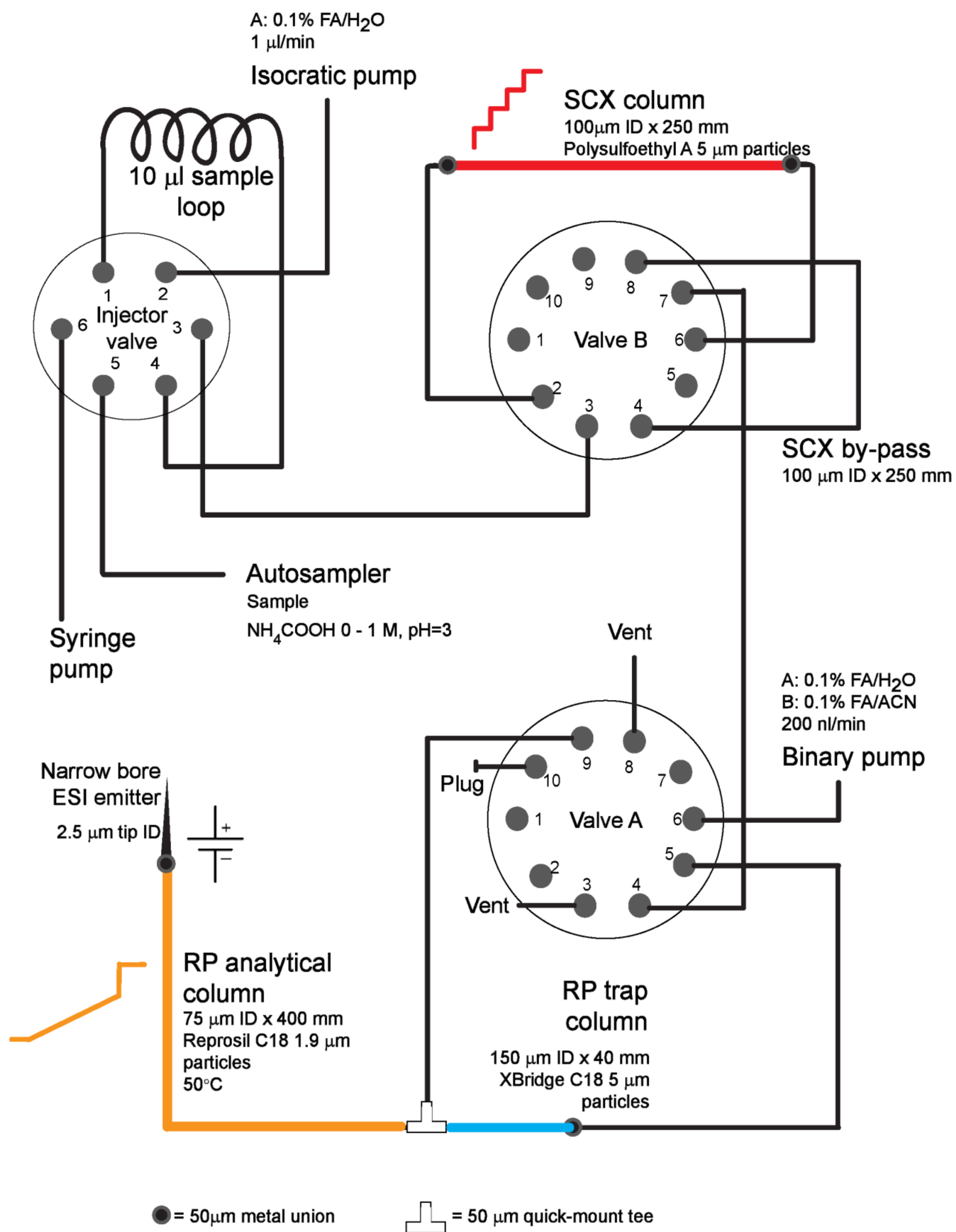
- Sample or mobile phase contain TFA or other modifier that interfere with peak focusing by SCX. Suggested solution: use ammonium formate and formic acid as mobile phase modifiers, or ammonium acetate.
- Isocratic (mass action) elution is the main mode of chromatographic separation. Suggested solution: avoid using more than 2 column volume to drive salt plugs. Verify accurate calibration of HPLC flow meters.

