Size exclusion chromatography

Superdex™ 75 Increase columns

Superdex 75 Increase prepacked columns are designed for rapid separation and analysis of proteins with molecular weights ranging from M_r 3000 to 70 000 by size exclusion chromatography (SEC), also called gel filtration (Fig 1). These versatile columns offer separations with high resolution for a variety of applications including preparative protein purification (µg to mg quantities), aggregate analysis, studies of complex formation, and screening of samples. Superdex 75 Increase belongs to the new generation of SEC columns that replaces the well-known Superdex 75 columns.

Superdex 75 Increase columns offer:

- Increased resolution compared with Superdex 75, for higher purity and improved analysis results
- Reduced runtime compared with Superdex 75, for results faster
- Versatile use in both preparative and analytical applications
- Tolerance to repeated harsh cleaning procedures at high pH, giving long column lifetime and minimal carry-over

Medium characteristics

Superdex 75 Increase medium (resin) is based on a high-flow agarose base matrix with good pressure/flow properties. The small bead size with a narrow size distribution allows for high-resolution separations. In addition, low nonspecific interactions permit high recovery of biological materials. The characteristics of the Superdex 75 Increase medium are shown in Table 1.

Table 1. Characteristics of Superdex 75 Increase medium (resin)

Fractionation range	M_r 3000 to 70 000 (globular proteins) M_p* 500 to 30 000 (dextrans)
Exclusion limit	Approx. M_r 100 000 (globular proteins)
pH stability	
long term short term	3 to 12 1 to 14
Operating temperature	4°C to 40°C
Storage temperature	4°C to 30°C
Matrix	Composite of cross-linked agarose and dextran
Average bead size	9 µm

* Peak molecular weight

Fig 1. Superdex 75 Increase 3.2/300, Superdex 75 Increase 5/150 GL, and Superdex 75 Increase 10/300 GL columns.

Selectivity of the medium

Superdex 75 Increase and the related SEC media Superdex 200 Increase and Superose™ 6 Increase belong to the new generation of SEC media based on high-flow agarose with small beads. The media have different selectivities to complement each other (Fig 2). Compared with Superdex 200 Increase (recommended fractionation range M_r 10 000 to 600 000 for globular proteins), Superdex 75 Increase gives improved separation in the lower molecular weight range. Superose 6 Increase is specially designed for separation of large proteins and protein complexes (recommended fractionation range up to M_r 5 000 000).

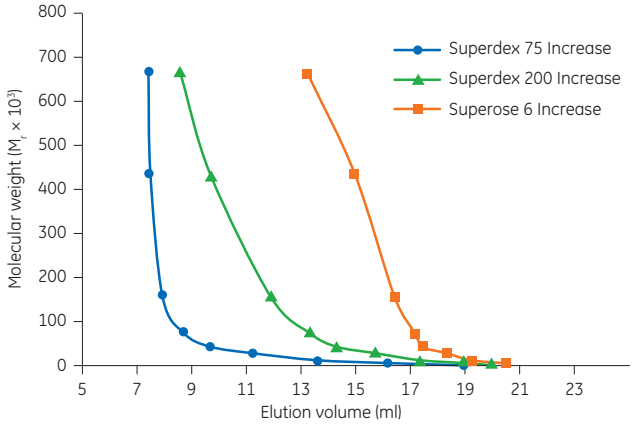


Fig 2. Elution volumes for globular proteins of various molecular weights on Superdex 75 Increase 10/300 (blue), Superdex 200 Increase 10/300 (green), and Superose 6 Increase 10/300 (orange). Note! The whole fractionation range of Superose 6 Increase is not covered in this diagram.

Table 2. Characteristics of columns prepacked with Superdex 75 Increase

Column	Bed dimensions diam. x height (mm)	Approximate bed volume (ml)	Column efficiency (theoretical plates m^{-1})	Recommended sample volume (µl)	Recommended flow rate H_2O at 25° (ml/min)*	Max. flow rate H_2O at 25° (ml/min)*	Typical pressure drop over column, H_2O at 25° (MPa)
Superdex 75 Increase 10/300 GL	10 × 300	24	> 43 000	25 to 500	0.8	1.6	3.0
Superdex 75 Increase 5/150 GL	5 × 150	3	> 38 000	4 to 50	0.45	0.75	3.0
Superdex 75 Increase 3.2/300	3.2 × 300	2.4	> 43 000	4 to 50	0.075	0.15	2.0

* Note! Flow rate needs to be decreased when working at low temperature or with viscous solutions, see product instructions for more details.

