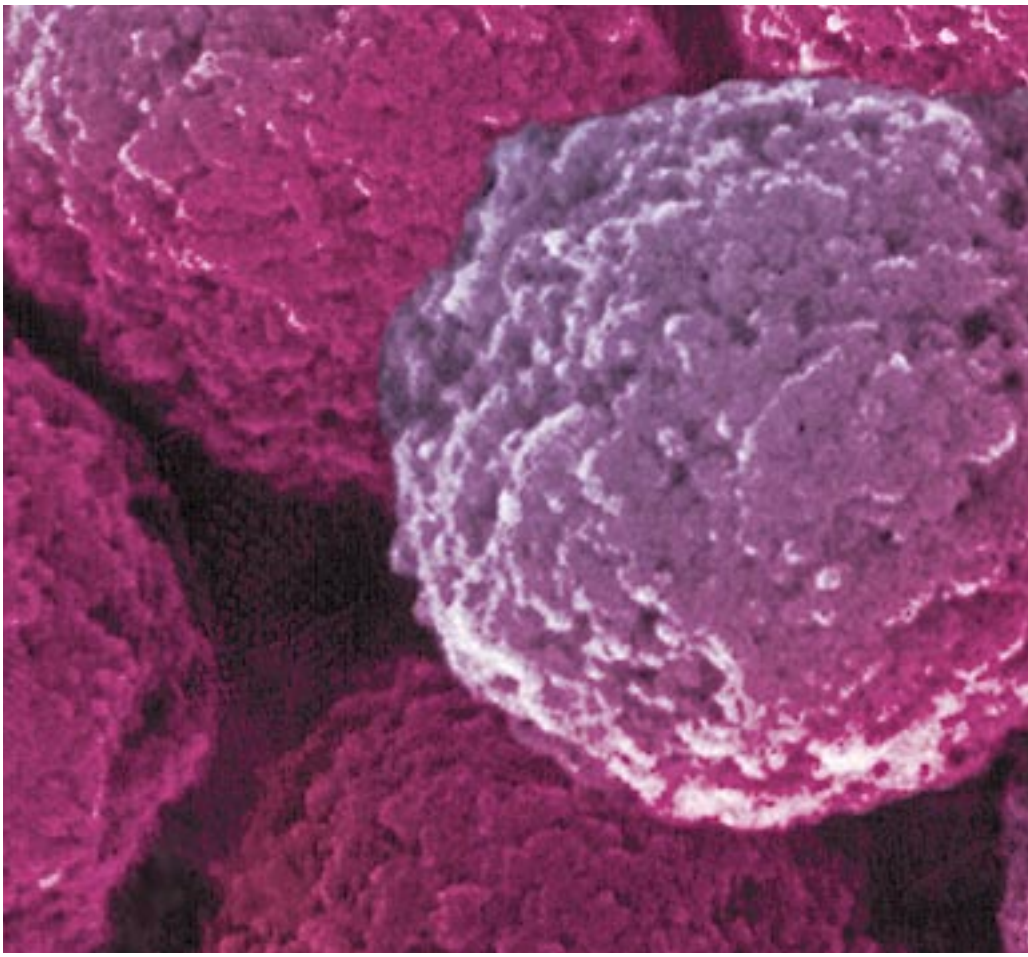
Macro-Prep®
Chromatography Supports

Ceramic Hydroxyapatite

BIO-RAD

Macro-Prep Ceramic Hydroxyapatite



The separation and purification of proteins and nucleic acids using hydroxyapatite (HT) has become a well established chromatographic technique since the work of Tiselius in 1956. There are several thousand references on the use of HT for the purification and concentration of protein extracts and fermentation broths, removal of DNA from samples, high resolution separation of IgG subclasses, and final separation of single peak eluting isozymes. HT is also commonly used to separate single-stranded DNA from double-stranded DNA.

