

## Protocol for off-line 2D LC

### 1. Materials

TT200PSC, TopTips (PolyLC) with POROS strong cation material (for 10-200  $\mu$ l samples (one box of 96) or 1 mL samples (one bag of 8)). Note: The large size seems more convenient to be used than the small size.

TT200MC18.96, TopTips (PolyLC) with carbon + C18 (mix mode) material, same formats as SCX TopTips.

Solvent A: 0.2% formic acid, 20% ACN.

Solvent B: 20% ACN.

Solvent C: 0.01M KCl, 20% ACN

Solvent D: 0.025M KCl, 20% ACN

Solvent E: 0.05M KCl, 20% ACN.

Solvent F: 0.075M KCl, 20% ACN

Solvent G: 0.1M KCl, 20% ACN.

Solvent H: 0.2M KCl, 20% ACN.

Solvent I: 0.35M KCl, 20% ACN.

Solvent J: 0.5M KCl, 1% NH<sub>3</sub>, 20% ACN.

Formic acid

Ammonia

pH papers

### 2. Procedures

1. Mixture (100-300  $\mu$ g of total protein digests) of iTRAQ- or TMT-labeled protein digests is diluted by solvent A and its pH is adjusted to be acidic (pH 2-5 by 5% formic acid) to the maximum volume of an Eppendorf tube (2.0 mL).

2. The SCX tip is preconditioned with 2 x 0.8 mL solvent A.

3. Run samples through the tip...Collect run-through as fraction 0.

4. Rinse the tip with 0.6 mL solvent B... fraction 1.

5. Rinse the tip with 0.6 mL solvent C...fraction 2.

6. Rinse the tip with 0.6 mL solvent D...fraction 3.

7. Rinse the tip with 0.6 mL solvent E...fraction 4.

8. Rinse the tip with 0.6 mL solvent F...fraction 5.

9. Rinse the tip with 0.6 mL solvent G...fraction 6.

10. Rinse the tip with 0.6 mL solvent H...fraction 7.

11. Rinse the tip with 0.6 mL solvent I...fraction 8.

12. Rinse the tip with 0.6 mL solvent J...fraction 9.

13. All the fractions are SpeedVac dried to remove ACN.
14. Dissolve all fractions in 0.2% formic acid (note: make sure that the pH of fraction 9 is turned into acidic after acidification) to final volume 1.5 mL.
15. Precondition the carbon+C18 tip with 3 x 0.8 mL 0.2% formic acid.
16. Load samples to the tip (If you are patient enough, collect the run-through and repeat the loading once).
17. Rinse the tip by 0.2% formic acid, 3-5 x 0.8 mL (low salt 3 times and high salt 5 times).
18. Elude peptides by 0.8 mL 60% ACN.
19. Wash the tip by 3 x 0.8 mL of 100% ACN.
20. Recondition the tip by 3 x 0.8 mL 0.2% formic acid for the next fraction desalting.
21. Repeat steps 16-20 for all the factions.
22. SpeedVac dry fractions to just completed dry (no over time).
23. Add 20  $\mu$ l of 0.2 % formic acid in each faction tube.
24. After votexing well, transfer the samples to autosampler vials.
25. Run LC/MS/MS triplicates with 2  $\mu$ l injection volume.