Insufficient retention of peptides on the trap column. Possible causes include:

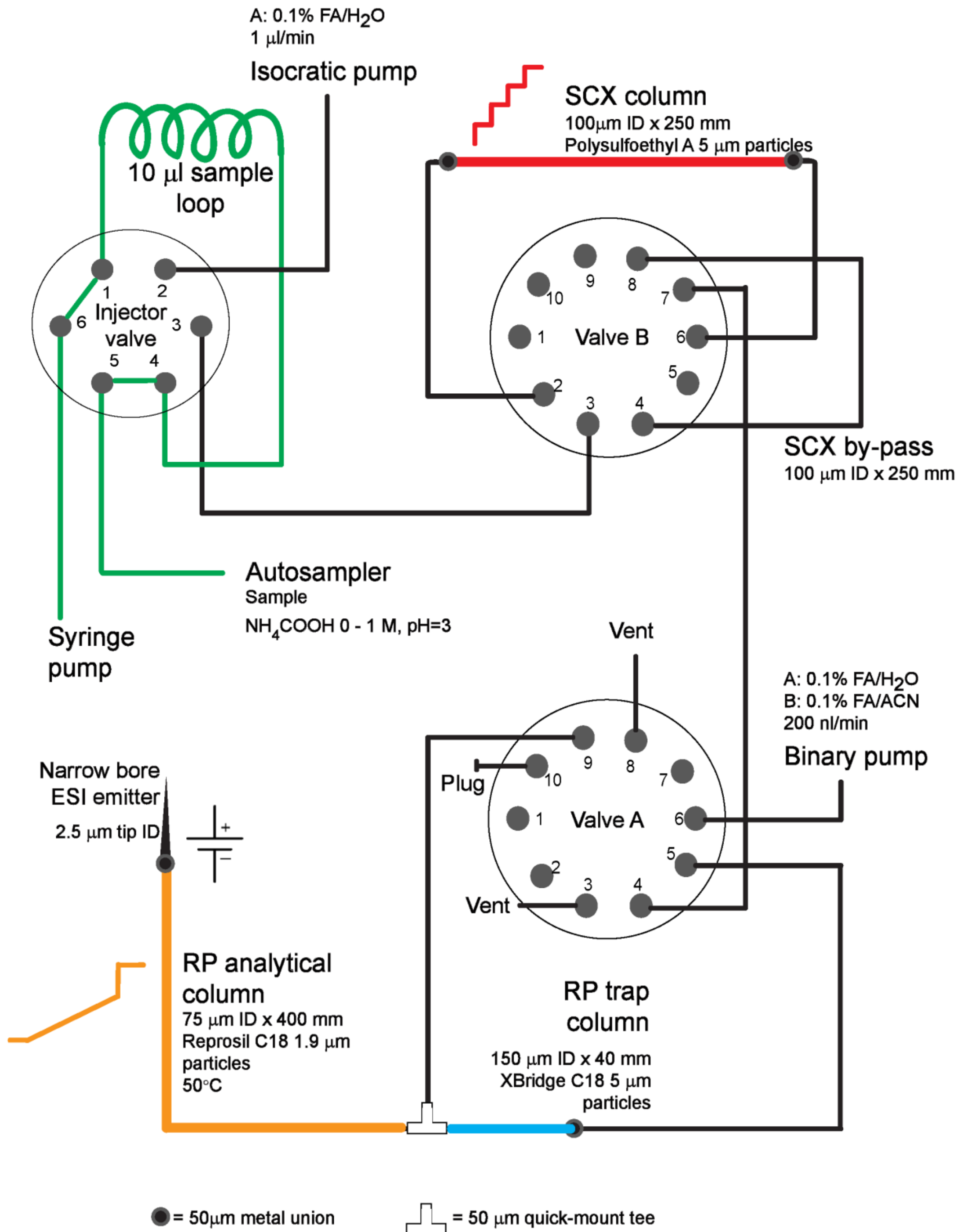
- The SCX mobile phase and/or the salt plugs contain organic solvents. Do not use more than 2% acetonitrile or methanol in the buffer resuspension buffer. Avoid adding any organic solvent to the isocratic pump mobile phase.
- The fractions eluting from the SCX column exceed the loading capacity of the reverse phase trap column. This may be determined by excessive sample amount or incorrect salt concentration in the SCX plugs. Suggested solution: carefully determine sample concentration. Prepare fresh ammonium formate solutions.

