

## Gel Filtration Chromatography with Biocompatible SigmaChrom GFC Columns

High performance SigmaChrom GFC-100 and SigmaChrom GFC-1300 gel filtration columns are well suited for separating and isolating peptides and proteins, in HPLC or FPLC systems. The two columns span an optimal separation range of 3000 to 600,000 dalton, with exclusion limits of 100,000 dalton and 1,300,000 dalton, respectively, for globular proteins. Constructed of inert polyetheretherketone polymer and filled with a polysaccharide-based packing, the biocompatible columns are excellent alternatives to silica-based stainless steel gel filtration columns. Typical applications and calibration curves for globular proteins are shown.

### Key Words:

- proteins ● peptides ● gel filtration chromatography
- size exclusion chromatography

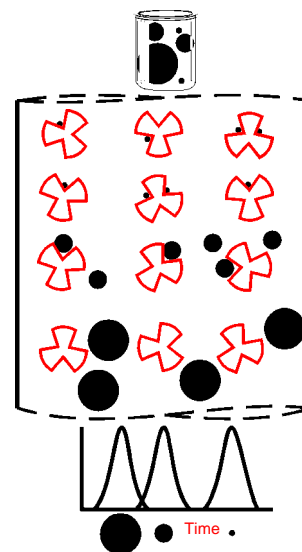
Size exclusion chromatography (SEC) separates molecules according to differences in their sizes. The two forms of size exclusion chromatography, gel filtration chromatography (GFC) and gel permeation chromatography (GPC), differ in the nature of the mobile phase used. An aqueous mobile phase is used in GFC and an organic mobile phase is used in GPC.

Pores in the column packing particles are within a controlled range of sizes, comparable to the sizes of molecules to be separated, and thus they govern the analytes' access to the pore volume inside the particles. Molecules larger than the *exclusion limit* of the packing are fully excluded from entering the pores and thus pass through the column in the void volume,  $V_0$ . Smaller analytes have, according to their size, partial to complete access to the pore volume. Passage of small molecules that have complete access to the pores defines the pore volume,  $V_i$ . Among these smaller analytes, the larger molecules elute first and the smallest molecules elute last (Figure A).

The *fractionation range* — the molecular weight range of the largest molecule that is fully included by the pores to the smallest molecule that is fully excluded — depends on the size of the pores in the particles and varies with analyte type and shape (e.g., globular proteins). Within the fractionation range, and for a packing material of a particular particle size, peak resolution is determined by the pore volume: the greater the pore volume the larger the number of proteins of similar molecular weight that can be resolved.

High performance SigmaChrom™ GFC-100 and SigmaChrom GFC-1300 columns have been designed for analytical-scale gel filtration separations and isolations of peptides and proteins. In these columns, the mobile phase occupies ~85% of the column

**Figure A. Analytes Separate by Size in Gel Filtration Chromatography**



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volume, and interparticle porosity is ~35%. Thus, the pores occupy half the total column volume. Constructed of polyetheretherketone (PEEK), an inert, biocompatible plastic, the SigmaChrom columns are well suited to analysis of biomolecules. The two columns span a separation range of 3000 to 600,000 dalton, with exclusion limits of ~100,000 dalton and ~1,300,000 dalton, respectively, for globular proteins. Additional physical and chemical characteristics of these columns are summarized in Table 1.

**Table 1. Characteristics of SigmaChrom GFC Columns**

Packing:	crosslinked polysaccharide, 12-15µm
Column:	polyetheretherketone, 30cm x 7.5mm
Plates/Meter:	>30,000
Peak Symmetry:	0.7-1.3
Empty Column Volume:	13.25mL
Flow Rate:	typical: 0.5mL/min maximum: 1.0mL/min
Maximum Back Pressure:	250psi (1.72MPa)
pH:	3-12
Maximum Protein Capacity:	5.5mg
Optimal Separation Range:	GFC-100: 3-70 x 10 <sup>3</sup> dalton GFC-1300: 10-600 x 10 <sup>3</sup> dalton
Protein Exclusion Limit:	GFC-100: 100 x 10 <sup>3</sup> dalton GFC-1300: 1300 x 10 <sup>3</sup> dalton
Typical Recovery:	Mass: >90% Enzyme Activity: >80%
Typical Separation Times:	15-30 min

## Using Calibration Curves

GFC is widely used for determining protein molecular weight. For compounds of similar molecular shape, a sigmoidal calibration curve is obtained by plotting the logarithm of molecular weight (MW) versus the elution volume ( $V_e$ ) for molecules of known weight (Figure B). The *optimal separation range* is defined by the linear portion of this curve. Once a calibration curve is prepared, the elution volume for a protein of similar shape, but unknown weight, can be used to determine the MW. Results are most accurate when the investigator prepares the calibration curve and determines the molecular weight of the unknown molecule on the same day, with the same mobile phases, etc.