New Macro-Prep Ceramic Hydroxyapatite

Until recently, the use of hydroxyapatite has been limited by the fragile nature of its crystalline form. The separation characteristics of the new Macro-Prep ceramic hydroxyapatite are virtually identical to crystalline hydroxyapatite, but the physical limitations of the old material have been overcome. The material is macroporous, spherical, and extremely stable, both chemically and physically. Ceramic hydroxyapatite (CHT) is exceptionally easy to work with. It can be packed into columns which can be cycled hundreds of times with no change in either separation or operating pressure. Ceramic hydroxyapatite can withstand high flow rates, high pressure, high pH, and exposure to various organic solvents.

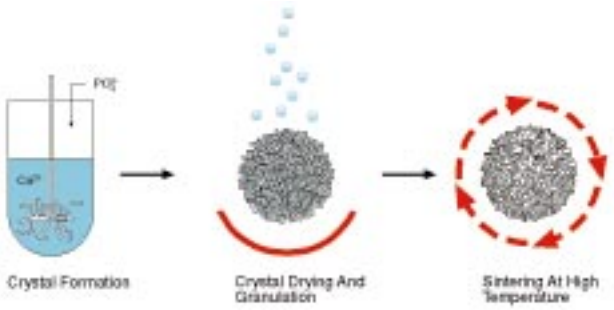


Fig. 2. Sintering of agglomerated hydroxyapatite crystals produces a hard, ceramic bead.

Macro-Prep ceramic hydroxyapatite is available in two types, type I and type II. The two types are chemically identical, but have been sintered at different times and temperatures. This difference in sintering conditions effects the surface properties of each type. The type I material exhibits a higher binding capacity for proteins, in particular acidic proteins. The type II material separates ss DNA and ds DNA better, and often gives a better separation of IgG subclasses. The retention time differences between sixteen proteins with isoelectric points between 3.9 and 10.6 for the two types are shown in Figure 3. The proteins were eluted using a linear gradient of sodium phosphate at pH 6, 7, 8, and 9. With its higher capacity, the type I hydroxyapatite is recommended as the first choice for protein purification.

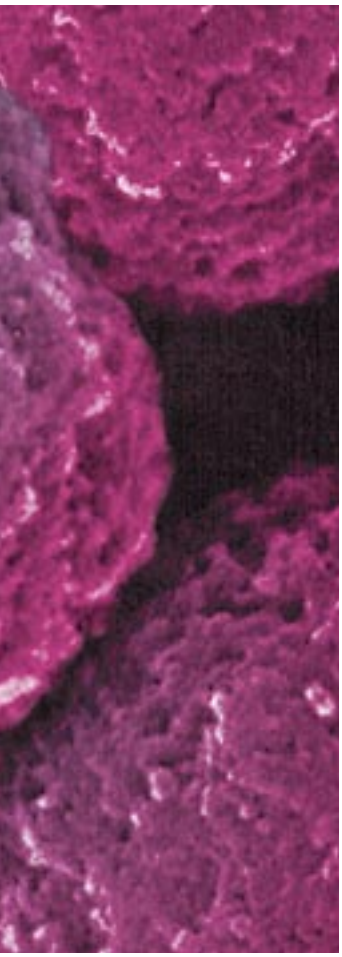


Fig. 1. Ceramic hydroxyapatite bead.

Mechanism of Action

Proteins are usually adsorbed to hydroxyapatite in a low ionic strength phosphate buffer at neutral pH (e.g., 10 mM at pH 6.8). Selective elution is accomplished with higher concentrations of phosphate and/or pH gradients. The unique selectivity of hydroxyapatite will often resolve components that other techniques fail to separate.

Experimental evidence (1) suggests that interaction between the charged moieties on the protein surface and the hydroxyapatite structure (Ca^{2+} ions at the positively-charged centers and PO_4^{3-} ions at the negatively-charged centers) results in essentially a "mixed-mode" ion exchange separation. Although the exact separation mechanism is not known, the separation is not primarily dependent on any single physical property of the protein, including isoelectric point, molecular weight, or charge density.

In general, hydroxyapatite demonstrates higher affinity for proteins with high pI's and lower affinity for proteins of low pI. Thus proteins tend to separate according to their isoelectric point, with more acidic proteins eluting early, or not binding at all, and neutral and basic proteins eluting later.

