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PolyCAT A™ Columns

Initial Use: PolyCAT A™ is a silica-based material with a bonded coating of polyaspartic acid. It is a weak cation-exchange (WCX) material. Columns are shipped in 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. A good conditioning solution is 40 mM EDTA.2Na (filtered, but pH not adjusted) at a low flow rate for 20-24 hours.

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-µm material. In some cases this has also caused the collapse of 5-µm, 200-Å column packings]. Alternatively, after flushing with water, condition the column for 2 hours with 0.2 M NaH_2PO_4 + 0.3 M sodium acetate. This solution conditions the coating but does not passivate metal surfaces.

Routine Use: Proteins can be eluted from PolyCAT A™ columns with salt and/or pH gradients. The most useful range for cation-exchange of proteins is pH 6-7. Phosphate and Bis-tris are good buffers in this range. The higher the pH, the weaker the retention. Avoid prolonged exposure to a pH above 8. For weakly basic peptides or for cation-exchange below pH 4, use PolySULFOETHYL A™, our strong cation-exchange (SCX) material.

Use ambient temperature (20-25°C), as this polypeptide-based coating is more sensitive to elevated temperatures than are other materials. Filter mobile phases and samples before use. Failure to do so may cause the inlet frit to plug. This frit can be replaced. At the beginning of the day, flush the column with 15 column volumes of the high-salt buffer before equilibration 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 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: After every 250 runs, invert the column and run it backwards overnight, at a low flow rate, with 40 mM EDTA.2Na. Continue using the column in this inverted direction for the next 250 samples, then repeat this treatment. If possible, open the inlet and fill in any voids with bulk PolyCAT A™ after running 500 samples.

Minimize Iron in the System: This coating chelates Fe^{+3}, which ruins its performance. If chloride-containing mobile phases are used regularly, passivate the column and the HPLC system every 4 weeks with the 40 mM EDTA.2Na solution as described above. NOTE: If the HPLC system has not been used for several days (e.g., over a weekend), then Fe^{+3} ions tend to accumulate in the fluid in the lines. When restarting the system, flush this fluid to waste offline before diverting flow through the column.

Volatile Solvents: Below pH 4 the coating loses its negative charge. Thus, peptides can be eluted by a gradient to dilute acetic acid.

Miscellaneous applications:
1) Hemoglobins: These are well separated by PolyCAT A™! See the specific Application Note.

2) Growth Factors or Protein Variant separations: Try an ammonium acetate gradient in 40% ACN. For separation of Asp- vs, isoAsp- variants, try mobile phases at pH 4.2.

3) Antibodies: Human: try pH 6.4-7. Murine (= mouse): try pH 7.2-8.0.

4) Chloride vs. acetate: Unlike chloride ion, acetate does not corrode stainless steel. However, it is not transparent below 230 nm, and 10% more acetate is required to match the eluting power of chloride.

5) Mixed-mode effects: When the mobile phase contains over 60% organic solvent, then hydrophilic interactions will be superimposed on the electrostatic effects. PolyCAT A™ can then resolve many peptides that differ in polarity but not charge (e.g., methylation of a Lys- residue). It may be necessary to use a gradient salt with good solubility in org. solvents, such as sodium perchlorate or triethylamine phosphate (TEAP).

Cation-Exchange HPLC with Volatile Mobile Phases

This method works with PolyCAT A™, our weak cation-exchange (WCX) material. A gradient to dilute acetic acid (HOAc) can uncharge the carboxyl- groups on the surface, leading to the elution of retained peptides. This gradient does not uncharge our strong cation-exchange (SCX) material, PolySULFOETHYL A™. Peptides are reliably retained on a WCX material only if they have three or more basic residues or two basic residues and a free N-terminus. Thus, most tryptic peptides are not well-retained. This method also works with nonpeptide basic solutes such as polyamines and aminoglycoside antibiotics. Elution is generally in order of least to most basic, but there is also some separation of sequence variants differing in nonbasic residues.