Table 3. Column choice for different applications

Type of application/column	10/300 GL	5/150 GL	3.2/300
Small-scale preparative runs (mg)	×		
Micro-scale preparative runs (µg) When sample amount is limited and small buffer consumption is important			×
High-resolution analysis (25 to 500 µl)	×		
High-resolution analysis (4 to 25 µl) When sample amount is limited and small buffer consumption is important			×
Purity check		×	
Rapid screening When time is limited and small buffer and sample consumptions are important		×	

Chemical stability of Superdex 75 Increase

Superdex 75 Increase medium can be used with aqueous solutions in the pH range 3 to 12. Solutions containing chaotropic agents (6 M guanidine hydrochloride, 8 M urea) and detergents such as SDS (at least up to 1%) can be used together with the medium without affecting its performance. Superdex 75 Increase medium also withstands the conditions used for cleaning in place (CIP) in the pH range 1 to 14. For storage conditions, see column instructions listed under Related literature.

Column characteristics and column selection

Superdex 75 Increase is available prepacked in Tricorn™ high-performance columns of two different sizes (10/300 GL and 5/150 GL) as well as in the 3.2/300 column format (Table 2). All columns are made of glass to allow easier visual inspection of the packed bed. The glass tube is coated with a protecting plastic film or protected with a plastic sleeve. Each column has its own application purpose (Table 3). Superdex 75 Increase 10/300 GL is suitable for both small-scale preparative purification and for analytical applications. Superdex 75 Increase 3.2/300 is an excellent choice when working with very small sample volumes and for high-resolution micro-scale separations. Superdex 75 Increase 5/150 GL is the first choice for rapid screening, and for purity checks.

Excellent reproducibility and durability

Reproducible results are essential in all research. The long working life and high reproducibility of Superdex 75 Increase prepacked columns are the result of optimized design, stable properties of the medium, and controlled production procedures.

Studies were performed on Superdex 75 Increase 10/300 GL columns to verify the chemical stability and robustness of the medium and prepacked column. The column efficiency and resolution were tested with acetone and standard proteins, respectively, before a series of 200 runs were conducted, each with application of a 600-µl sample of high IgG concentration. After every 10th run, a CIP procedure with NaOH was performed, followed by a new efficiency test. In addition, after every 20th run, a new resolution test with standard proteins was performed.

Peak areas and resolution were essentially unchanged during the study (Fig 3). Plate numbers remained high over time. The results illustrate the stable properties of the medium during a large number of runs, and the compatibility of the medium with repeated NaOH-exposure.

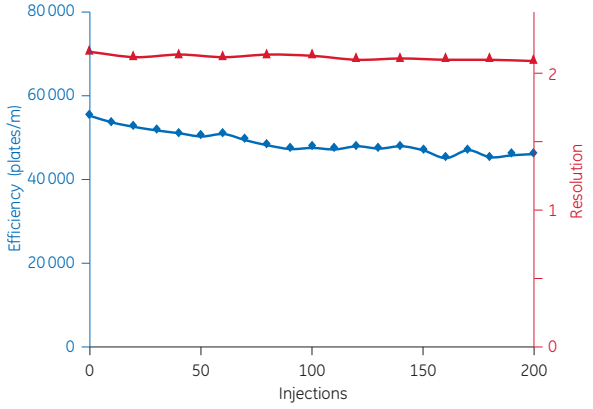


Fig 3. Example of results from a study with 200 IgG injections, including harsh CIP procedures after every 10th run (1.5 column volumes of 0.5 M NaOH). Efficiency test conducted every 10th run after CIP (blue). Resolution determined every 20th run after CIP by separation of ovalbumin and carbonic anhydrase (red).

Improved resolution compared with Superdex 75

Compared with its predecessor Superdex 75, Superdex 75 Increase has improved properties, both in terms of resolution and flow rate. In Figure 4, the higher resolution of Superdex 75 Increase 10/300 GL compared with Superdex 75 10/300 GL is shown. In Figure 5 it is shown that approximately the same resolution was achieved with a 3 to 4 times faster separation using Superdex 75 Increase compared with Superdex 75.