High Chemical Stability

The Macro-Prep CHT supports are excellent choices for long term use requiring cleaning and sanitization. The Macro-Prep CHT is stable from pH 5.5–14, and supports can withstand repeated cleaning and sanitation with up to 2 M NaOH, with negligible changes in performance. Macro-Prep supports can be sterilized by autoclaving.

The stability of the CHT supports is demonstrated in Figure 4. A purified protein mixture (myoglobin, ribonuclease A, α-chymotrypsinogen, cytochrome c, and BSA) was injected before and after one hundred 20 ml gradient cycles with no loss in resolution and negligible increase in back pressure.

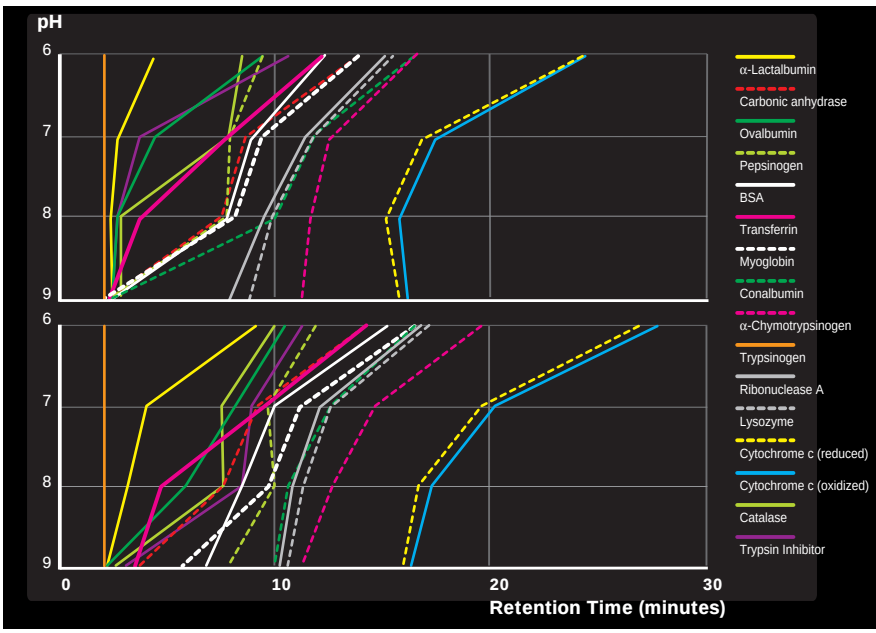


Fig. 3. Elution vs. pH, Macro-Prep ceramic hydroxyapatite, type I (bottom) and II (top).

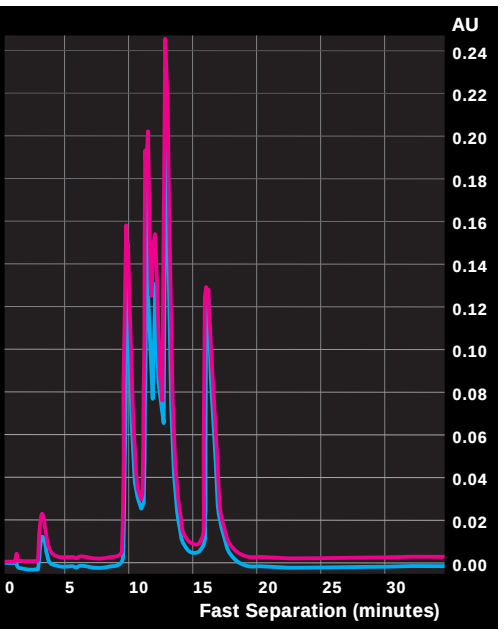
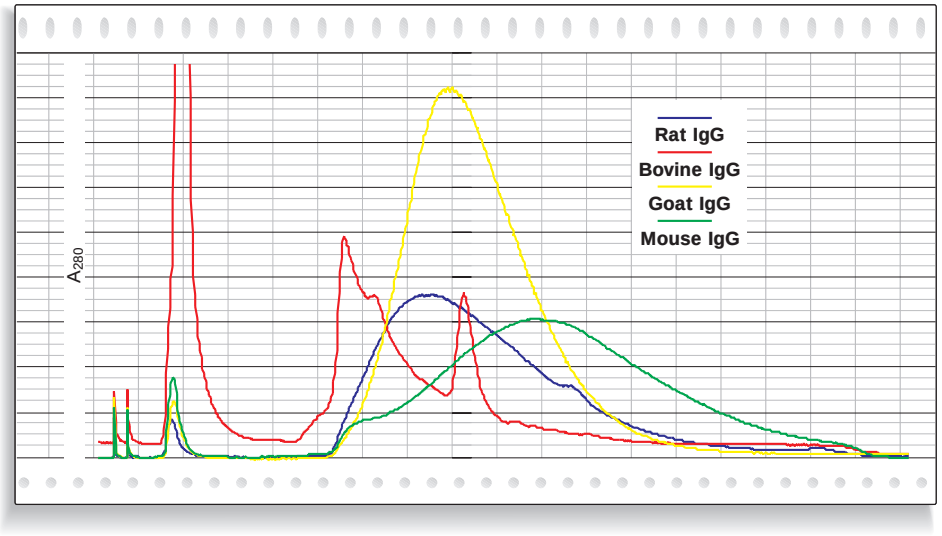


