Gel Filtration Calibration Kit

Instructions for Use

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Read these instructions carefully before using the products.

Intended use

The products are intended for research use only, and shall not be used in any clinical or *in vitro* procedures for diagnostic purposes.

Safety

For use and handling of the products in a safe way, refer to the Safety Data Sheets.

1 Handling

Safety warnings and precautions

Not recommended or intended for diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals.

All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes, wash immediately with water. See Safety Data Sheet(s) and/or safety statement(s) for specific advice.

Storage

The kit should be stored at 2°C to 8°C. It is recommended that each vial of protein is dissolved in a buffer with a pH of 6 to 8 and an ionic strength of ≥ 0.15 (e.g., 50 mM phosphate, 0.15 M NaCl, pH 7.2)

Expiry

For expiry details see outer packaging.

2 Components

Components in Gel Filtration Calibration kits LMW and HMW

Table 1. The content of Gel Filtration Calibration Kit LMW (low molecular weight), Product code 28403841.

Protein (weight per vial)	**Molecular weight (M_r)**	**Stoke's radius[1] (Å)**	**Source**
Aprotinin (10 mg)	6500	NA	Bovine lung
Ribonuclease A (50 mg)	13 700	16.4	Bovine pancreas
Carbonic anhydrase (15 mg)	29 000	NA	Bovine erythrocytes
Ovalbumin (50 mg)	44 000	30.5	Hen egg white
Conalbumin (50 mg)	75 000	NA	Chicken egg white
Blue Dextran 2000			

Table 2. The content of Gel Filtration Calibration Kit HMW (high molecular weight), Product code 28403842.

Protein (weight per vial)	**Molecular weight (M_r)**	**Stoke's radius[1] (Å)**	**Source**
Ovalbumin (50 mg)	44 000	30.5	Hen egg white
Conalbumin (50 mg)	75 000	NA	Chicken egg white
Aldolase[2] (50 mg)	158 000	48.1	Rabbit muscle
Ferritin[2] (15 mg)	440 000	61.0	Horse spleen
Thyroglobulin (50 mg)	669 000	85.0	Bovine thyroid
Blue Dextran 2000			

[1] Reference: CRC Practical Handbook of Biochemistry and Molecular Biology G.D. Fasman, ed., CRC Press, 1989, 601 pp.

[2] These proteins are supplied mixed with sucrose or mannitol to maintain stability and aid their solubility.

Common abbreviations

SEC	size exclusion chromatography
HMW	high molecular weight
HR	high resolution
K_{av}	partition coefficient
LMW	low molecular weight
M_r	relative molecular weight
pg	prep grade
R_{St}	Stoke's radius of solute
V_0	void volume
V_c	geometric column volume
V_e	elution volume
V_t	total liquid volume

3 Introduction

Two Gel Filtration Calibration Kits are available for protein molecular weight determination by size exclusion chromatography (SEC), also called gel filtration. The Low Molecular Weight Kit contains 5 proteins with molecular weights in the range 6500 to 75 000 and Blue Dextran 2000. The High Molecular Weight Kit contains 5 proteins with molecular weights in the range 44 000 to 66 9000 and Blue Dextran 2000.

The use of SEC for the determination of the molecular weight and size of proteins is well documented. The technique is based on the well-established ability of SEC resins, such as Superdex™, Superose™, Sephacryl™, Sephadex™ and Sepharose™ to separate molecules according to size. Prepacked columns are available (*Table 3, on page 7*) and can be run on chromatography systems such as ÄKTA™ design.

Molecular weight determination by SEC are carried out by comparing an elution volume parameter, such as K_{av} of the protein of interest, with the values obtained for several known calibration standards. In practice it is found that for homologous series of compounds a sigmoid relationship exists between their various elution volume parameters and the logarithm of their molecular weights.

The molecular weight of an unknown protein can be determined from the calibration curve (plot of K_{av} versus the logarithm of molecular weight) once its K_{av} value is calculated from its measured elution volume.