Columns: Superdex 75 Increase 10/300 GL (A)
Superdex 75 10/300 GL (B)

Sample:
1. Conalbumin (M_r 75 000), 2.9 mg/ml
2. Ovalbumin (M_r 44 000), 3.7 mg/ml
3. Carbonic anhydrase (M_r 29 000), 1.4 mg/ml
4. Cytochrome C (M_r 12 300), 1.0 mg/ml
5. Aprotinin (M_r 6 500), 2.0 mg/ml
6. Vitamin B12 (M_r 1 300), 0.25 mg/ml

Sample volume: 100 µl

Buffer: PBS (10 mM phosphate buffer, 140 mM NaCl, pH 7.4)

Flow rate: 0.8 ml/min

System: ÄKTA™ pure 25

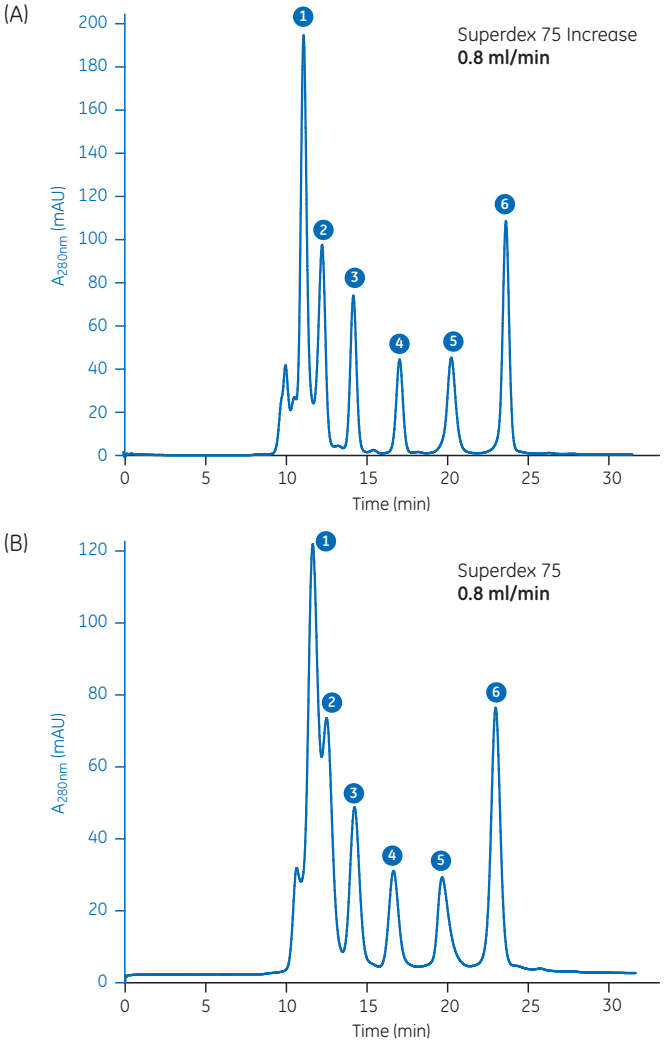


Fig 4. Chromatograms showing high-resolution SEC at 0.8 ml/min of a mixture of six standard components on (A) Superdex 75 Increase 10/300 GL and (B) Superdex 75 10/300 GL. Note that increased resolution gives higher peaks, with higher concentrations. In the comparison here shown, the resolution improvement with the new generation column was 40-120%, with the largest improvement for the higher-M_r proteins.

Columns: Superdex 75 Increase 10/300 GL (A)
Superdex 75 10/300 GL (B)

Sample:
1. Conalbumin (M_r 75 000), 2.9 mg/ml
2. Ovalbumin (M_r 44 000), 3.7 mg/ml
3. Carbonic anhydrase (M_r 29 000), 1.4 mg/ml
4. Cytochrome C (M_r 12 300), 1.0 mg/ml
5. Aprotinin (M_r 6 500), 2.0 mg/ml
6. Vitamin B12 (M_r 1 300), 0.25 mg/ml

Sample volume: 100 µl

Buffer: PBS

Flow rates: 1.5 ml/min (A)
0.4 ml/min (B)