The calibration curves in Figure B demonstrate the excellent separation characteristics of the SigmaChrom GFC columns — a function of their very large pore volumes. The large pore size and pore volume for the GFC-1300 column allow separations of medium to large proteins. The linear portion of the curve extends from cytochrome c to thyroglobulin. The capability for separating a very broad MW range of proteins makes the GFC-1300 column the preferred general purpose column, providing best results for large proteins. The smaller pores in the GFC-100 column packing provide optimum resolution for large peptides and small proteins, from about 3000 to 70,000 dalton. Despite similar weights, cytochrome c, ribonuclease A and myoglobin elute in the expected order in the middle of the linear portion of the curve.

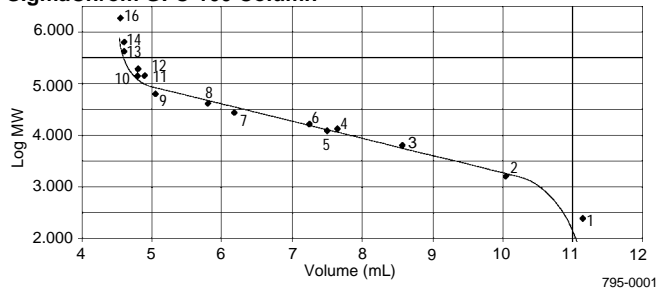
**Figure B. Molecular Weight Calibration Curves for SigmaChrom GFC Columns (Globular Proteins)**

Columns: **30cm x 7.5mm ID, 12-15 $\mu$ m particles**  
 Cat. Nos.: **54750-U (GFC-100), 54751 (GFC-1300)**  
 Mobile Phase: 50mM Tris-HCl/100mM KCl, pH 7.5  
 Flow Rate: 0.5mL/min  
 Det.: UV, 280nm  
 Inj.: 20 $\mu$ L, 1-10mg/mL each analyte

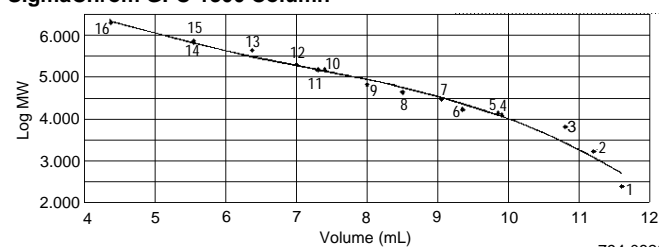
Molecule	MW	Molecule	MW
1. Cytidine	243	9. Albumin	66,000
2. Neurotensin	1673	10. Alcohol dehydrogenase	150,000
3. Aprotinin	6500	11. $\square$ Globulins	150,000
4. Cytochrome c	12,400	12. $\square$ -Amylase	200,000
5. Ribonuclease A	13,690	13. Apoferritin	443,000
6. Myoglobin	16,900	14. Thyroglobulin	669,000
7. Carbonic anhydrase	29,000	15. $\square_2$ -Macroglobulin*	750,000
8. Ovalbumin	43,500	16. Blue dextran	2,000,000

\*GFC-1300 column only

**SigmaChrom GFC-100 Column**

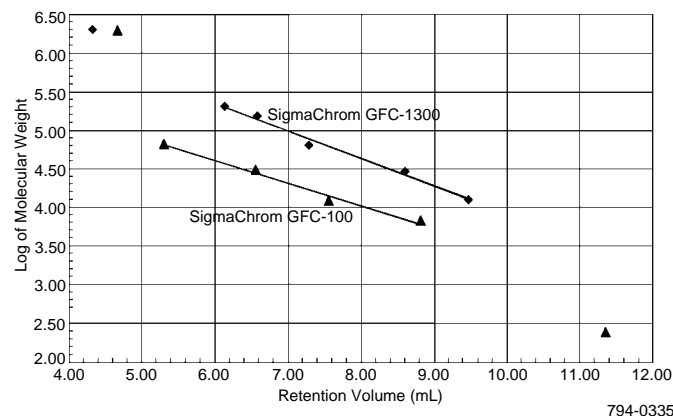


**SigmaChrom GFC-1300 Column**



Researchers desiring to determine a globular protein molecular weight do not need to use all the proteins used to plot Figure B. Generally, a standard molecular weight kit, representative of the column's fractionation range, can be used. We used kits containing four or five proteins, plus blue dextran as a large MW marker, and added cytidine as a marker for the total pore volume. Figure C shows the resulting simple linear calibration curves for the SigmaChrom columns.