Fig. 4. Stability test on the Bio-Scale CHT2-I column.

Ideal Support for Antibody Purification



The ceramic hydroxyapatite type I support (CHT-I) are particularly well suited for the purification of antibodies, enzymes, and other proteins. The unique selectivity of this newly-developed support allows the bio-chromatographer to purify antibodies from different sources. The gentle elution conditions using neutral sodium phosphate buffer preserve protein activity with high protein recovery. A major feature of the CHT support is that it can be used to purify monoclonal antibodies that will not bind to Protein A.

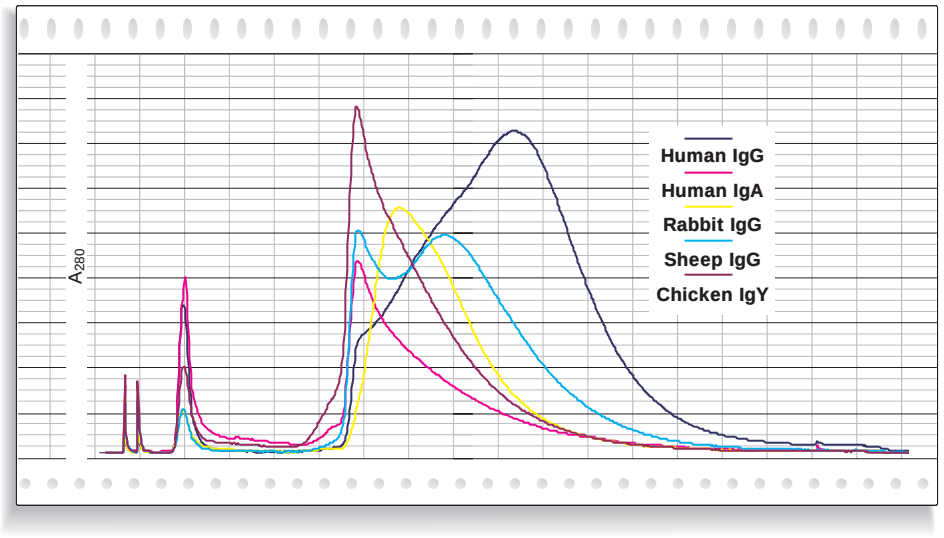


Fig. 5. Eight different purified IgG and IgA were individually injected into a Bio-Scale CHT2-I column. The results demonstrate that all polyclonal antibodies were bound at 10 mM sodium phosphate, pH 6.80, and were eluted by a linear gradient of 250 mM sodium phosphate, pH 6.80. The separations were performed using the Automated BioLogic System. Polyclonal antibodies elute as several peaks due to the differences in glycosylation. In some cases, this gives the impression of a broad peak, when in reality it is a series of overlapping peaks.