System: ÄKTA pure 25

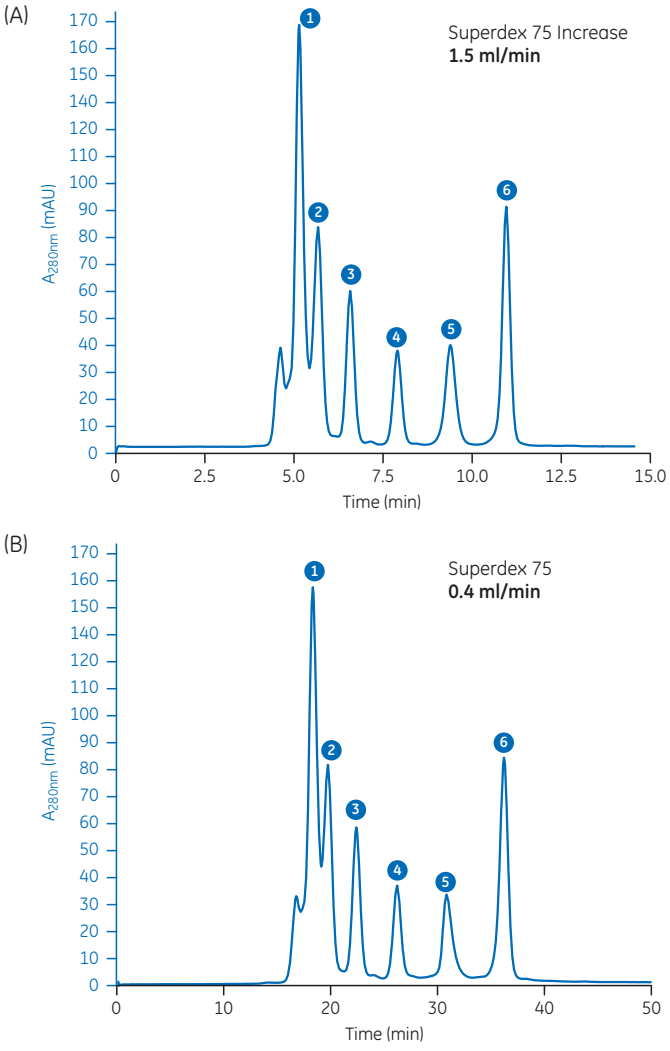


Fig 5. Chromatograms showing high-resolution SEC of a mixture of six standard components on (A) Superdex 75 Increase 10/300 GL at 1.5 ml/min and (B) Superdex 75 10/300 GL at 0.4 ml/min. Note that approximately the same resolution was achieved with a 3 to 4 times faster separation using Superdex 75 Increase 10/300 GL.

Superdex 75 Increase 10/300 GL vs 5/150 GL column: resolution vs speed

The Superdex 75 Increase 10/300 GL column is designed for high-resolution SEC while the smaller 5/150 GL column is designed for rapid screening, yet with sufficient resolution. While speed is preferable in applications such as purity and size homogeneity analyses, this comes at the cost of decreased resolution. As can be seen in Figure 6, SEC separation of a sample mixture using the 10/300 GL column gave a high-resolution separation in 25 min. The separation time on the 5/150 GL column was only 6 min, however, with a significantly lower peak resolution. For each separation purpose, the use of the 5/150 column for rapid screening should be optimized by choosing a flow rate that combines sufficient resolution with fast separations. Even though our tests have shown that the loss of resolution for the 5/150 column is small when going from, for example, 0.3 to 0.6 ml/min, the effect of flow rate changes should be investigated for each separation application.

Columns:	Superdex 75 Increase 10/300 GL and Superdex 75 Increase 5/150 GL
Sample:	1. Conalbumin (M_r 75 000), 2.9 mg/ml 2. Ovalbumin (M_r 44 000), 3.7 mg/ml 3. Carbonic anhydrase (M_r 29 000), 1.4 mg/ml 4. Cytochrome C (M_r 12 300), 1.0 mg/ml 5. Aprotinin (M_r 6 500), 2.0 mg/ml 6. Vitamin B12 (M_r 1 300), 0.25 mg/ml
Sample volumes:	100 µl, (10/300 GL column) 10 µl, (5/150 GL column)
Buffer:	PBS
Flow rates:	0.8 ml/min (10/300 GL) 0.45 ml/min (5/150 GL)
System:	ÄKTA pure 25

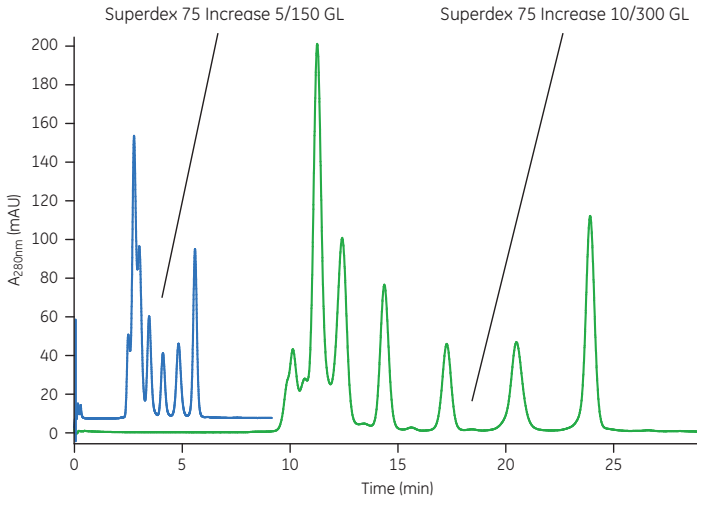


Fig 6. Overlay chromatograms with a shared time axis, showing separations of a six-component mixture using Superdex 75 Increase 5/150 GL (blue) and 10/300 GL columns (green).