**Figure C. Log MW vs. Retention Volume for SigmaChrom GFC Columns**

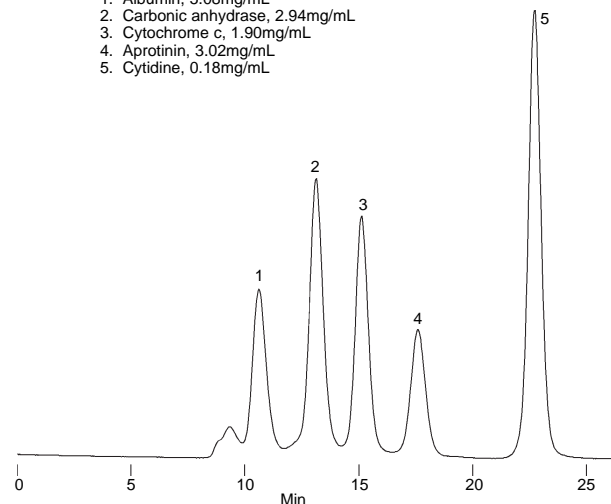


Buffer: 50mM Tris HCl, + 100mM KCl, pH 7.5  
 Markers for GFC-100: dextran, bovine serum albumin, carbonic anhydrase, cytochrome c, aprotinin, cytidine.  
 Markers for GFC-1300: dextran  $\square$  -amylase, alcohol dehydrogenase, bovine serum albumin, carbonic anhydrase, cytochrome c.

**Figure D. Protein Molecular Weight Standards**

Column: **GFC-100, 30cm x 7.5mm ID, 12-15 $\mu$ m particles**  
 Cat. No.: **54750-U**  
 Mobile Phase: 50mM Tris-HCl + 100mM KCl, pH 7.5  
 Flow Rate: 0.5mL/min  
 Det.: UV, 280nm  
 Inj.: 10 $\mu$ L

1. Albumin, 5.08mg/mL
2. Carbonic anhydrase, 2.94mg/mL
3. Cytochrome c, 1.90mg/mL
4. Aprotinin, 3.02mg/mL
5. Cytidine, 0.18mg/mL