For accurate determination of molecular weight, the calibration standards must have the same relationship between molecular weight and molecular size as the substance of interest. Cytiva Calibration Kits provide highly purified, well- characterized, globular protein standards for protein molecular weight determination.

Table 3. Prepacked columns for SEC.

Product	Fractionation range (M_r of globular protein)	V_c (mL)	Recommended sample volume (µL) during calibration
Superdex 75 Increase 10/300 GL	3×10^3 to 7×10^4	24	100
Superdex 75 Increase 5/150	3×10^3 to 7×10^4	3	12.5
Superdex 75 Increase 3.2/300	3×10^3 to 7×10^4	2.4	10
Superdex 200 Increase 10/300 GL	1×10^4 to 6×10^5	24	100
Superdex 200 Increase 5/150	1×10^4 to 6×10^5	3	12.5
Superdex 200 Increase 3.2/300	1×10^4 to 6×10^5	3	10
HiLoad 16/600 Superdex 75 pg	3×10^3 to 7×10^4	120	500
HiLoad 26/600 Superdex 75 pg	3×10^3 to 7×10^4	320	1000
HiLoad 16/600 Superdex 200 pg	1×10^4 to 6×10^5	120	500
HiLoad 26/600 Superdex 200 pg	1×10^4 to 6×10^5	320	1000
HiLoad 16/600 Superose 6 pg	5×10^3 to 5×10^6	120	500
Superose 6 Increase 10/300 GL	5×10^3 to 5×10^6	2.4	10
Superose 6 Increase 5/150 GL	$5 x 10^3$ to $5 x 10^6$	3	12.5
Superose 6 Increase 3.2/300	$5 x 10^3$ to $5 x 10^6$	2.4	10
HiPrep 16/60 Sephacryl S-100 HR	1×10^3 to 1×10^5	120	500
HiPrep 26/60 Sephacryl S-100 HR	1×10^3 to 1×10^5	320	1000
HiPrep 16/60 Sephacryl S-200 HR	5×10^3 to 2.5×10^5	120	500
HiPrep 26/60 Sephacryl S-200 HR	5×10^3 to 2.5×10^5	320	1000
HiPrep 16/60 Sephacryl S-300 HR	1×10^4 to 1.5×10^6	120	500
HiPrep 26/60 Sephacryl S-300 HR	1×10^4 to 1.5×10^6	320	1000
HiPrep 26/60 Sephacryl S-400 HR	2×10^4 to 8×10^6	320	1000
HiPrep 26/60 Sephacryl S-500 HR	4×10^5 to 2×10^7 *	320	1000

* dextran

Critical parameters

- Select buffer with pH 6 to 8 and ionic strength ≥ 0.15 (e.g., 0.15 M NaCl)
- Use prepacked columns
- Use recommended flow rate
- Use a sample volume that is 0.1% to 2% of total column volume
- Select a SEC resin with suitable fractionation range for sample (*Table 3, on page 7*).
- Minimize the delay volume in the chromatography system from injection valve to column and from column to detector.

4 Procedure

Step	Action
1	**Selection of SEC column** Select a column with a fractionation range so that the expected molecular weight of your sample falls approximately in the middle of the range for that column. Superose 6 Increase can be used for a preliminary, quick approximation of the sample molecular weight. A bed length of 30 to 60 cm is sufficient for most determinations.

Step	Action
2	**Equilibration of the column** If the column has been stored in 20% ethanol, wash the column first with 2 column volumes of distilled water. Equilibrate the column with 2 column volumes of buffer. A buffer with a pH of 6 to 8 and an ionic strength ≥ 0.15 is suggested. A typical buffer is 50 mM phosphate in 0.15 M NaCl, pH 7.2.
3	**Choice of calibration kit proteins** Include Calibration Kit proteins of a higher molecular weight and of a lower molecular weight than that of the sample. The proteins listed in *Table 4, on page 9* may be mixed to give resolved peaks.

