PolyLC INC.
9151 Rumsey Rd, Suite 180, Columbia, MD 21045 USA
Phone: (410) 992-5400 Fax: (410) 730-8340 www.PolyLC.com

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Isolation of Phosphopeptides via ERLIC*

*(Electrostatic Repulsion-Hydrophilic Interaction Chromatography)

ERLIC is a new, general-purpose mode of chromatography; a column of the same electrostatic charge as the solutes is run in the HILIC mode. With ERLIC, **phosphopeptides** can be isolated selectively from tryptic digests.

At pH 2.0, phosphate groups in peptides retain some of their negative charge. This does not permit the isolation by **anion-exchange chromatography (AEX)** of singly phosphorylated peptides from tryptic digests, since the electrostatic attraction is not sufficient to overcome the electrostatic repulsion from the N-terminus and the C-terminal Lys- or Arg- residue. When an AEX column is run in the **ERLIC mode**, though, then the combination of electrostatic attraction and hydrophilic interaction does suffice to pull singly phosphorylated peptides away from the nonphosphorylated peptides in tryptic digests. Unlike the situation with high-affinity media such as IMAC or titania, phosphopeptides can be well-resolved from each other in ERLIC. This permits their convenient separation into numerous fractions, an important tool in **phosphoproteomics** for identifying the sequences of thousands of phosphopeptides from a single sample. Peptides with multiple phosphate groups are retained so strongly that a salt gradient is necessary for elution.

Example 1: Separation with the Same Column in the ERLIC and AEX Modes

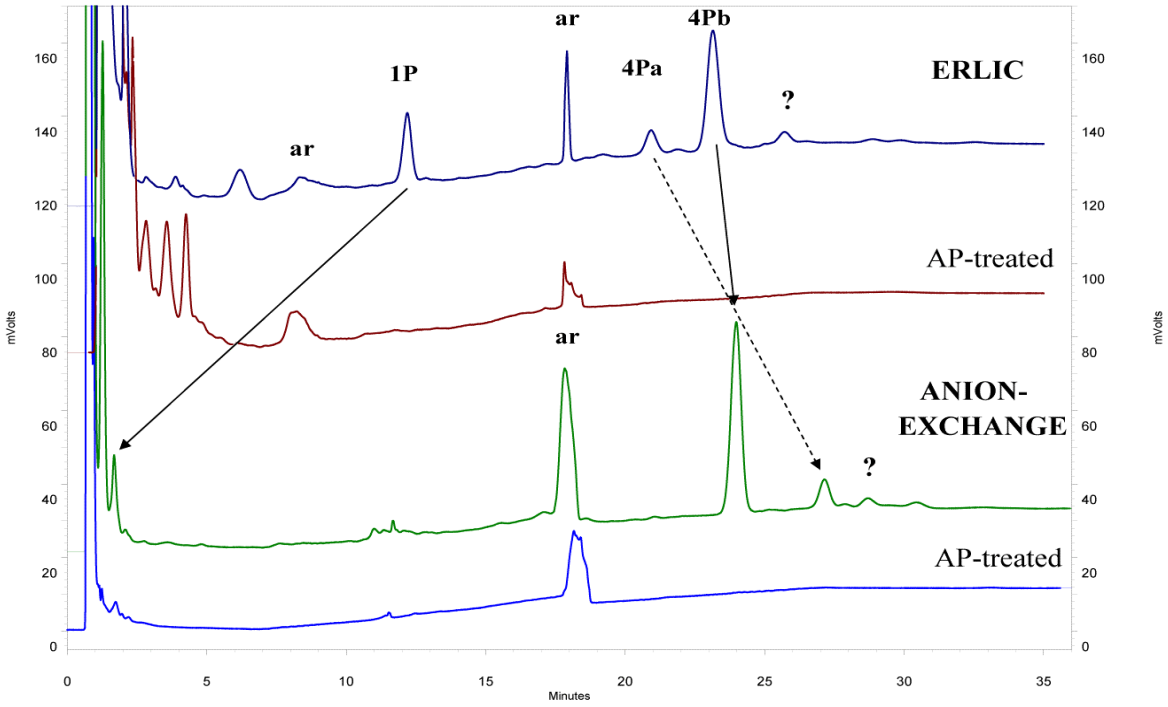
A 100x4.6-mm column of PolyWAX LP™ (5-µm, 300-Å) was used (item# 104WX0503). Note the poor retention of the singly phosphorylated fragment in the anion-exchange mode.