FIGURES

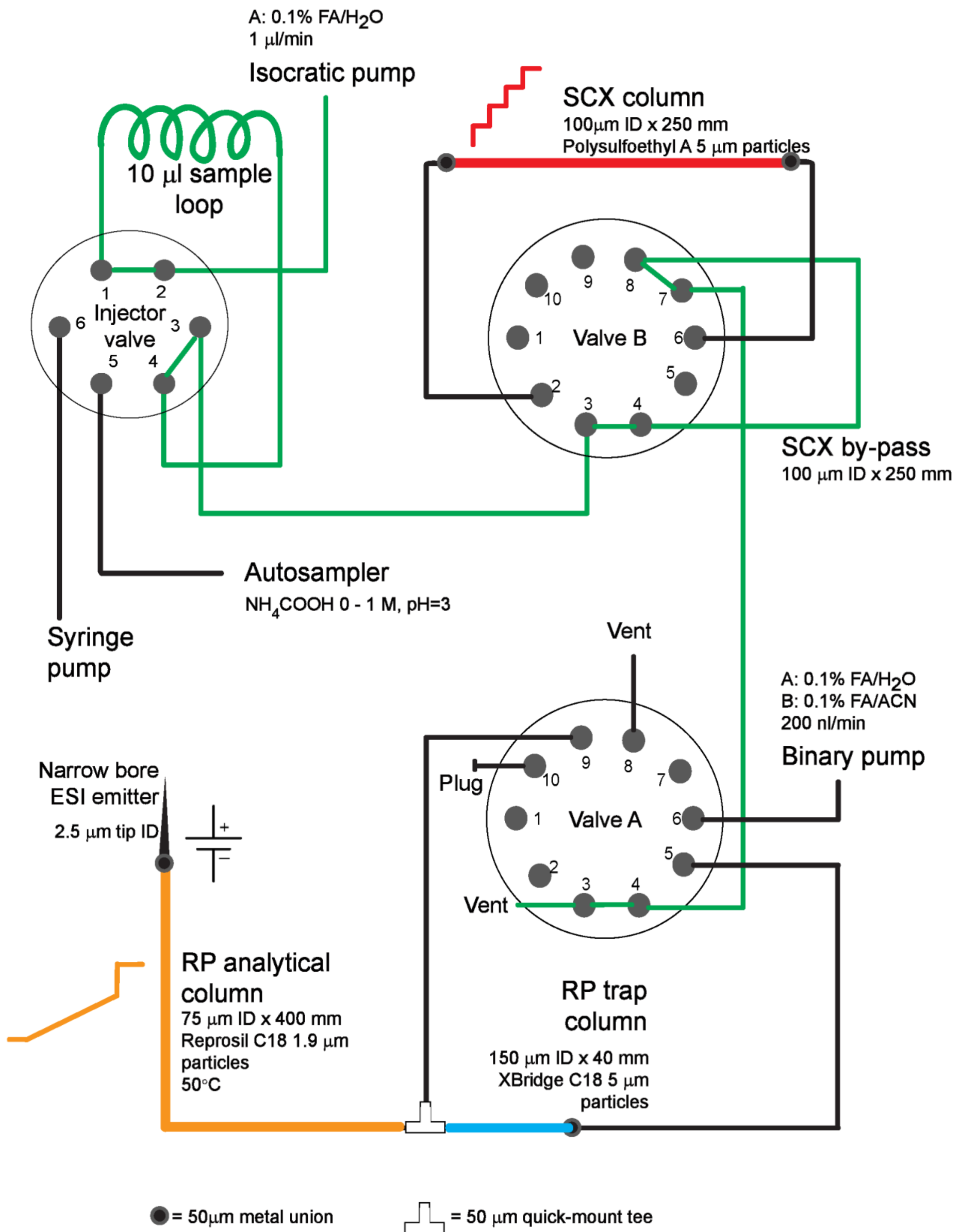
Figure 1: Overview of the plumbing scheme.