High sensitivity for small sample volumes

Superdex 75 Increase 3.2/300 gives high resolution and good sensitivity (high peaks) for small sample volumes. To compare column sizes, a 10 µl sample mixture was applied to both 3.2/300 and 10/300 GL columns. As shown in Figure 7, the detection signal from the run with Superdex 75 Increase 3.2/300 was considerably higher compared with the more diluted peaks from using Superdex 75 Increase 10/300 GL.

Columns:	Superdex 75 Increase 3.2/300 (A) Superdex 75 Increase 10/300 GL (B)
Sample:	1. Conalbumin (M_r 75 000), 2.9 mg/ml 2. Ovalbumin (M_r 44 000), 3.7 mg/ml 3. Carbonic anhydrase (M_r 29 000), 1.4 mg/ml 4. Cytochrome C (M_r 12 300), 1.0 mg/ml 5. Aprotinin (M_r 6 500), 2.0 mg/ml 6. Vitamin B12 (M_r 1 300), 0.25 mg/ml
Sample volume:	10 µl
Flow rates:	0.075 ml/min (A) 0.75 ml/min (B)
Buffer:	PBS
System:	ÄKTA pure 25

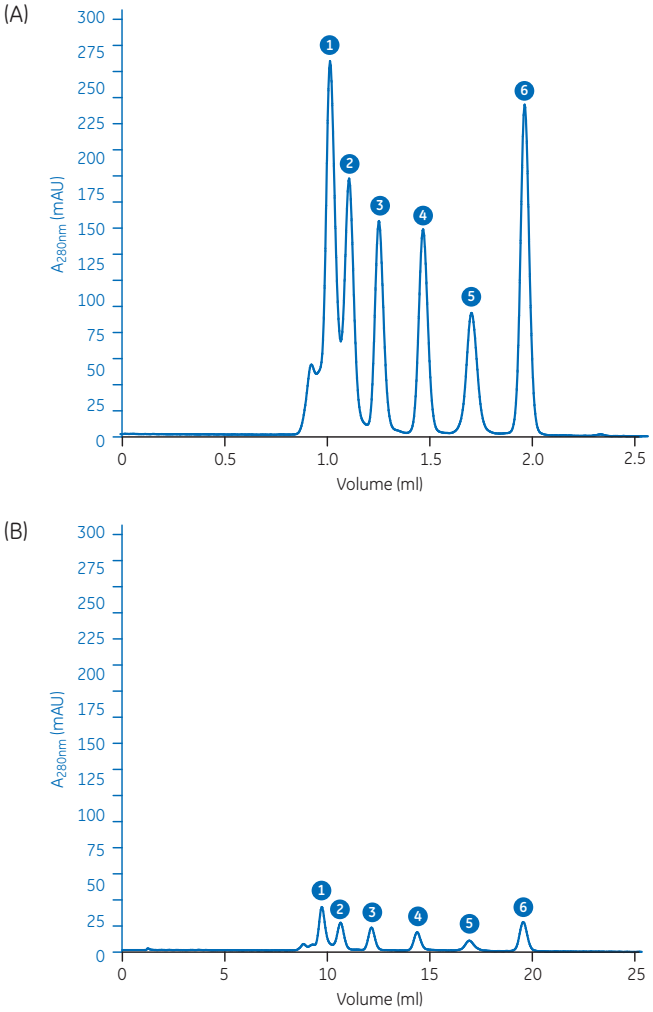


Fig 7. A small volume (10 µl with a total of 110 µg protein) of a sample mixture containing six components was separated on (A) Superdex 75 Increase 3.2/300 and (B) Superdex 75 Increase 10/300 GL. Both chromatograms show the same scale for UV detection.

Operation

Choice of eluents

The sample should be fully soluble in the selected eluent. The eluent should also be chosen so that downstream applications are simplified. Chaotropic agents and detergents, for example, for membrane proteins, can be used. To further minimize the low ionic interactions with the matrix, it is recommended to add at least 0.15 M NaCl to the eluent or to use a buffer with equivalent ionic strength.