Tryptic Digest of β-Casein; ERLIC vs. Anion-Exchange

1P: FQ**S**EEQQQTEDELQDK **ar** = artifact

4Pa: ELEELNVPGEIVE**S**L**SSS**EESITR **AP** = Alkaline Phosphatase

4Pb: RELEELNVPGEIVE**S**L**SSS**EESITR



Example 2: Elution of Ideal Tryptic Monophosphopeptide Standards with a Volatile Mobile Phase: ↑ [Salt] vs. ↓ [ACN]

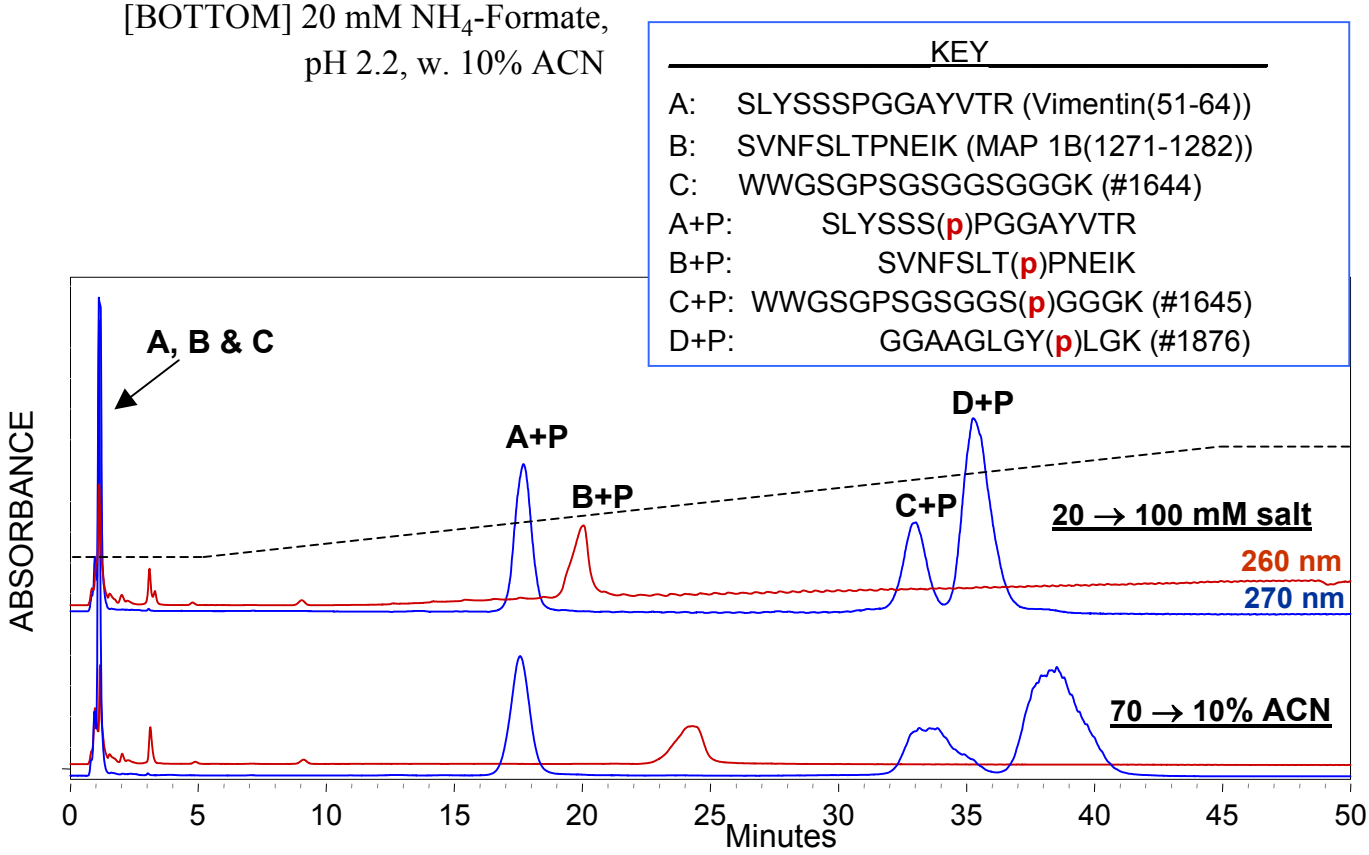
Column: 104WX0503 Flow rate: 1 ml/min. Detection: As noted