Table 4. Suitable protein mixtures for calibration of SEC columns.

Resin	Mix A	Mix b	Mix c
Superdex 200 Increase	F+C+CA+R	Ald+O+R+Apr	
Superdex 75 Increase	C+CA+R+Apr	O+R+Apr	
Superose 6 Increase	T+Ald+CA+Apr	F+O+R	
Sephacryl 300	T+Ald+CA	F+O+R+Apr	
Sephacryl 200	Ald+CA+R+Apr	C+CA+R	O+CA+R
Sephacryl 100	Ald+CA+R+Apr	C+CA+R	O+CA+R

Apr - Aprotinin, R - Ribonuclease A, CA - Carbonic Anhydrase, O - Ovalbumin, C - Conalbumin, Ald - Aldolase, F - Ferritin, T - Thyroglobulin

Step	Action
4	**Individual protein preparation** We recommend that the proteins are dissolved in high concentration (20 mg/mL) and diluted with buffer before use. Dissolve the content of the vial in a buffer with a pH of 6 to 8 and an ionic strength of ≥ 0.15 (e.g., 50 mM phosphate, 0.15 M NaCl, pH 7.2). Ferritin and aldolase are supplied mixed with sucrose or mannitol to maintain stability and aid their solubility. For these two proteins, it is particularly important to dissolve the full content of the vial to get an homogeneous solution. It is recommended that carbonic anhydrase is dissolved in distilled water to avoid the formation of aggregates during freezing and thawing.

Step	Action
5	**Protein mix preparation**

Dilute the proper combination of calibration kit proteins in the buffer. To obtain peaks with similar heights at 280 nm, use the concentrations in *Table 5, on page 11*. The concentrations have been calculated with an assumed applied volume of 0.5% of the geometrical column volume (V_c). See Appendix for a thorough explanation how to prepare a typical protein mix sample.

Note:

If precipitation of the proteins occurs upon mixing we recommend brief centrifugation to clarify the protein mixture before applying it to the column.

Table 5. Suggested protein concentrations for producing peaks of similar height.

Kit	Protein	Protein concentration
LMW	Aprotinin	3 mg/mL
LMW	Ribonuclease A	3 mg/mL
LMW	Carbonic Anhydrase	3 mg/mL
LMW, HMW	Ovalbumin	4 mg/mL
LMW, HMW	Conalbumin	3 mg/mL
HMW	Aldolase[1]	4 mg/mL
HMW	Ferritin[1]	0.3 mg/mL
HMW	Thyroglobulin	5 mg/mL

[1] ***Note:***
These proteins are supplied mixed with sucrose or mannitol to maintain stability

Step	Action
6	**Size of sample volume** Apply Calibration Kit proteins to the column. To get good resolution, the sample size should not exceed 2% of the geometric column volume, V_c. ($V_c = r^2 \times \pi \times l$ where r is radius and l is column length).
7	**Determination of elution volume (V_e)** From the UV curve, determine the elution volumes (V_e) for the Calibration Kit proteins by measuring the volume of the eluent from the point of injection to the center of the elution peak, see *Fig. 1, on page 14*.

Step	Action
8	**Determination of void volume (V_o)** The elution volume for Blue Dextran 2000 is equal to the column void volume (V_o). Prepare a fresh solution of Blue Dextran 2000 (1.0 mg/mL) in the buffer. The rate of solubilization of the Blue Dextran 2000 may be increased by heating the buffer to 50°C before adding the Blue Dextran 2000. Apply a sample to the column (sample size, 0.5% of the geometric column volume) to determine the void volume (V_o). The elution of Blue Dextran can be conveniently monitored using absorbance at wavelengths of 254, 280 or 620 nm. It is strongly recommended that the Blue Dextran 2000 is run alone, not mixed with the Calibration Kit or sample proteins, as the fraction of Blue Dextran is broad and may overlap the protein peaks. Always calculate the void volume from the first eluted peak from Blue Dextran.

