The following procedure is the second step in a three-step process for the preparation and enrichment of phosphopeptides using immobilized metal affinity chromatography (IMAC) for the identification of the phosphopeptides by LC-MS/MS. This procedure describes the construction of microchromatographic columns, or micro-tips. This protocol is modified from Erdjument-Bromage H, Lui M, Lacomis L, et al. Examination of micro-tip reversed-phase liquid chromatographic extraction of peptide pools for mass spectrometric analysis. *J Chromatogr A* (1998) Nov 27;826(2):167-81. Although others have reportedly reused columns, we pack a fresh column before each use.

**Procedure**
1. Prepare a piece of trifluoroacetic acid (TFA)-treated cartridge filter by using a micropipette tip to core out a small circle from the filter disk.
2. Pipette 20 µl of absolute ethanol using an Eppendorf geloader tip, and keep the liquid inside the tip.
3. Push the cored circle down into the lower end of the Eppendorf GELoader tip, and let the circle stick to form a frit.
4. Cut the top of the tip column with a razor blade to about 1 cm above the packing.
5. Prepare a 20- to 200-µl pipette tip with about 3 mm cut from the bottom. Sleeve the geloader tip column with this larger pipette tip. Cut another 3 mm from the bottom.
6. Pipette 20 µl of POROS 20 MC bead slurry (2:1; ethanol:beads) on top of the frit, and apply ethanol to the tip several times to pack the beads tightly. The micro-tip column is now ready to be activated with metal for use in the enrichment of phosphopeptides using IMAC.
**Figure 1.** Schematic diagram of a “micro-tip” column. (A) Micro-tip ready for sample application. (B) Micro-tip, cut with razor blade at top and bottom, inserted in Universal fit pipette tip (cut at bottom); ready for elution. This figure is adapted from Erdjumend-Bromage H, Lui M, Lacomis L, et al. Examination of micro-tip reversed-phase liquid chromatographic extraction of peptide pools for mass spectrometric analysis. *J Chromatogr A* (1998) Nov 27;826(2):167-81.

**Reagents and Materials**

Trifluoroacetic acid (TFA)-treated cartridge filter: Applied Biosystems; catalog no. 400379

Micropipette tip, 0.5 to 20 µL: Rainin; catalog no. GPS-L10

Ethanol, absolute: AAPER Alcohol and Chemical Co.; catalog no. 061300

Eppendorf GELoader tip, 0.5 to 10 µL: Eppendorf; catalog no. 0030 001.222

Pipette tip, 20 to 200 µL: Rainin; catalog no. L-200

POROS 20 MC bead: Applied Biosystems; catalog no. 1-5428-02

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