Gradient: 0-5': 0%B; 5-45': 0-100% B; 45-50': 100%B

MP A: 20 mM NH_4-Formate, pH 2.2, with 70% ACN

MP B: [TOP] 100 mM NH_4-Formate, pH 2.2, w. 64% ACN;

[BOTTOM] 20 mM NH_4-Formate, pH 2.2, w. 10% ACN



[***TOP***] Increasing the salt content of the mobile phase is a standard way to elute solutes in ion-exchange, and works well here. Selectivity and peak shapes are quite good.

[***BOTTOM***] An alternative gradient of decreasing [ACN] was also tried, switching the mode from ERLIC to AEX, since tryptic monophosphopeptides are not well-retained in the AEX mode. The selectivity was retained although peak shapes deteriorated to some extent. The ACN gradient has three significant advantages:

1) Since the salt concentration doesn't vary, the absorbance baseline is steady. This suggests the possibility of monitoring ~ 220 nm for peptides that lack aromatic residues; the baseline would be ~ 0.4 AU but steady.

2) The use of only 20 mM ammonium formate would be quite convenient for direct flow to a mass spectrometer. Of course, a salt gradient would be necessary to elute peptides with more than one phosphate.

3) Nonideal tryptic phosphopeptides, with more than one basic residue, elute easily with this gradient but not with the gradient of increasing NH4-formate concentration.