Scaling Up with the Macro-Prep CHT

The availability of the Macro-Prep CHT in four particle sizes (10, 20, 40, and 80 µm) provides unrivaled ability for scaling up purification protocols from laboratory scale to process scale. It is no longer necessary to do the development work on one type of high resolution analytical support, only to be forced to transfer it to another type of preparative chromatographic support. The following scale-up application shows the maintained resolution obtained from a yeast enzyme concentrate using different formats of the Macro-Prep CHT-I support with simple a linear gradients of sodium phosphate.

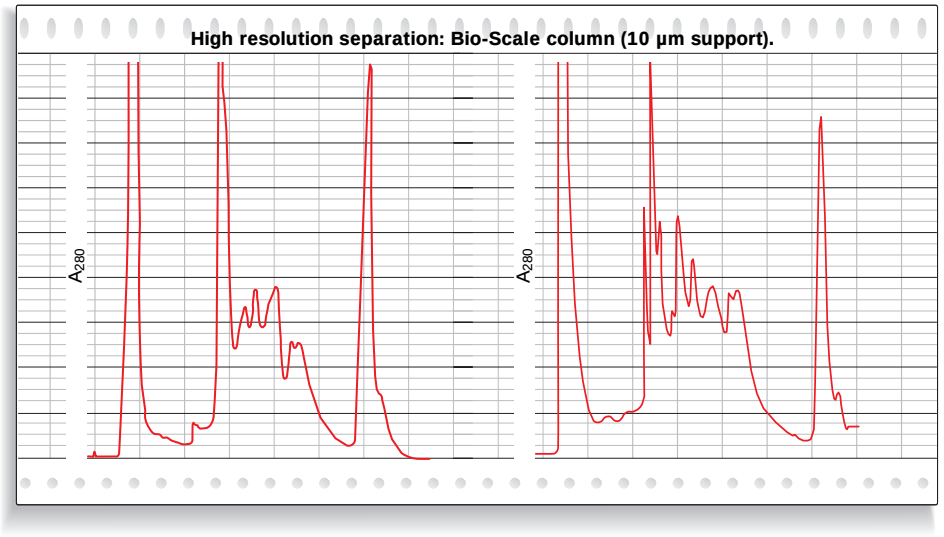


Fig. 6. Bio-Scale CHT5-I column, 5 ml. Sample: 25 mg. Flow rate: 1.0 ml/min (80 cm/h).

Fig. 7. Bio-Scale CHT20-I column, 20 ml. Sample: 100 mg. Flow rate: 2.3 ml/min (80 cm/h).

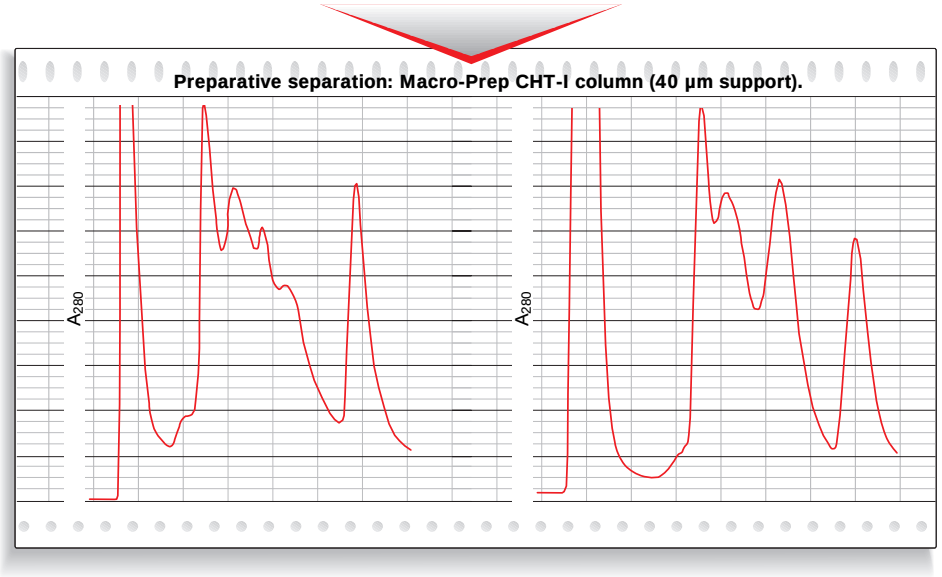


Fig. 8. Macro-Prep CHT-I column, 50 ml. Sample, 200 mg. Flow rate, 6.5 ml/min (80 cm/h).

