

# PolyLC INC.

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

## PolyWAX LPTM Columns

**Initial Use:** PolyWAX LPTM is a silica-based material with an adsorbed, cross-linked coating of linear polyethyleneimine. It is a weak anion-exchange (WAX) material. The capacity is high for both small and large solutes. Columns are shipped filled with methanol. Flush new columns with at least 15 column volumes of water (30 ml for a 200 x 4.6-mm) then condition with a salt solution prior to initial use. The hydrophilic coating imbibes a layer of water of hydration and swells, especially in the presence of salt solutions. This causes a modest, irreversible increase in the column backpressure. Since the swelling increases the surface area of the coating, this increases the binding capacity for proteins as well. This process should be hastened by eluting the column with a concentrated salt solution for two hours prior to its initial use. A convenient solution for the purpose is 0.2 M  $\text{NaH}_2\text{PO}_4$  + 0.3 M sodium acetate. Alternatively, if the column is to be used for proteins or peptides that may interact with heavy metals (e.g., phosphopeptides), then the column should be passivated by elution for 20-24 hours with 40 mM EDTA.2Na at a low flow rate.

New HPLC columns sometimes absorb small quantities of proteins or phosphorylated peptides in a nonspecific manner. The sintered metal frits have been implicated in this. Eluting the column for 20-24 hr. at a low flow rate with 40mM EDTA.2Na usually solves the problem. This passivates all metal surfaces in the HPLC system, as well as the column [CAUTION: This treatment can affect the integrity of the frits in some cases, and should probably be avoided with columns packed with 3- $\mu\text{m}$  material. In some cases this has also caused the collapse of 5- $\mu\text{m}$ , 200-Å column packings].

**Routine use:** Columns should be used at ambient temperatures. Filter mobile phases and samples before use. Failure to do so may cause the inlet frit to plug. If a salt gradient is being used, flush the column with 15 ml of the high-salt buffer before equilibrating with the low-salt buffer. At the end of the day, flush the column with 15 column volumes of water and plug the ends.

**Loading Capacity:** The loading capacity of a 4.6mm ID column is about 4-5 mg of protein/injection, depending on the strength of the protein's binding to the support.

**Storage:** 1) Overnight: 100% mobile phase A. 2) Several days: Store in water. 3) Longer periods: Store in water in the refrigerator, with the ends plugged. **ACN can be added to the storage solvent (e.g., ACN:Water = 80:20) to retard microbial growth.**

### Column maintenance:

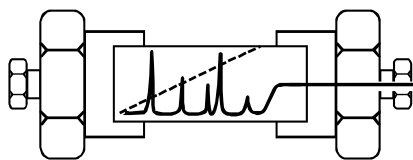
- 1) After several hundred operating hours, reverse the direction of flow and elute the column for several hours with a high-salt buffer. Continue using the column in the reverse direction.
- 2) Silica-based columns are slowly degraded at basic pH. Transient exposure to > pH 8.0 should have minimal effect on the column. If prolonged use above pH 8.0 is expected, then inserting a saturator column of inexpensive silica in-line before the injector valve will prolong column lifetime.

**Anion-exchange of peptides:** In contrast to proteins, acidic peptides can be run at lower pH down to the range where Asp- and Glu-residues start to lose charge (@ pH 4). Retention reaches a maximum around pH 4.5. Peak shape and selectivity can sometimes be improved by inclusion of 10% organic solvent (MeOH or ACN) in the mobile phase.

**Anion-exchange of proteins:** This is typically performed in the pH range 7.0-8.0, using either increasing salt or decreasing pH gradients. The higher the pH, the stronger the binding of proteins. Tris is a good buffer in this range. Since halide salts tend to corrode stainless steel, acetate salts are often used to generate the gradient. However, it may be necessary to use a chloride salt gradient if the absorbance is to be monitored below 235 nm. If so, flush with 15 column volumes of water at the end of the day to preclude corrosion. It is also helpful to passivate the column every 3-4 weeks with EDTA.2Na (@ above) if chloride salts are used with regularity.

- For proteins < 15 KDa, a 300-Å material can be used. For larger proteins, a 1000-Å column is recommended.