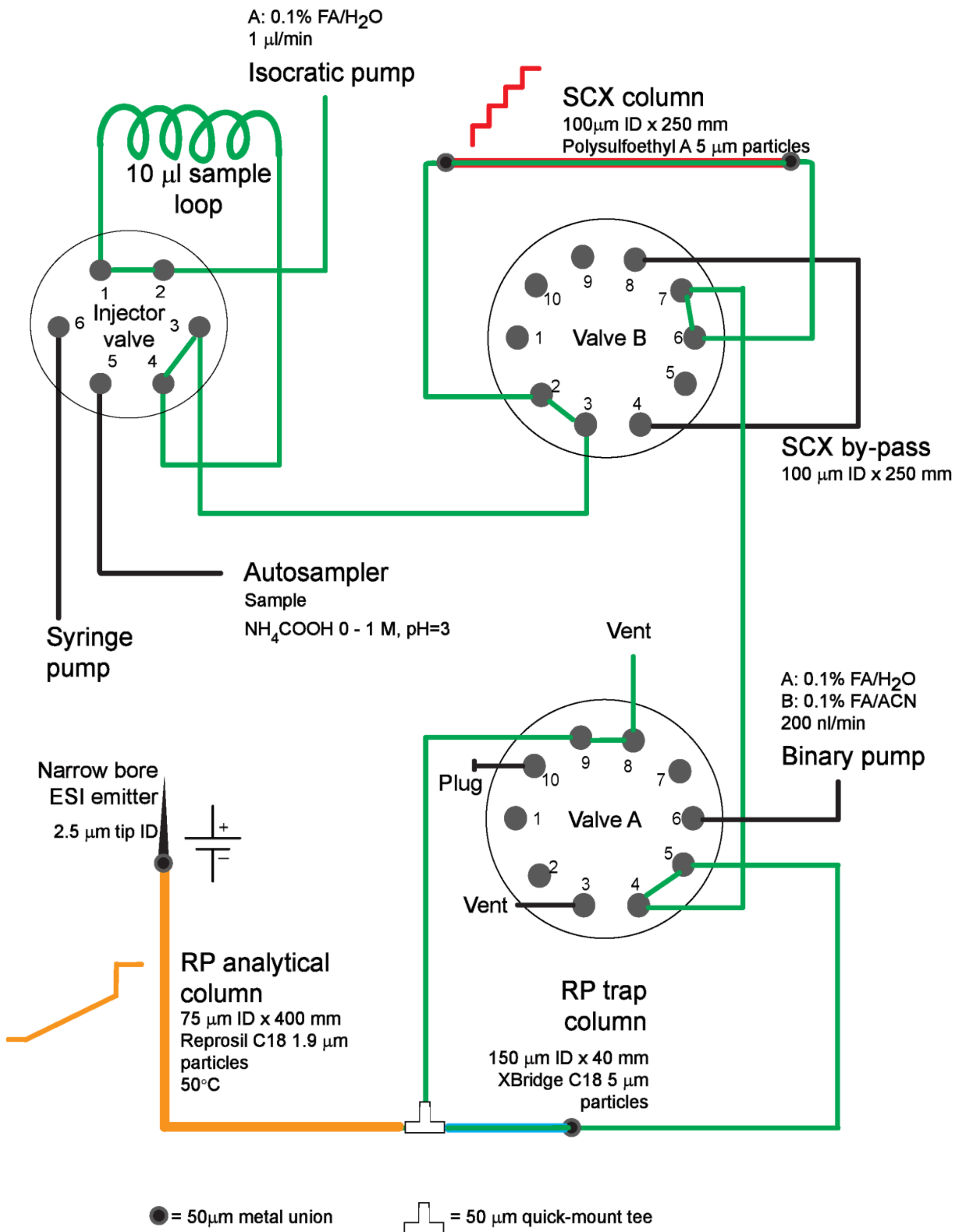
Supplementary Figure 1a: Sample or elution plug loading. The syringe pump loads either peptide sample or SCX elution plugs from autosampler vials. In the first case, the syring pump aspires into the loop only the user define volume (e.g. 2 µl), flanked by two elements of 0.1% formic acid in water. In the latter, the injection loop is completely filled with salt solution. The active flow path is showed in green.