Sample volumes, flow rates, and system dead volumes

For protein concentrations below 10 mg/ml, the impact of sample viscosity is generally low or zero. To achieve high resolution, the sample volume should be less than 2% of the column volume. Sample volumes between 0.1% and 1.0% of the column volume give the highest resolution. By decreasing the flow rate, increased resolution can be achieved. For maximal resolution in SEC, all extra volumes should be minimized. This applies to dead volumes both before the column (sample injector to column inlet) and after the column (column outlet to UV cell or fraction collector). Decrease the dead volume by using a suitable small-volume column valve (or no valve) and use short, narrow-diameter capillaries. For optimization examples and further information, see column instructions and Cue Card listed under Related literature.

Applications

Preparative SEC purification

Preparative SEC is a powerful method for obtaining size-homogeneous protein preparations. As only a limited number of separations are often performed, flow rates can be kept low to obtain maximal resolution. An ultrafiltration step can be included to increase sample concentrations so that more protein can be applied in minimal volume.

Figures 8 and 9 show an example where 5 mg target protein, essentially free of oligomers and aggregates, was obtained in a single SEC run. The start material was a preparation of partially purified histidine-tagged protein that had oligomerized during storage and freeze-thawing. Using the Superdex 75 Increase 10/300 GL column, 500 µl of the preparation was purified at a low flow rate (0.2 ml/min) and fractions of 0.25 ml were collected. The preparative run gave essentially two peaks, with the later peak being the desired one. Before deciding which fractions to pool, 25 µl samples of the fractions were rapidly analyzed by SEC to assess size-homogeneity before pooling (not shown). Note that SEC analysis can reveal size-heterogeneity that cannot be seen with SDS-PAGE, as the SDS generally dissociates any non-covalent dimers and oligomers.

Columns:	Superdex 75 Increase 10/300 GL for preparative SEC (A) Superdex 75 Increase 5/150 GL for analysis (B)
Samples:	Concentrated, partially purified histidine-tagged protein (A) Samples of SEC fractions and from pooled peaks (B)
Sample volumes:	500 µl (A) 25 µl (B)
Buffer:	PBS
Flow rates:	0.2 ml/min (A) 0.45 ml/min (B)
System:	ÄKTA pure 25 with 2 mm UV cell to extend the linear absorbance range

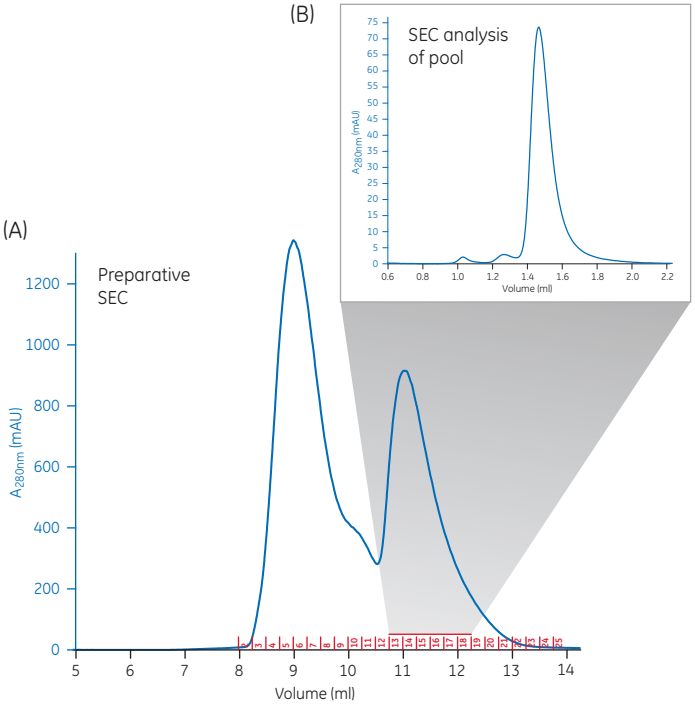
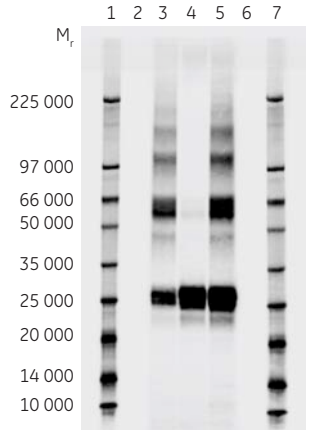


Fig 8. (A) Preparative SEC purification of a partially purified and oligomerized histidine-tagged protein. With rapid SEC analysis of fresh fractions (not illustrated), it was revealed that fraction no. 13, but not fraction no. 12, was sufficiently size-homogeneous to be included in the pool. The final pool (fractions marked with red bar) contained approximately 5 mg target protein. (B) SEC analysis of the final pool.



