A. Single-Phase Fused-Silica 100μm Microcapillary Column

1. Place about 40 cm of 100 μm i.d. x 365 μm o.d. fused silica (Polymicro Technologies) into a Sutter Instrument Co. Model P-2000 laser puller.

2. Use heating/pulling cycle settings such as to pull the capillary to about a 5 μm opening. A typical four-step parameter setup for pulling approx 3 to 5-μm tips from a 100 μm i.d. x 365 μm o.d. fused silica capillary is:

   [Heat = 290, Velocity = 40, and Delay = 200]
   [Heat = 280, Velocity = 30, and Delay = 200]
   [Heat = 270, Velocity = 25, and Delay = 200]
   [Heat = 260, Velocity = 20, and Delay = 200]

   with all other values set to zero.

3. Make a slurry of 5 μm Aqua C18 Reverse Phase (Aqua, Phenomenex) at about 15 mg/mL in 500 µL of methanol (this concentration roughly corresponds to an amount of resin powder covering the tip of a small spatula, about 5-7 mm³).

4. Pack the fused silica column with 9-10 cm of 5 μm C₁₈ Reverse Phase using a high pressure loading device (using a black background behind the capillary being packed helps seeing the resin levels inside the column).

5. Wash with Methanol for at least 5min.

6. Equilibrate in buffer A for at least 30min.
B. DOUBLE-PHASE FUSED-SILICA 250µM MICROCAPILLARY COLUMN

1. Place an inline micro filter assembly with a 2-µm filtered union (UpChurch Scientific, Oak Harbor, WA) and a microtight sleeve on one end of a 250 µm i.d. x 360 µm o.d. fused silica capillary cut to about 20 cm in length.

2. Make slurries of 5 µm Aqua C18 Reverse Phase (Aqua, Phenomenex) and 5-µm strong cation exchange material (Luna, Phenomenex or Partisphere SCX, Whatman), both at about 30 mg/mL in 1 mL of methanol (this concentration roughly corresponds to an amount of resin powder covering the tip of a small spatula, about 10-15 mm³)

3. Pack the 250 µm capillary assembly with 3-4 cm (i.e. about 1 cm past the end of the green microtight sleeve) of 5 µm strong cation exchange resin using a high pressure loading device.

4. Wash with methanol for at least 5 min; mark resin level in column with a marker.

5. Pack fused silica column with 1-2 cm of 5 µm Aqua C18 RP (Figure 4B); mark resin level in column with a marker.

6. Wash with Methanol for at least 5 min.

7. Equilibrate in buffer A for at least 30 min.
C. Off-Line Loading and Desalting

① Spin samples down @ 14,000rpm for 30min.
② Transfer to new tube.
③ Load samples to 250μm column.
④ Wash with Buffer A (1.5ml). Note that it is not recommended to wash columns extensively after loading if one’s goal is to detect phosphorylated peptides.
D. CONNECTING 100 µM RESOLUTIVE COLUMN WITH PEPTIDE-LOADED 250 µM CAPILLARY

1. Connect 250µm i.d. column to equilibrated 100µm double-phase column.

2. Install the loaded and washed split-three-phase microcapillary column on the nanoelectrospray stage.

3. Connect the microcapillary column, quaternary HPLC pump, gold wire through which a 2.4 kV voltage is applied to the liquid phase, and overflow tubing using two microtight tees (UpChurch Scientific, Oak Harbor, WA). Applying the voltage via the microtight tee most distal to the microcapillary column helps stabilize the electrospray.

4. Keep the HPLC flow rate constant at 0.1 mL/min throughout the chromatography. However, to achieve a slower flow rate at the tip of the column of about 200-300 nL/min, split the flow using a waste line consisting of 50 µm i.d. fused silica capillary cut to about 40 cm (i.e. back pressure of ~40 bar on Agilent 1100).