Step	Action

Column:	HiLoad 16/60 Superdex 200 pg
Geometric column volume (V_C):	120 mL
Sample volume:	500 µL
Buffer:	50 mM phosphate, 150 mM NaCl, pH 7.2
Flow rate:	1 mL/min
System:	ÄKTAexplorer™ 10
Detection:	Absorbance at 280 nm

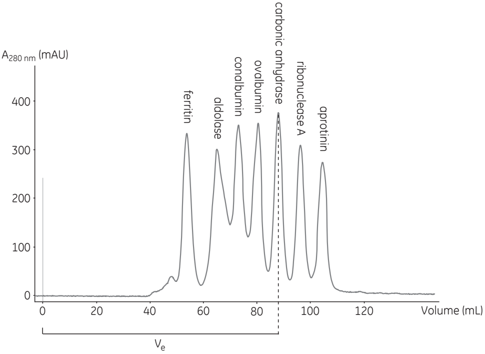

Fig 1. Elution profiles of Calibration Kit proteins on HiLoad 16/600 Superdex 200 pg column. Elution volumes (Ve) are found at maximum peak height of each respective protein, see for example, carbonic anhydrase.

Step **Action**

9 **Preparation of calibration curve**

Calculate the K_{av} values for the Calibration Kit proteins using the equation:

$$K_{av} = \frac{V_e - V_o}{V_c - V_o}$$

where V_o = column void volume, V_e = elution volume, and V_c = geometric column volume.

Prepare a calibration curve of K_{av} versus log molecular weight either on semi-logarithmic paper or with a calculation program. It should be possible to fit a curve to the data points, see *Fig. 2, on page 18* to *Fig. 9, on page 22*.

Step	Action
10	**Molecular weight determination** Apply the unknown sample (volume 0.1% to 2% of V_c) and determine the elution volume (V_e) of the compound of interest. Adjust the concentration of the sample taking into consideration that a sample of 0.5% of V_c will be diluted 5 to 15-fold during the run. Calculate the corresponding K_{av} for the component of interest and determine its molecular weight from the calibration curve prepared using the Calibration Kit proteins. Note that the molecular weight determinations using the molecular weights of glycoproteins, lipoproteins, non-globular proteins, or other polymers may not correlate well to the calibration curves established for globular proteins by the Calibration Kit proteins. For such compounds, useful information can be obtained by relating their elution volume data to a molecular size parameter, such as Stoke's radius (R_{St}), rather than to molecular weight values. Plots of $\sqrt{(-\log K_{av})}$ vs. R_{St} have been used successfully to determine the Stoke's radius of proteins and our Calibration Kit proteins may be used for these plots too.

5 Typical results

Method used for figures 2 to 11

Sample:	Proteins from Gel Filtration Calibration Kits LMW and HMW: aprotinin (Apr), RNAse A (R), carbonic anhydrase (CA), ovalbumin (O), conalbumin (C), aldolase (Ald), ferritin (F) and thyroglobulin (T)	
Sample vol.:	Figure 2 to 4:	100 µL
	Figure 5, 7, 9-11:	500 µL
	Figure 6:	2.7 mL
	Figure 8:	1 mL
Buffer:	Figure 5, 9-11: 50 mM phosphate buffer, 150 mM NaCl, pH 7.2	
	Figure 2-4, 6-8: 10 mM phosphate buffer, 140 mM NaCl, 2.7 mM KCl, pH 7.4	
Flow rate:	Figure 2-4, 7, 8 and 9	0.5 mL/min
	Figure 5	1 mL/min
	Figure 6:	2.6 mL/min
System:	Figure 5, 9-11: ÄKTAexplorer 10	
	Figure 2-4, 6-8: ÄKTA pure	
Detection:	Absorbance at 280 nm	

Results

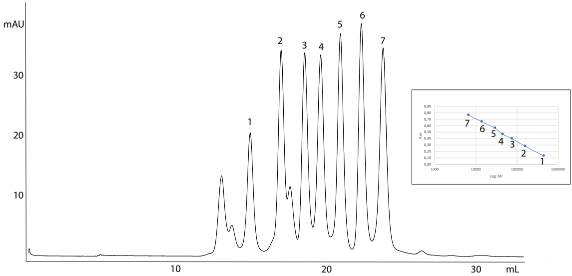

Fig 2. Chromatographic separation for some of the standard proteins on Superdex 200 Increase 10/300 GL column.