Lanes

1 and 7.	Molecular weight marker
2 and 6.	Blanks
3.	Pool of first peak (discarded) of the preparative SEC (fractions 3 to 12, diluted 1:5)
4.	Pool of the second peak of the preparative SEC (fractions 13 to 18, diluted 1:5)
5.	Start material (diluted 1:10)

Fig 9. Analysis of samples from the preparative SEC purification. SDS-PAGE separation of fluorescent prestained samples on an 8%–18% gel using Amersham™ PhastSystem™ electrophoresis unit. The system allows automatic scanning of the gel in its run position.

Analysis of protein-protein interaction

Separately purified proteins can be mixed and then analyzed by SEC to quickly determine any tendency of interaction between them. This approach can also be used to determine under which conditions two proteins interact. By mixing separately purified proteins under certain buffer conditions, and performing SEC separation under the same buffer conditions, rapid screening of a number of different potential binding conditions can be conducted.

The example shown in Figure 10 involves two bacterial proteins: a transcription factor and an adaptor protein. The proteins were separately expressed with histidine tags in *E. coli*, and then purified by immobilized metal ion affinity chromatography (IMAC) followed by tag cleavage, tag removal, and preparative SEC. The analytical SEC results for each protein, and after mixing the two proteins are shown as an overlay plot. When the two proteins were mixed, a single peak appeared at an elution position that corresponds to the molecular weight of a 1:1 complex between the two proteins.

Column:	Superdex 75 Increase 10/300 GL
Samples:	Separately purified transcription factor (M_r 16 000), adaptor protein (M_r 34 000) and a mix of the two
Sample volume:	100 µl
Buffer:	50 mM Tris-HCl, 150 mM NaCl, pH 8.0
Flow rates:	0.75 ml/min
System:	ÄKTA avant 25

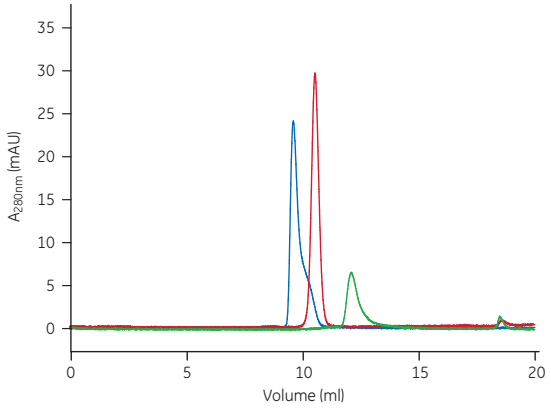


Fig 10. Overlay chromatograms of analytical SEC on a Superdex 75 Increase 10/300 GL column. Transcription factor (green) and adaptor protein (red) were separately analyzed. A mixture of the proteins was also analyzed (blue), showing interaction under the conditions used. Image and experimental data are published with permission from Drs Annika Rogstam and Claes von Wachenfeldt, Lund Protein Purification Platform (LP3), Department of Biology, Lund University, Sweden.

Analysis of protein storage stability

In structural and functional protein studies, it is essential that the prepared protein does not aggregate, oligomerize, or degrade. With analytical SEC, aggregation tendencies and other size changes can be studied under different conditions, for example, for storage stability analyses of biopharmaceuticals.

In the examples in Figure 11, Superdex 75 Increase 5/150 GL was used to monitor small changes in size homogeneity of two proteins stored under different conditions and over different time periods (weeks). The used HPLC system equipped with autosampler allowed many samples to be analyzed during long series of unattended over-night SEC runs.

Column:	Superdex 75 Increase 5/150 GL
Samples:	Stored protein preparations
Sample volume:	10 µl
Flow rate:	0.5 ml/min
Buffer:	PBS
System:	HPLC system equipped with autosampler