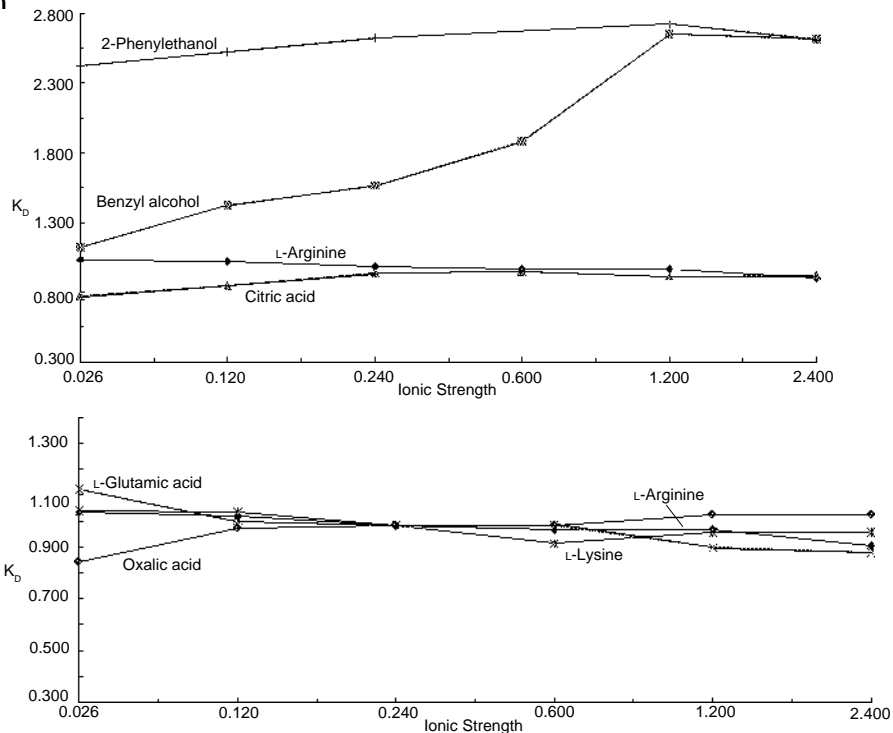


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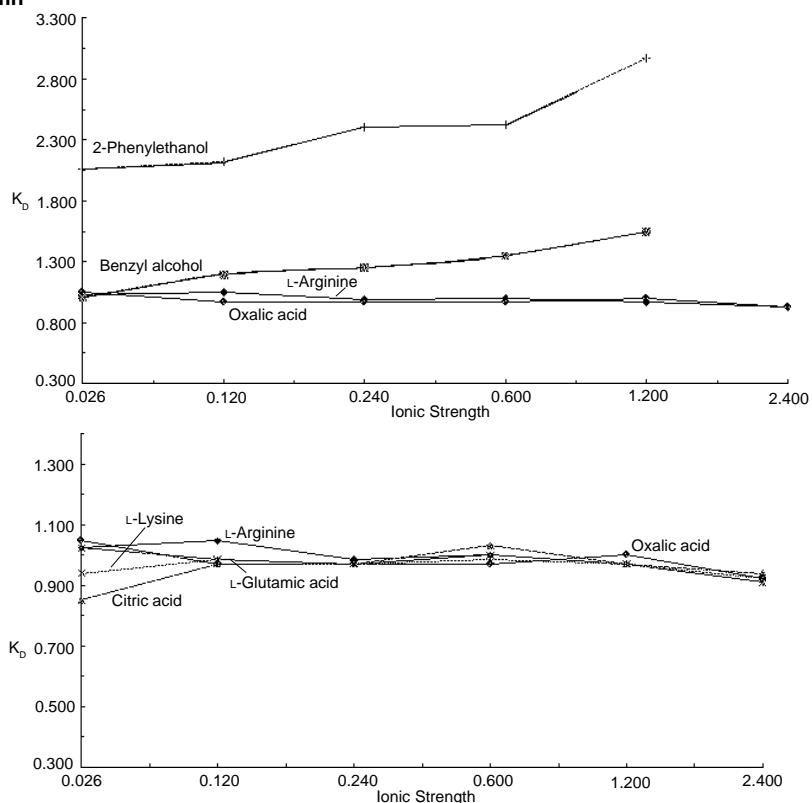
## Figure E. Effects of Ionic Strength on Distribution Coefficient ( $K_D$ ): Small Molecules

Columns: 30cm x 7.5mm ID, 12-15 $\mu$ m particles  
 Cat. Nos.: 54750-U (GFC-100), 54751 (GFC-1300)  
 Mobile Phase: K phosphate, pH 7.0  
 Flow Rate: 0.5mL/min  
 Det.: UV, 280nm  
 Inj.: 20 $\mu$ L, 10 $\mu$ L/mL or 5mg/mL each analyte

### SigmaChrom GFC-100 Column



### SigmaChrom GFC-1300 Column



Glutamic acid and 2-phenylethanol exhibited poor solubility at lower ionic strengths.

794-0827, 0828, 0829,0830

As mentioned previously, calibration curves should be generated using analytes whose shape is similar to that of the unknown molecule. Hydrodynamic volume and shape of the standards, in addition to their molecular weight, play a role in retention on GFC columns (and thus in calibration). For example, different standards were required for water-soluble globular proteins and detergent-soluble membrane proteins (1). Membrane proteins often are excluded or retarded. Accordingly, peptide standards should be used for determining the MW of a peptide (2), and MW determination of a denatured protein should be performed on a calibration curve of denatured protein standards.

Figure D shows the separation of the standards in Sigma® Protein Molecular Weight Kit MW-GF-70 on a SigmaChrom GFC-100 column. The small, complex peak eluting before albumin most likely is aggregated species. The instructions for using the Sigma molecular weight kit explain that aggregates can form when the protein standards are mixed together. The elution volumes of the remaining peaks agree well with the SigmaChrom GFC-100 calibration curve shown in Figure B. In preparing calibration curves for GFC, you can save time by mixing protein standards together, but be aware of the possibility of aggregate formation, and that not all aggregates will be as evident as that in Figure D.