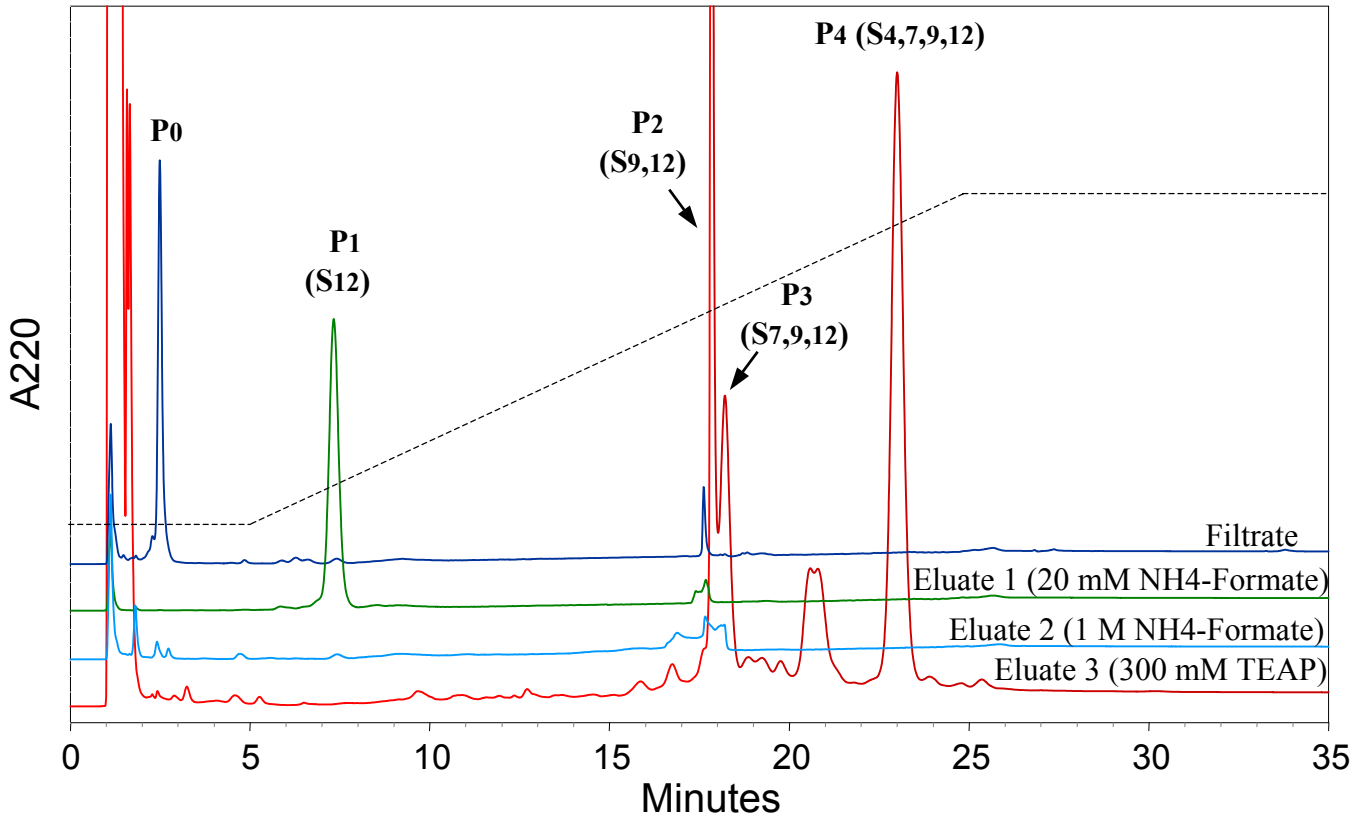
Example 3: ERLIC-SPE Fractionation of Phosphopeptide Standards

SPE cartridge: PolyWAX LP TopTip™, item# TT200WAX

Sample: WWGSGPSGSGGSGGGK with 0-4 phosphate groups on the serines

Binding solvent: 20 mM NH4-formate, pH 2.2, w. 70% ACN. Eluting solvents (all 10% ACN): 1) 20 mM NH4-formate, pH 2.2; 2) 1 M NH4-formate, pH 2.2,; 3) 300 mM TEAP, pH 2.0 (desalted for HPLC)

HPLC analysis: PolyWAX LP column (item# 104WX0503) with Na-MePO4 – TEAP gradient per standard ERLIC method