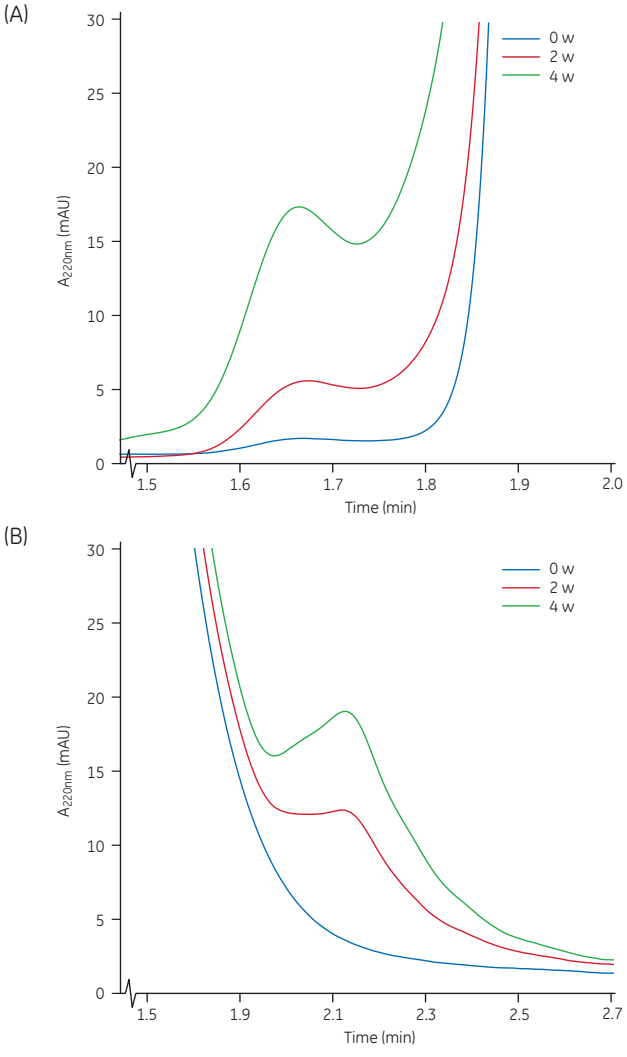


Fig 11. Monitoring of size-homogeneity changes of proteins during storage (0, 2, and 4 weeks) using Superdex 75 Increase 5/150 GL for rapid analyses of two purified proteins. In (A), higher M_r variants were detected, whereas in (B), degraded products were observed. The area under the curves for the much larger main peaks (not shown) decreased over time, corresponding to the area-increase seen for the small heterogeneity shoulders in (A) and (B).

Ordering information

Product	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

Related products

Superdex 200 Increase 10/300 GL	1	28990944
Superdex 200 Increase 5/150 GL	1	28990945
Superdex 200 Increase 3.2/300	1	28990946
Superose 6 Increase 10/300 GL	1	29091596
Superose 6 Increase 5/150 GL	1	29091597
Superose 6 Increase 3.2/300	1	29091598
Gel Filtration LMW Calibration Kit	1	28403841
Gel Filtration HMW Calibration Kit	1	28403842

Accessories

Tricorn 10 Filter Kit	1	29053612
Tricorn 5 Filter Kit	1	29053586
Filter Tool	1	18115320
Fingertight connector, 1/16" male	10	18111255
Tricorn storage/shipping device	1	18117643

Related literature

Handbook: Size-exclusion chromatography, principles and methods	1	18102218
Selection guide: Prepacked chromatography columns for ÄKTA systems	1	28931778
Instrument management handbook: ÄKTA laboratory-scale chromatography systems	1	29010831
Procedure: Maintenance and cleaning of size exclusion chromatography columns	1	29140760
Cue card: Optimal configuration of ÄKTA pure 25 for small-scale SEC	1	29181181
Instruction Superdex 75 Increase 5/150 GL/ Superdex 75 Increase 10/300 GL	1	29163059
Instruction Superdex 75 Increase 3.2/300	1	29163060

Acknowledgement

We thank Drs Annika Rogstam and Claes von Wachenfeldt, Lund Protein Purification Platform (LP3), Department of Biology, Lund University, Sweden for sharing of data.

GE Healthcare UK Ltd.
Amersham Place, Little Chalfont
Buckinghamshire, HP7 9NA
UK

www.gelifesciences.com

GE Healthcare Bio-Sciences AB, Björkgatan 30, 751 84 Uppsala, Sweden
GE Healthcare Europe GmbH, Munzinger Strasse 5, D-79111 Freiburg, Germany
GE Healthcare Bio-Sciences Corp., 100 Results Way, Marlborough, MA 01752, USA
GE Healthcare Dharmacon Inc., 2650 Crescent Dr, Lafayette, CO 80026, USA
HyClone Laboratories Inc., 925 W 1800 S, Logan, UT 84321, USA
GE Healthcare Japan Corp., Sanken Bldg., 3-25-1, Hyakunincho Shinjuku-ku, Tokyo 169-0073, Japan
For local office contact information, visit www.gelifesciences.com/contact

29187961 AA 04/2016