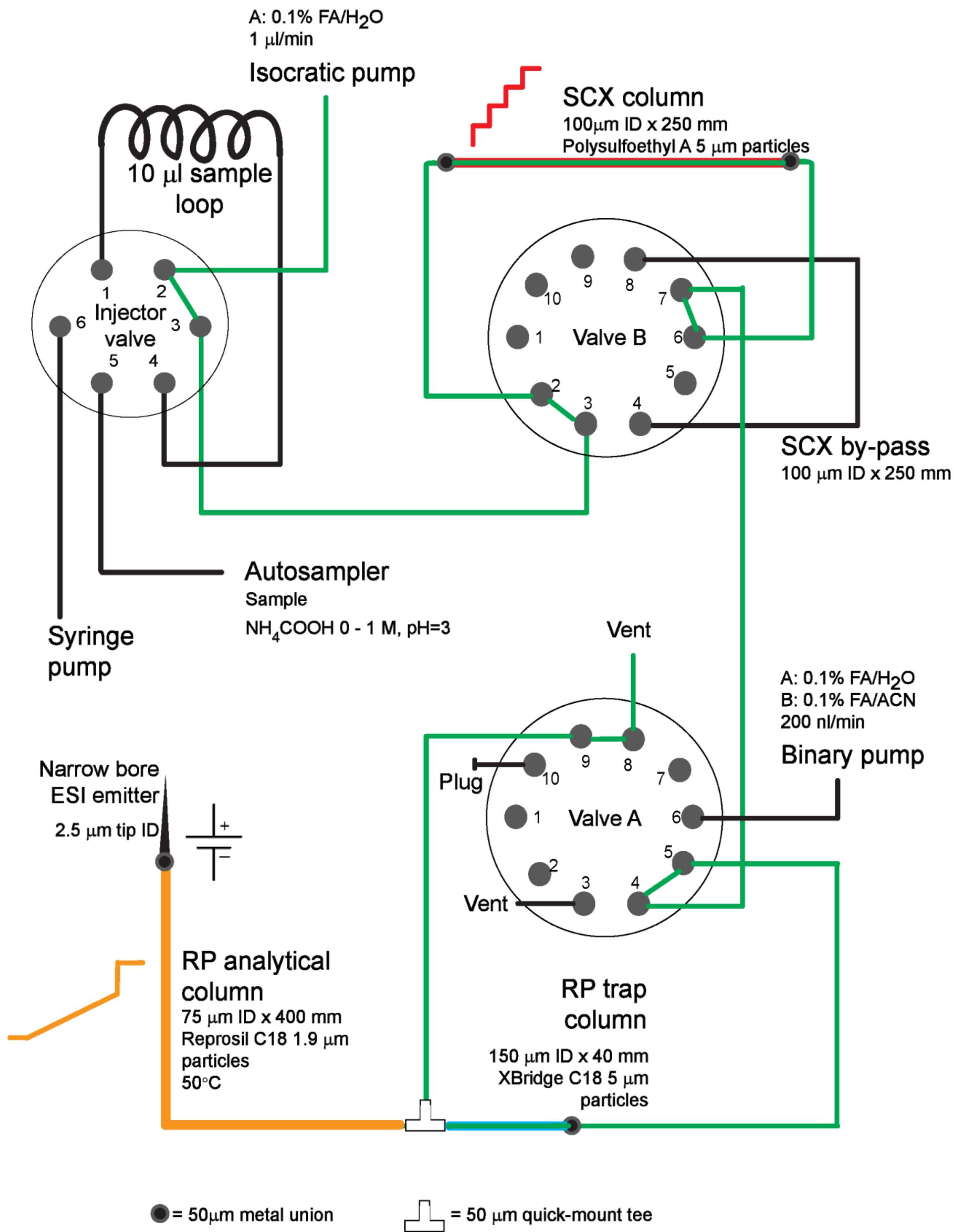
Supplementary Figure 1b: Lines equilibration. The isocratic pump is used to drive the content of the injection loop. The lines leading to the SCX column are equilibrated with the elution buffer, while ensuring that flow-rate stabilizes. Both the SCX and the trap columns are excluded from the flow path. The active flow path is showed in green.