Adsorption: Apply the mixture to a PolyCAT A column equilibrated with 10 mM ammonium acetate, pH 5-5.5. Binding capacity is approx. 4 mg. peptide for a 4.6-mm i.d. column.

Elution: Run a linear gradient to 15% aq. HOAc. Some extremely basic peptides or polyamines have required as much as 30% HOAc for elution.

Detection: Absorbance detection below 240 nm is not possible with these mobile phases. Thus, absorbance detection is confined to peptides with aromatic residues; 254 nm (for Phe-), 270 nm (for Tyr-), or 280 nm (for Trp-). Suitable alternatives include mass spectroscopy or an evaporative light scattering detector (ELSD). Alternatively, just collect fractions for bioassay after lyophilization or drying in a SpeedVac.

Notes:

1) Extremely basic solutes: If elution requires over 20% HOAc, try PolyCAT A™ with 1000-Å pore diameter instead of 300-Å. This can decrease by 3x the concentration of HOAc required.

2) Synthetic peptides: This is a convenient way to clean up a crude synthetic product. Basic peptides are retained while deprotection fragments are not. This eliminates the need for an ether precipitation step (and potential oxidation of labile side chains).

3) Unusually hydrophobic peptides: To promote solubility, @ 20% organic solvent can be included in both mobile phases. Use of > 50% organic solvent will result in hydrophilic interactions being superimposed on the electrostatic effects.

4) Counterions: This method yields peptides with acetate counterions. This is compatible with bioassays, unlike the trifluoroacetate counterion frequently contributed by reversed-phase HPLC.

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Analysis of Hemoglobins using PolyCAT A™ columns

The following protocols were developed by Cheryl Rognerud and Ching-Nan Ou at Texas Children's Hospital (Houston):

For rapid screens or routine analysis use i**tem# 3.54CT0510; 5µ, 1000Å.**

Flow rate: Routine Screen 1.7mL/min

Gradient:	Time (min)	%B
	0	10
	8	40
	12	100
	13	10*

Mobile Phase A: 20mM Bis-tris + 2mM KCN, pH 6.96.
Mobile Phase B: 20mM Bis-tris + 2mM KCN + 200mM NaCl, pH 6.55.
Data Collection: *Collect data through 15'. Ready for a new sample at 20'.
**Collect data through 8.5'. Ready for a new sample at 10'.

NOTE: Initial %B may vary depending on column & reagent lot as well as individual instrument configuration. Ending %B is set to the same value as @ time 0'.

Flow rate: Rapid Screen 3.4mL/min

Gradient:	Time (min)	%B
	0	10
	4	40
	6	100
	7	10**

For analysis of a small number of samples or confirmatory run for identifying a variant use **item# 204CT0510; 5µ, 1000Å.**

Flow rate: Routine Screen 1.0mL/min

Gradient:	Time (min)	%B
	0	14
	16	50
	22	100
	24	100
	26	14

Mobile Phase A: 20mM Bis-tris + 2mM KCN, pH 6.96.
Mobile Phase B: 20mM Bis-tris + 2mM KCN + 200mM NaCl, pH 6.55.
Data Collection: Collect data through 32'. Ready for a new sample at 44'.

For rapid screens or routine analysis use **item# 3.54CT0315; 3µ, 1500Å.**
This column has replaced item# 3.54CT0510 in most labs.

Flow rate: Routine Screen 1.7mL/min

Gradient:	Time (min)	%B
	0	10
	8	40
	15	100
	16	10

Mobile Phase A: 20mM Bis-tris + 2mM KCN, pH 6.96.
Mobile Phase B: 20mM Bis-tris + 2mM KCN + 200mM NaCl, pH 6.55.
Data Collection: Collect data through 15'. Ready for a new sample at 20'.

Rapid Screen TBD

Faster throughput can be obtained with a faster flow rate and gradient schedule, but keep back pressure < 2000psi.

For superior resolution of difficult variants use i**tem# 104CT0315; 3µ, 1500Å.**
This column has replaced item# 204CT0510 in most labs.