Fig. 9. Macro-Prep CHT-I column, 50 ml. Sample, 1.0 gram. Flow rate, 6.5 ml/min (80 cm/h).

Several Formats and Particle Sizes Available

The Macro-Prep CHT supports are available in four particle sizes (10, 20, 40, and 80 µm) and in a variety of package sizes. This provides total flexibility for

- High resolution laboratory scale protein purification using the 10 µm support
- Rapid methods development, without changing to different support materials when scaling-up
- Low pressure liquid chromatography using the 20 µm support
- Process scale chromatography and industrial production of biomolecules using the 40 and 80 µm support

High Resolution Protein Purification

The prepacked Bio-Scale CHT-I columns contain the 10 µm Macro-Prep ceramic hydroxyapatite type I support. These columns can be used for rapid and reproducible high resolution separation of biomolecules with the BioLogic System or any medium or high pressure system. Four different column sizes (2, 5, 10, and 20 ml) provide unrivaled flexibility for economical and predictable scale-up of separation and purification protocols without sacrificing resolution due to overloading. Other features of the Bio-Scale columns include

- Biocompatible materials preserve protein integrity
- Fingertight fittings eliminate the need for tools
- Adjustable bed support eliminates column voids
- Top-off resin kit (optional) extends column life

Low Pressure Liquid Chromatography

The 20, 40, and 80 µm Macro-Prep CHT supports are available in 100 gram bottles for packing low pressure columns of any size. The supports pack easily and quickly-without the use of special packing devices, and form stable column beds which provide long trouble free use.

For maximum convenience, the 20 µm Macro-Prep CHT-II support is available in 1 ml and 5 ml prepacked Econo-Pac® cartridges. The Econo-Pac cartridges simplify chromatography because there is no gel to prepare and no column to pack. The cartridges may be used with Bio-Rad's Econo System and other low pressure LC systems and pumps, and are easily adapted for use with the BioLogic System, or any medium or high pressure systems. Up to three Econo-Pac cartridges can be connected in series for instant scale-up with no loss of resolution. For simple step wise or isocratic elution, the cartridges can be used with a syringe.

Process Scale Chromatography

The 20, 40, and 80 µm Macro-Prep ceramic hydroxyapatite type I and type II supports are also available in 1 kg and 5 kg packages for scale-up and process scale chromatography applications. The physical and chemical properties of the Macro-Prep CHT supports make them ideally suited for larger scale separations. Bio-Rad is registered under ISO 9901. All Macro-Prep supports are manufactured under cGMP and have Drug Master Files.

Performance and Properties

Table 1. Bio-Scale CHT-I Column

	CHT2-I	CHT5-I	CHT10-I	CHT20-I
Column volume (ml)	2	5	10	20
Recommended maximum protein loading (mg)	20	50	100	200
Recommended flow-rates (ml/min)	0.5–3.0	0.5–5.0	0.5–7.0	0.5–10.0
Dynamic protein binding capacity (mg lysozyme/per column)	50	125	250	500
Average particle size (µm)	10 ± 2	10 ± 2	10 ± 2	10 ± 2
Column Dimensions (mm)	7 x 52	10 x 64	12 x 88	15 x 113
Maximum operating pressure (psi/bar)	1,000/70	750/50	600/40	500/34
Back-pressure at maximum recommended flow-rate (psi/bar)	360/24	320/21	290/19	320/21

Table 2. Econo-Pac CHT-II Cartridge

Cartridge volume (ml)	1	5
Recommended maximum protein loading (mg)	3 mg BSA, 6 mg lysozyme	15 mg BSA, 30 mg lysozyme
Recommended flow rates (ml/min)	0.5–0.8	0.5–1.0
Average particle size (µm)	20 ± 10	20 ± 10
Cartridge dimensions (mm)	0.6 x 3.8	1.24 x 3.8
Maximum operating pressure (psi/bar)	50/3	50/3
Back-pressure at maximum recommended flow-rate	<10 psi	<10 psi