**Anion-exchange of oligonucleotides and oligonucleotide analogs:** For oligos < 25 bases long, use 300-Å material. For oligos > 25 bases long and dsDNA, use of a 1000-Å column will afford elution without excessive amounts of salt. It is helpful to include 25-50 % acetonitrile or propanol in both mobile phases. Large oligos may require over 1 M salt for elution. It is advisable to use a chaotropic salt (e.g., NaBr or  $\text{NaClO}_4$ ) for the gradient in cases where there could be a tendency for self-association like GC-rich sequences; otherwise, ammonium acetate can be used.



# PolyLC INC.

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

## Using PolyWAX LP™ in the ERLIC Mode

ERLIC (Electrostatic Repulsion-Hydrophilic Interaction Chromatography) involves the use of an ion-exchange column with the same charge as what would normally be the best-retained solutes in a sample. Their retention is selectively antagonized by electrostatic repulsion, throwing their elution into the same time frame as the other solutes in the sample. If this is done with > 60% ACN (acetonitrile) or other organic solvent in the mobile phase, then hydrophilic interaction will be strong enough for solutes to be retained by the column despite the electrostatic repulsion. General principles:

- 1) The anion used in the mobile phase plays an important role in selectivity. Methylphosphonate promotes retention of acidic solutes such as phosphates and aspartic acid while phosphate promotes retention of basic solutes. Effects of other ions have not been worked out as yet.
- 2) In order for the electrostatic repulsion effect to be significant, chromatography of proteins, peptides and amino acids should be performed at ~ pH 2.0. At higher pH, Asp- and Glu- residues and C-termini will acquire negative charge and the solutes will be retained via electrostatic attraction plus any hydrophilic interaction that may be present. This may result in loss of control over the selectivity of the separation.

**Isolation of Tryptic Phosphopeptides:** Under the following conditions, most peptides lacking a phosphate group will elute in or near the void volume while most singly phosphorylated peptides will elute isocratically within 3-8 column volumes. Most peptides with 2 or more phosphates will elute during a subsequent salt gradient.

Mobile phases: A) 20 mM sodium methylphosphonate (Na-MePO<sub>4</sub>), pH 2.0, with 70% ACN.  
B) 200 mM triethylamine phosphate (TEAP), pH 2.0, with 60% ACN.

Prepare A by dissolving methylphosphonic acid in water, then adding NaOH to reach pH 2.0. Filter. It is advisable to prepare a stock solution of higher concentration and then use aliquots of this to prepare the mobile phase. The 20 mM figure refers to the concentration overall in the mobile phase; it does not mean a mobile phase containing 30% of a solution containing 20 mM Na-MePO<sub>4</sub> and 70% ACN (i.e., 20 mM x 0.30 = 6 mM overall).

Flow rate: 0.8-1.0 ml/min for a column of 4.6-mm i.d. Flow rates should be adjusted proportionately for other sizes.

Gradient: It is advisable to stay at 100% A for at least the first 5' before starting the gradient, in order to distribute the elution of singly phosphorylated peptides over a larger number of fractions. Thereafter, run a linear gradient to 100% B in no less than 20' if the sample is likely to contain peptides with at least 3 phosphate groups. For analysis of tryptic digests containing large numbers of phosphate groups, running a longer gradient and collecting more fractions will result in significantly more phosphopeptides being identified. An example would be a 40-50' linear gradient and collection of 40-50 fractions. With complex samples such as digests of whole cell lysates, the fractions eluting after 70% B are liable to contain some peptides with 4 or more phosphates. Identification of these requires an appropriate mass spectrometer and identification program (i.e., not SEQUEST).

Reequilibration: In order to insure good separation of singly phosphorylated peptides from the nonphosphopeptides, one should reequilibrate an ERLIC column for a longer period than with other modes of chromatography. As an example, a 100x4.6-mm column should be reequilibrated for at least 60' between runs at 1 ml/min or 30' at 2 ml/min (if the latter, then reduce the flow rate to 1 ml/min before injecting the sample and starting the next run).

Carryover: Peptides with 4 or more phosphates may interact with heavy metals in the HPLC system and small amounts carried over to the next run. If this is a significant concern, then it would be a good idea to perform at least one blank run between sample runs and preferably two such blank runs.

Reagents: Use the purest grade of methylphosphonic acid available, since impurities in the reagent can accumulate on the column during the reequilibration step and then elute in an artifact peak at the start of the gradient. At this time, 98+% methylphosphonic acid from Fluka appears to be better than the reagent from other suppliers. Use HPLC-grade triethylamine and phosphoric acid as well.