Peak	Protein	Peak	Protein
1	Ferritin	5	Carbonic anhydrase
2	Aldolase	6	RNase A
3	Conalbumin	7	Aprotinin
4	Ovalbumin		

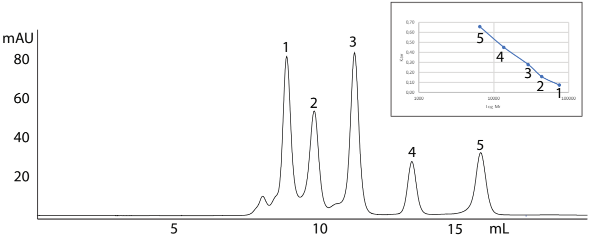

Fig 3. Chromatographic separation for some of the standard proteins on Superdex 75 Increase 10/300 GL column.

Peak	Protein	Peak	Protein
1	Conalbumin	4	RNase A
2	Ovalbumin	5	Aprotinin
3	Carbonic anhydrase		

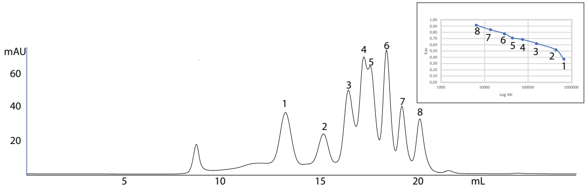

Fig 4. Chromatographic separation for some of the standard proteins on Superose 6 Increase 10/300 GL column.

Peak	Protein	Peak	Protein
1	Thyroglobulin	5	Ovalbumin
2	Ferritin	6	Carbonic anhydrase
3	Aldolase	7	RNase A
4	Conalbumin	8	Aprotinin

Note: *Thyroglobulin may be excluded from the calculation of* K_{av} *due to none-linear behavior of thyroglobulin on this column. Thyroglobulin may however, be included in a plot of √ (-log* K_{av}*) vs. Stoke's radius (*R_{St}*).*

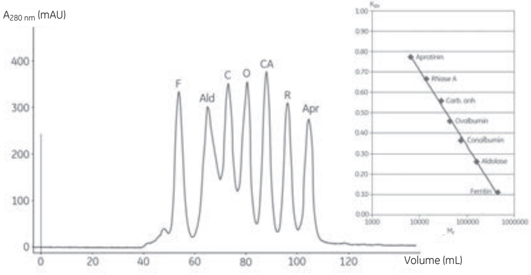

Fig 5. Chromatographic separation and calibration curve for some of the standard proteins on HiLoad 16/600 Superdex 200 pg column.

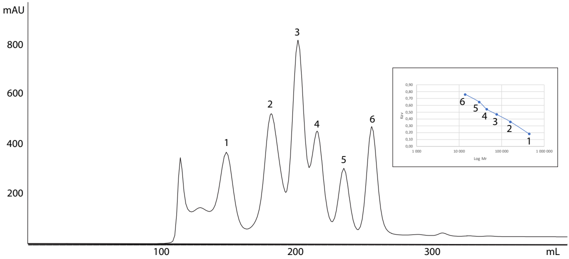

Fig 6. Chromatographic separation and calibration curve for some of the standard proteins on HiLoad 26/600 Superdex 200 pg.