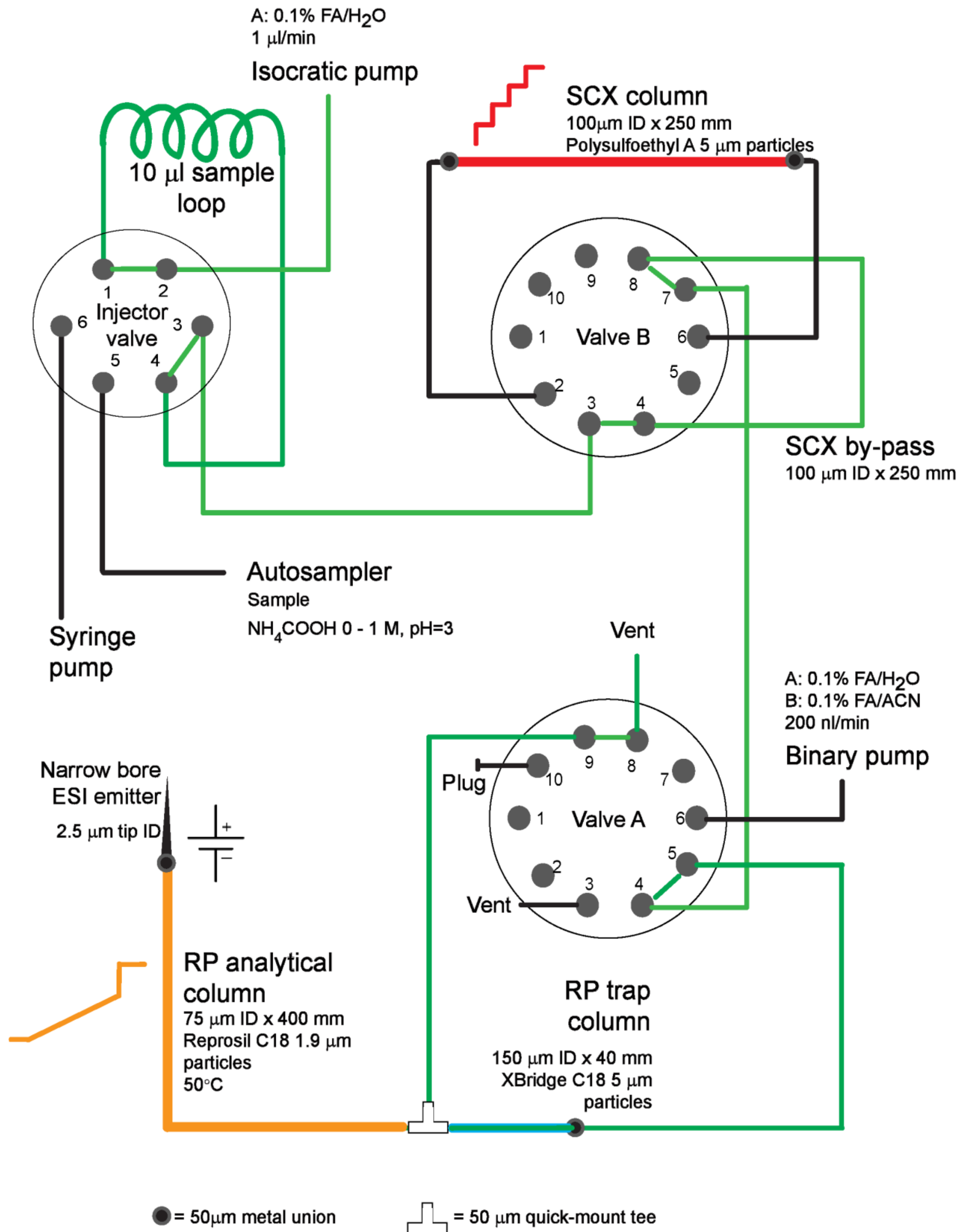
Supplementary Figure 1c: Delivery to the SCX column. The SCX column is included in the flow path, while the isocratic pump drives the content of the injection loop. For SCX sample loading, the entire content of the loop is injected onto the SCX column. Eluted and not retained peptides are loaded onto the trap column. The active flow path is showed in green.