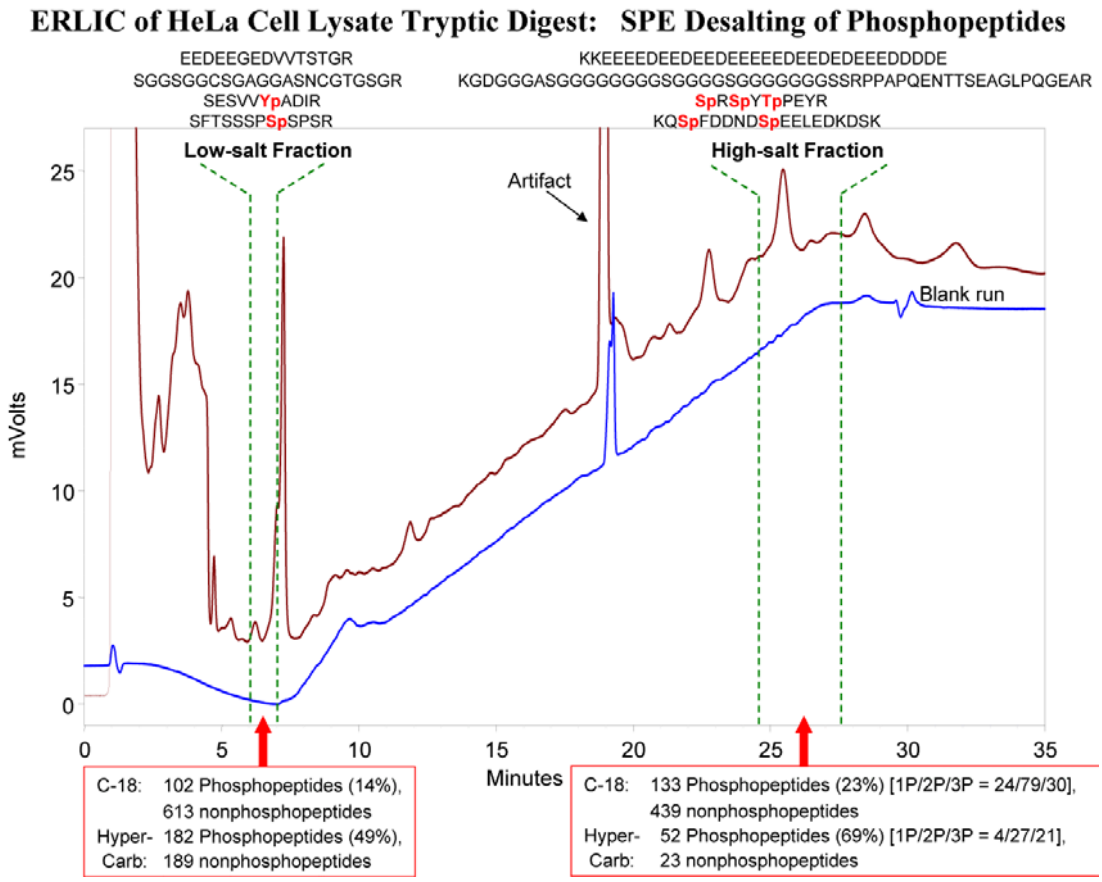


The nonphosphopeptide eluted in the filtrate, as expected in the ERLIC mode.

The monophosphopeptide eluted with the step from 70-10% ACN in 20 mM NH4-formate. Since peptides with 1 phosphate represent ~ 82% of all phosphopeptides in complex tryptic digests, then this fraction would be of greatest interest in general for phosphoproteomics. It could prospectively be rerun on a PolyWAX LP capillary with a shallow gradient of decreasing [ACN] with elution directly into a mass spectrometer.

The di-, tri- and tetraphosphopeptides only eluted with a step to TEAP, a nonvolatile salt. Multiphosphopeptide fractions will have to be desalted prior to MS analysis. We recommend a sequence of C-18 and HyperCarb® TopTips for maximum recovery.

Example 4: Fractionation of the Tryptic Digest of HeLa Cell Lysate



Again, PolyWAX LP column# 104WX0503 was used here. Monophosphopeptides elute in the early fractions, multiply phosphorylated peptides in later fractions, and nonphosphopeptides in or near the void volume. It should be noted that recovery of phosphopeptides is highest if low-salt fractions are desalted using HyperCarb®; high-salt fractions, using C-18 silica.

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Buying the products used in these examples:

PolyWAX LP column, 100x4.6-mm, 5-µm, 300-Å (item# 104WX0503): $ 455.
PolyWAX LP TopTip, 10-200 µl (item# TT200WAX): $ 25/pack of 10, $ 225/box of 96

Other sizes of PolyWAX LP columns are available. TopTips are available for samples 1-10 µl, 10-200 µl, and 200-1000 µl. Regular SPE cartridges (0.5-5 ml) of PolyWAX LP are also available. Please consult our Web site: www.PolyLC.com.