Table 3. Macro-Prep Ceramic Hydroxyapatite Support

	Type I	Type II
Functional group	Ca^{2+},PO_4^{-3}	Ca^{2+},PO_4^{-3}
Dynamic protein binding capacity (mg lysozyme/g)	>25mg	>12.5 mg
Nominal particle size (µm)	20, 40, and 80	20, 40, and 80
Nominal pore size(Å)	500–1,000	500–1,000
Recommended maximum linear flow rate	5,000 cm/hour	5,000 cm/hour
Autoclavable (121 °C, 30 min)	yes	yes
2.0 M NaOH (20 °C)	excellent	excellent
pH stability	5.5–14	5.5–14
Regeneration	Trisodium phosphate	Trisodium phosphate
Sanitization	1–2 M NaOH	1–2 M NaOH

Note: Many chromatography support manufacturers choose to publish a static binding capacity value. However, expressing the binding capacity in terms of dynamic loading capacity more realistically reflects the practical loading limits in order to achieve maximal resolution under flow conditions.

Reference: 1. Gorbunoff (1984), *Anal. Biochem*. **136**, 425-432.

Larger volumes are available upon request

Econo-Pac cartridges simplify protein purification

Ordering Information

Catalog Number	Product Description
732-0081	**Econo-Pac CHT-II Cartridge,** 1 x 5 ml
732-0085	**Econo-Pac CHT-II Cartridge,** 5 x 5 ml
732-0083	**Econo-Pac CHT-II Cartridge,** 5 x 1 ml
751-0021	**Bio-Scale CHT2-I Column,** 2 ml
751-0023	**Bio-Scale CHT5-I Column,** 5 ml
751-0025	**Bio-Scale CHT10-I Column,** 10 ml
751-0027	**Bio-Scale CHT20-I Column,** 20 ml
751-0029	**Top-Off Resin Kit CHT-I,** 1 ml
157-0020	**Macro-Prep Ceramic Hydroxyapatite,** type I, 20 µm, 100 g
157-0021	**Macro-Prep Ceramic Hydroxyapatite,** type I, 20 µm, 1 kg
157-0025	**Macro-Prep Ceramic Hydroxyapatite,** type I, 20 µm, 5 kg
157-0040	**Macro-Prep Ceramic Hydroxyapatite,** type I, 40 µm, 100 g
157-0041	**Macro-Prep Ceramic Hydroxyapatite,** type I, 40 µm, 1 kg
157-0045	**Macro-Prep Ceramic Hydroxyapatite,** type I, 40 µm, 5 kg
157-0080	**Macro-Prep Ceramic Hydroxyapatite,** type I, 80 µm, 100 g
157-0081	**Macro-Prep Ceramic Hydroxyapatite,** type I, 80 µm, 1 kg
157-0085	**Macro-Prep Ceramic Hydroxyapatite,** type I, 80 µm, 5 kg
157-2000	**Macro-Prep Ceramic Hydroxyapatite,** type II, 20 µm, 100 g
157-2100	**Macro-Prep Ceramic Hydroxyapatite,** type II, 20 µm, 1 kg
157-2500	**Macro-Prep Ceramic Hydroxyapatite,** type II, 20 µm, 5 kg
157-4000	**Macro-Prep Ceramic Hydroxyapatite,** type II, 40 µm, 100 g
157-4100	**Macro-Prep Ceramic Hydroxyapatite,** type II, 40 µm, 1 kg
157-4500	**Macro-Prep Ceramic Hydroxyapatite,** type II, 40 µm, 5 kg
157-8000	**Macro-Prep Ceramic Hydroxyapatite,** type II, 80 µm, 100 g
157-8100	**Macro-Prep Ceramic Hydroxyapatite,** type II, 80 µm, 1 kg
157-8500	**Macro-Prep Ceramic Hydroxyapatite,** type II, 80 µm, 5 kg

See the current Bio-Rad catalog for additional information. Specifications are subject to change without notice.

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