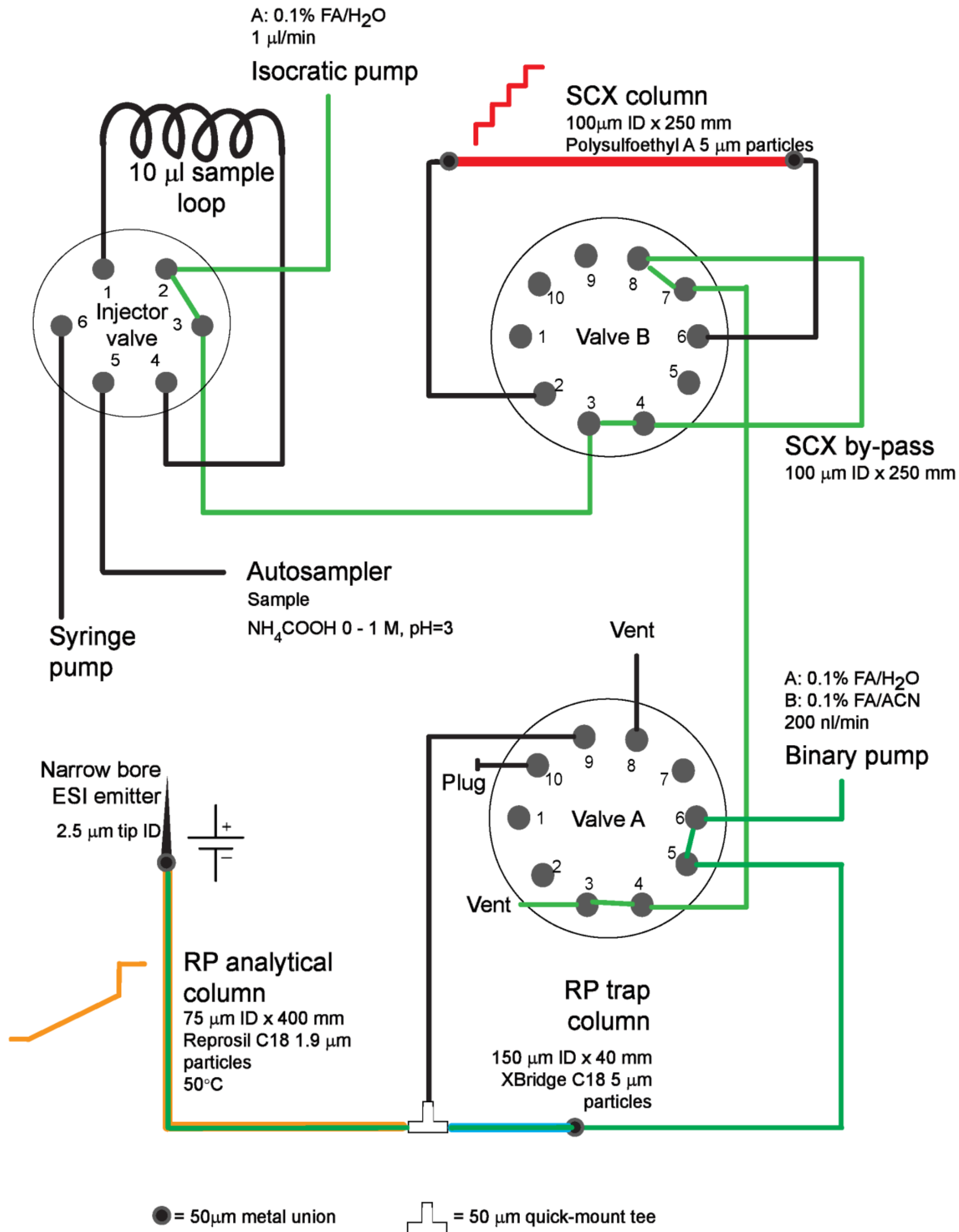
Supplementary Figure 1d: Eluate drive and desalt. After the time necessary to deliver the exact volume of elution buffer, the injection loop is excluded from the flow-path. Eluted peptides are driven by the isocratic pump into the trap column, where excess salt is not retained. The active flow path is showed in green.



Supplementary Figure 1e: Single dimension sample loading. The isocratic pump drives the entire content of the injection loop onto the trap column. The SCX column is by-passed maintaining the void volume constant.



Supplementary Figure 1f: Final dimension reverse phase and SCX line wash. The trap column is set on line with the analytical reverse phase, where SCX-fractionated peptides are resolved, prior to electrospray ionization and MS analysis. In parallel, the injection loop and the line up- and down-stream the SCX column are flushed with 0.1% formic acid to prevent salt precipitation. The active flow path is showed in green.



Supplementary Figure 1g: Columns regeneration. SCX and reverse phase columns are washed and re-equilibrated. The SCX column is discharged using 10 µl (5 column volumes) of 1M ammonium formate at pH9, followed by re-equilibration with 50 µl 0.1% formic acid pH3. Flow-through is directed to waste. In parallel, the reverse phase columns are discharged in 80% ACN, and re-equilibrated in 3% ACN.