### Secondary Interactions

Under ideal size exclusion chromatography conditions, all molecules elute in an elution volume larger than the void volume, but smaller than the total mobile phase volume ( $V_0 + V_i$ ). The distribution coefficient,  $K_D$ , for elution by ideal size exclusion chromatography is presented in equation [1], in which  $K_D$  varies from zero for a fully excluded molecule to one for a small molecular weight molecule that is capable of penetrating all the pores.

$$V_e = V_0 + K_D V_i \quad [1]$$

Despite advances in the synthesis of polymer-based particles with hydrophilic surface properties and the use of elaborate bonding procedures, all commercially available GFC supports show some deviation from ideal size exclusion behavior for peptides and proteins. Secondary interactions can be ionic and/or hydrophobic in nature. Silica- and polymer-based supports have slight residual negative or positive charges. Increasing the ionic strength of the mobile phase can overcome the ionic interactions between sample analytes and packing particles. However, hydrophobic interactions increase with increasing ionic strength. Thus, a balance must be struck between the need to reduce ionic interactions and the need to limit hydrophobic interactions. Most supports exhibit ideal size exclusion chromatography with aqueous systems at neutral pH in the presence of electrolyte.

In practice, hydrophobic interaction is not a strong component of protein retention in GFC, because the hydrophobic side chains of the amino acids are predominantly located in the interior of the protein. The addition of 5-20% of a non-denaturing solvent, such as ethylene glycol, to a high ionic strength mobile phase can eliminate hydrophobic interactions. In contrast to the case for proteins, hydrophobic interaction can be significant in GFC of peptides. High concentrations of organic solvents may be needed to obtain a retention of some of these molecules by size exclusion alone (3,4).

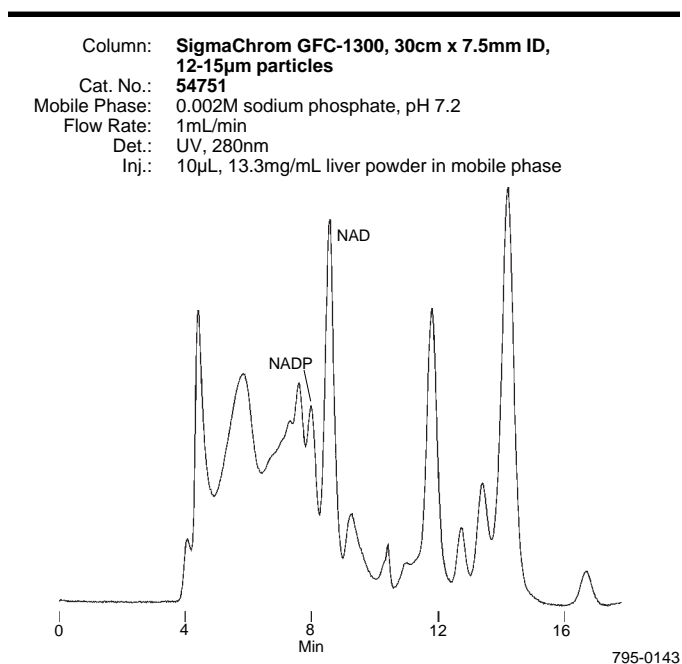
Following earlier work with silica-based GFC columns (5), we

determined the effect of ionic strength on the distribution coefficients for small molecular weight compounds on the SigmaChrom GFC columns. Plots for  $K_D$  versus mobile phase ionic strength (Figure E) indicate that the SigmaChrom support has a slight negative charge: positively charged small molecules (arginine, lysine, glutamic acid) are retarded and negatively charged small molecules (citric and oxalic acids) are repelled at low ionic strength (i.e., 0.026). The minimum ionic strength required to avoid ionic interactions between the analytes and the medium is 0.120 (e.g., 0.05M potassium phosphate). 2-Phenylethanol and benzyl alcohol exhibit some hydrophobic interaction with the support (note that the interaction increases with increasing ionic strength). Uncharged hydrophobic small molecules are used to characterize the hydrophobicity, to avoid additional interactions due to charge.

Hydrophobic interactions can be overcome by adding alcohol to the mobile phase. As detailed above, our experiments have shown hydrophobic interaction to be a problem only with small hydrophobic analytes (e.g., peptides). In general, our results agree with the findings of Barth et al. (5), who used similar small molecular weight probes. Other investigators obtained similar results for secondary retention of proteins on silica- and polymer-based packing materials (6,7).