Flow rate: Routine Screen 1.2mL/min

Gradient:	Time (min)	%B
	0	18
	8	45
	12	100
	13	18

Mobile Phase A: 40mM Bis-tris + 2mM KCN, pH 6.5.
Mobile Phase B: 40mM Bis-tris + 2mM KCN + 200mM NaCl, pH 6.8.
Data Collection: Collect data through 18'. Ready for a new sample at 25'.

Notes: **Do not degas the buffers with helium or vacuum!** Bypass any vacuum degasser in the system. Hemoglobin tetramers exchange dimers in solution in a dynamic fashion. In the absence of oxygen, this process is much slower.
Results: 1) Unnatural subunits are stabilized, resulting in artificial peaks
2) Artifacts can result from use of KCN or NaCN over 10 years old,
3) Artifacts are also observed with poorly set automatic injector valves. Assess this by using a manual injector valve if you suspect problems.

Loading Capacity: The loading capacity of a 4.6mm ID column is about 4 mg of protein/injection, depending on the strength of the protein's binding to the support. If a PolyCAT A™ column is to be used for preparative isolation of hemoglobins, then it is necessary to use 5 – 10mM KCN in the sample solvent and mobile phases.

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: After every 250 runs, invert the column and run it backwards overnight, at a low flow rate, with 40 mM EDTA.2Na. Continue using the column in this inverted direction for the next 250 samples, and then repeat this treatment. If possible, open the inlet and fill in any voids with bulk PolyCAT A™ after running 500 samples.

Preparation of Hemolyzates

1) Draw 1-2 ml of blood into a purple-capped tube (i.e., with EDTA). Alternatively, draw 100-200 µl with a fingerstick.

2) Washing away plasma proteins: Add 50 µl whole blood to a 1.5-ml microcentrifuge tube with 1 ml isotonic saline (= 0.9% NaCl = 154 mM) and spin it for 30-60" @ 13,000 rpm. Discard supernatant.

3) Lysing the erythrocytes: Add water equal to 2-3x the volume of the packed cells. Let sit 5-10' to lyse the erythrocytes. The following are aids in lysing the cells: a) Vortexing; b) Including Triton X-100 in the lysis solution; c) A freeze-thaw cycle.

4) Isolation of the hemolyzate: Centrifuge 5' @ 13,000 rpm to spin down the erythrocyte ghosts. The visible pellet should be grayish and may occupy up to 1/3rd of the volume of the solution, with the clear red lysate on top. This lysate is the hemolyzate. NOTE: If the precipitate is red, then the erythrocytes have not been lysed.

5) Preparation of samples for HPLC analysis: Add 20 µl lysate to 250 µl mobile phase A. Inject 8-10 µlper analysis with a 200 x 4.6-mm column or 4-8 µl for a 35 x 4.6-mm column. A partially filled loading loop is acceptable. If this solution is to serve as a standard, then it can be stored in a freezer in aliquots of 10-20 µl for future analysis.

Notes:
1) Smaller injections of more concentrated samples afford better resolution than larger, more dilute samples.

2) Dot blot analyses from neonates: Prick the heel of the neonate and blot a single drop of blood onto a piece of filter paper. This can be mailed to the lab that has the PolyCAT A™ column. A circle of the blood blot is cut out with a standard size paper puncher. This circle is soaked for 2 hr. in 200 µl of Mobile Phase A containing 4 mM KCN (not 2 mM). The resulting solution is centrifuged for 1' to remove particulates and 25-50 µl is injected for analysis; the optimum volumes depends on the percentage of Hb F and the number of other peaks present.

3) Destroying labile glycated hemoglobins prior to A1c analysis: Alternatives:

a) In Step 2 above, incubate the washed erythrocytes in 0.9% NaCl for 1 hr. at 37°C before lysis.

b) Same as a), but leave washed erythrocytes overnight at ambient temperature.

c) Leave the washed erythrocytes overnight in mobile phase A (which lyses the cells, see Step 5 above).

It usually isn’t necessary to destroy labile glycated hemoglobins prior to analysis, since these are separated from stable A1c on PolyCAT A™ columns and elute in a “pre-A1c” peak.