Peak	Protein	Peak	Protein
1	Ferritin	4	Ovalbumin
2	Aldolase	5	Carbonic anhydrase
3	Conalbumin	6	RNase A

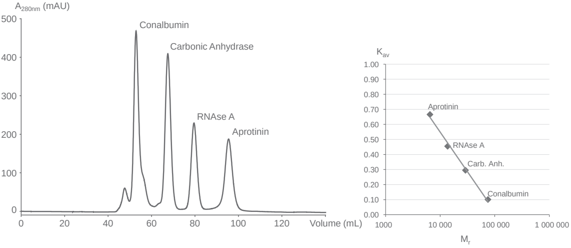

Fig 7. Chromatographic separation and calibration curve for some of the standard proteins on HiLoad 16/600 Superdex 75 pg column.

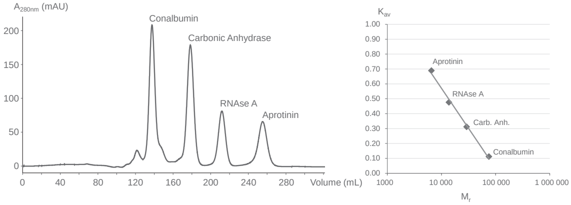

Fig 8. Chromatographic separation and calibration curve for some of the standard proteins on HiLoad 26/600 Superdex 75 pg column.

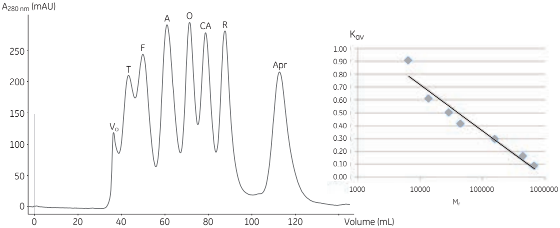

Fig 9. Chromatographic separation and calibration curve for some of the standard proteins on HiPrep 16/60 Sephacryl S-300 HR column.

Note: *Aprotinin may be excluded from the calculation of K_{av} due to non-linear behavior of aprotinin on this column.*

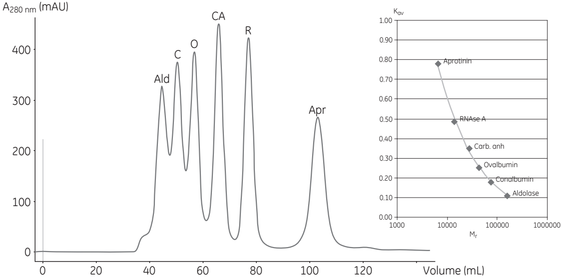

Fig 10. Chromatographic separation and calibration curve for some of the standard proteins on HiPrep 16/60 Sephacryl S-200 HR column.

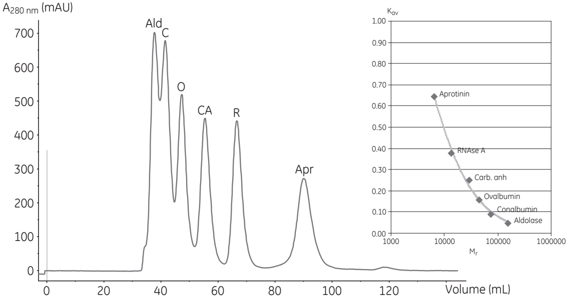

Fig 11. Chromatographic separation and calibration curve for some of the standard proteins on HiPrep 16/60 Sephacryl S-100 HR column.

6 Important notes

The use of the calibration kits with denaturing solvents

The molecular weight ranges given in *Table 3, on page 7* are for globular proteins in their native conformations. The use of denaturing agents, such as sodium dodecyl sulfate (SDS), chaotropic salts and guanidine hydrochloride (GuHCl) and hydrogen bond disrupting agents, such as urea, may alter the molecular conformation of proteins often greatly increasing their hydrodynamic volumes. Since separations by SEC are based on molecular size, the molecular weight ranges change when the proteins assume extended conformations.