Ideal size exclusion chromatography is critical for accurate determination of molecular weight, but secondary interactions can be helpful in isolating and/or purifying analytes (2). Figure F shows that, with a mobile phase of low ionic strength, secondary interactions can be used to separate two analytes of similar weight, NADP (MW 765) and NAD (MW 663). The negatively charged molecules are repelled from the matrix and are separated by differences in their hydrophobicity. Under ideal SEC conditions, these analytes would have eluted from the column in the inclusion volume (~11.6mL) and would not have been separated. Note that under the conditions in Figure F other compounds are retained on the column past the inclusion volume, due to hydrophobic interactions with the support.

**Figure F. GFC with Secondary Interactions Resolves NAD from NADP**



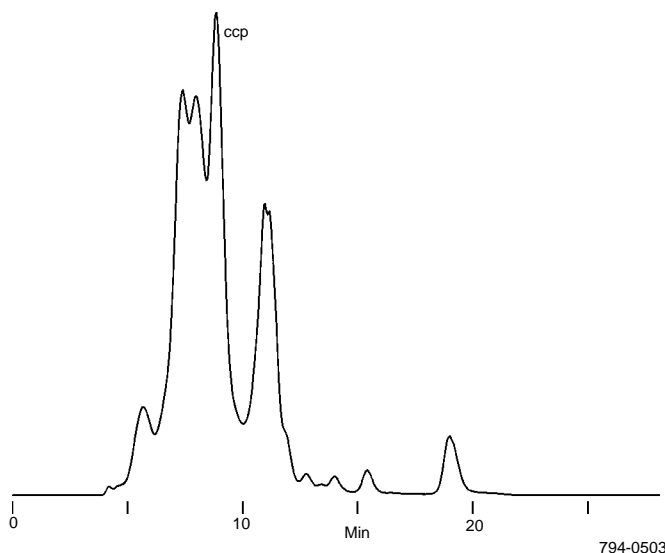
## Additional Applications for SigmaChrom GFC Columns

We have used SigmaChrom GFC-1300 columns in a semi-preparative isolation of cytochrome c peroxidase (ccp), traditionally isolated from bakers' yeast by low pressure techniques. The final step of the protocol is GFC separation of ccp from flavins. Relative to the low pressure protocol, the procedure shown in Figure G is faster and eliminates several steps. The collected ccp peak in Figure G is pure.

Biocompatible SigmaChrom GFC-100 and SigmaChrom GFC-1300 columns are excellent alternatives to silica-based stainless steel gel filtration columns, for separating or isolating a wide range of biomolecules in HPLC and FPLC® systems. Each lot of packing is tested to ensure quality, and each column is individually tested for efficiency and peak symmetry.

### Figure G. Semi-Preparative Isolation of Cytochrome c Peroxidase by GFC

Column: **SigmaChrom GFC-1300, 30cm x 7.5mm ID, 12-15µm particles**  
Cat. No.: **54751**  
Mobile Phase: 50mM Tris-HCl/100mM KCl, pH 7.2  
Flow Rate: 1mL/min  
Det.: UV, 280nm  
Inj.: 100µL concentrated crude ccp preparation from DEAE Sepharose® CL-6B



## Ordering Information:

SigmaChrom GFC-100 Column	54750-U
SigmaChrom GFC-1300 Column	54751
GFC-100 Top-Off Gel, 1mL	54771
GFC-1300 Top-Off Gel, 1mL	54772
Replacement Frits, pk. of 2	54770

## Molecular Weight Standards Kits

1 vial each of 5-7 proteins in MW range indicated.

Kit MW-GF-70 (MW 6500-66,000)	MW-GF-70
Kit MW-GF-200 (MW 12,000-200,000)	MW-GF-200
Kit MW-GF-1000 (MW 29,000-700,000)	MW-GF-1000

For descriptions of kits and for individual molecular weight standards, refer to the Supelco catalog.

## Recommended Reading

<i>Gel Filtration: Principles &amp; Methods</i> Pharmacia (1991), 102 pp, p691	23578
<i>Handbook of Size Exclusion Chromatography</i> Chi-san Wu, Ed., Marcel Dekker (1995), 453 pp	23629

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- References not available from Supelco.

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