Desalting collected fractions prior to subsequent RPC and/or mass spectrometry via solid-phase extraction (SPE): Fractions collected with < 50 mM salt should be desalted with HyperCarb® or some other form of graphitic carbon. Our HyperCarb TopTips™

are effective for this purpose, with ~ 50% more phosphopeptides recovered than when C-18 silica is used for desalting. Fractions with 50-150 mM salt are probably best desalted with titania TopTips, while fractions with > 150 mM salt are best desalted with C-18 cartridges. If you want to trap and desalt ERLIC fractions online, the organic solvent probably limits your choice to titania.

**CAUTION:** Samples should contain the same salt as the starting mobile phase if possible. A significant disparity in composition between the sample solvent and the mobile phase can result in skewed peaks. In extreme cases, one can get elution of a pure solute in two well-separated peaks connected by a continuum as the solute exchanges counterions on-column.

**ERLIC of Amino Acids:** PolyWAX LP™ columns with pore diameter of 100 Å are best for this. The limiting factor in isocratic separation of amino acids is the prolonged retention of acidic amino acids, especially aspartic acid. One can separate 16-17 amino acids isocratically with 10 mM TEAP, pH 2.0, with 65% ACN. If separation of all 20 amino acids is required, then it will be necessary to start with a higher level of ACN, such as 75%, in order to separate the more hydrophobic amino acids completely. Run a gradient to a lower concentration of ACN.

**CAUTION:** At pH 2.0, glutamine is converted to pyroglutamic acid with a halflife of about 24 hours. Also, as above, try to match the composition of the sample solvent and starting mobile phase.

**Isocratic Separation of Peptides:** Use a mobile phase of 20 mM Na-MePO<sub>4</sub>, pH 2.0, with 70% ACN. At higher levels of ACN, very basic peptides may elute too late for isocratic conditions to be practical. Phosphorylated peptides may also elute well out of the time frame of other peptides under these conditions. If a sample contains phosphopeptides and it's important to resolve it isocratically, then treat it with alkaline phosphatase prior to the chromatography. These conditions should be suitable for peptides in addition to tryptic digests, including extracts from natural products. This is also a convenient, general-purpose way to assess the purity of synthetic peptides. A convenient way to perform method development is to assess the chromatography isocratically at 60% ACN, then increase the ACN level in increments of 3-5% until the elution window is wide enough for satisfactory resolution of the sample. As the % ACN increases, the selectivity will shift, with basic peptides becoming better retained relative to the others. Tryptic digests are unlikely to contain unusually basic peptides and so can be run with > 70% ACN for isocratic elution.

**CAUTION:** As above, try to match sample solvent and mobile phase as closely as possible.

**Separation of Proteins:** As in HILIC, proteins that are most likely to respond well to ERLIC include histones, integral membrane proteins, and other proteins not normally found in aqueous solution. Try method development as with isocratic elution of peptides (III, above). Propanol is sometimes a better solvent for intact proteins than is ACN, and a blend of the two may be better than either solvent alone. Solubilizing agents such as HFIP (hexafluoro-2-propanol) can be effective in small amounts in keeping intact proteins in mostly organic solvents, but tend to antagonize retention in either the HILIC or ERLIC modes.

**CAUTION:** As above, try to match sample solvent and mobile phase as closely as possible.

**Fractionation of tryptic digests:** ERLIC excels at this application. Initially, try the following gradient:

A) 90% ACN with 0.1% acetic acid (overall concentration)

B) 30% ACN with 0.1% formic acid (overall concentration)

Gradient: 5', 0% B; 25', 0-36% B; 20', 36-100% B; 10', 100% B. For a 200x4.6-mm column, use a flow rate of 1 ml/min.

Peptides will elute in order both of increasing polarity and decreasing isoelectric point (pI). This method does not have selectivity for peptides with post-translational modifications such as phosphate or carbohydrate groups. Peptides elute with exceptionally uniform distribution among the collected fractions. Some tryptic peptides do not dissolve well in the starting mobile phase. In such cases, try dissolving the tryptic digest in 85% ACN with 0.1% acetic acid + 10 mM ammonium acetate.

Preliminary data suggests that our PolySAX LP™ material is more retentive than the PolyWAX LP material in this mode and so Mobile Phase A can contain 85% ACN instead of 90%. This alleviates the peptide solubility problem.