Superdex 200 Increase has the most useful molecular weight range and flow properties in solvents where proteins are completely denatured (exclusion limit is approximately 120 000 for completely denatured proteins). The Low Molecular Calibration Kit is suitable for the calibration of columns in denaturing solvents. Each protein in the kit comprises of a single polypeptide chain therefore, their molecular weights do not change when they are exposed to denaturants (although their Stoke's radii do change).

Dimer and oligomer formation in calibration kit proteins

The ribonuclease A, conalbumin, aldolase, ferritin and thyroglobulin standards may contain small amounts of apparent dimers or oligomers that elute in the void volume or slightly before the true peak. Dimers can be used to produce more calibration points, however, pure dimer formation has to be determined before calculations.

Electrophoresis calibration kits

Gel Filtration Calibration Kits (HMW and LMW) contain protein standards for use in SEC only. Kits containing protein standards for molecular weight determination by polyacrylamide gel electrophoresis are also available from Cytiva. Please visit, cytiva.com

7 Appendix

Protein mix preparation for calibrating HiLoad 26/600 Superdex 200 pg

Geometric column volume (V_c) = 320 mL Recommended sample volume = 1.6 mL (0.5% V_c)

Preparation of “Mix a” in Table 4 (Ferritin, Conalbumin, Carbonic Anhydrase and Ribonucelase A) for Superdex 200 Increase

Prepare each individual protein as described in *Chapter 4 Procedure, on page 8* to a concentration of 20 mg/mL

The recommended protein concentrations for the different proteins in a mix are according to *Table 5, on page 11*.

Ferritin: 0.3 mg/mL

Conalbumin: 3 mg/mL

Carbonic Anhydrase: 3 mg/mL

Ribonuclease A: 3 mg/mL

To prepare a 2 mL protein mix, make the following calculations:

Calculate the volume (X) of each protein needed from the 20 mg/mL solution in order to achieve the desired protein concentration.

Ferritin:

20 mg/mL* X mL= 2 mL * 0.3 mg/mL

X = 0.03 mL

All other proteins:

20 mg/mL * X mL= 2 mL * 3 mg/mL

X = 0.3 mL

Finally mix 0.03 mL Ferritin, 0.3 mL Conalbumin, 0.3 mL Carbonic Anhydrase and 0.3 mL Ribonucelase and dilute with 1.07 mL buffer to get 2 mL final volume.

Inject 1.6 mL of the protein mix onto the column.

8 Ordering information

Gel Filtration Calibration Kits	Product code
Low molecular weight	28403841
High molecular weight	28403842

Related products	Quantity	Product code
Superdex 75 Increase 10/300 GL	1	29148721
Superdex 75 Increase 5/150 GL	1	29148722
Superdex 75 Increase 3.2/300	1	29148723
Superdex 200 Increase 10/300 GL	1	28990944
Superdex 200 Increase 5/150 GL	1	28990945
Superdex 200 Increase 3.2/300	1	28990946
HiLoad 16/600 Superdex 75 pg	1	28989334
HiLoad 26/600 Superdex 75 pg	1	28989334
HiLoad 16/600 Superdex 200 pg	1	28989335
HiLoad 26/600 Superdex 200 pg	1	28989386
HiLoad 16/600 Superose 6 pg	1	29323952
Superose 6 Increase 10/300 GL	1	29091596
Superose 6 Increase 5/150 GL	1	29091597
Superose 6 Increase 3.2/300	1	29091598
HiPrep 16/60 Sephacryl S100 HR	1	17116501
HiPrep 26/60 Sephacryl S100 HR	1	17119401
HiPrep 16/60 Sephacryl S200 HR	1	17116601

Related products	Quantity	Product code
HiPrep 26/60 Sephacryl S200 HR	1	17119501
HiPrep 16/60 Sephacryl S300 HR	1	17116701
HiPrep 26/60 Sephacryl S300 HR	1	17119601

Reference literature	Product code
Selection guide: Gel Filtration Columns and Media	18112419
Handbook: Gel Filtration Principles & Methods	18102218

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