About: TSKgel SW Series Size Exclusion Columns

TSKgel UP-SW3000 columns are the latest addition to the popular TSKgel SW column series, the gold standard for QC analysis of antibody therapeutics. TSKgel UP-SW3000 columns feature the same pore size as the well-established TSKgel G3000SWXL columns. Hence methods developed using TSKgel G3000SWXL columns can easily be transferred to TSKgel UP-SW3000 columns on conventional HPLC systems as well as on UHPLC systems.

The TSKgel SW mAb columns meet the growing demand for the higher resolution and high throughput separation of monoclonal antibody (mAb) monomer and dimer/fragment, as well as higher resolution of mAb aggregates. While mAbs can be analyzed using many different modes of HPLC, size exclusion is best for aggregation, dimer, and fragmentation, making it the best method for heterogeneity studies.

TSKgel SW series SEC columns contain a large pore volume per unit column volume. This is critical in SEC, because the more pore volume per unit column volume, the better two proteins of different molar mass are separated. TSKgel UP-SW3000, SW mAb, SW, SWXL, and SuperSW columns are based on highly porous silica particles, the surface of which has been shielded from interacting with proteins by derivatization with ligands containing diol functional groups. TSKgel SW series columns stand out from other silica- or polymer-based high performance size exclusion columns by virtue of their large pore volumes and low residual adsorption.

TSKgel UP-SW3000, SW mAb, SW, SWXL, and SuperSW columns are stable from pH 2.5 to 7.5 and can be used in 100% aqueous conditions. The different pore sizes of the TSKgel SW series columns result in different exclusion limits for globular proteins, polyethylene oxides and dextrans, as summarized in Table 2. Furthermore, different particle sizes, column dimensions and housing materials are available for each of the TSKgel SW series columns.

The column internal diameter of TSKgel SuperSW columns has been reduced from 7.8 mm ID to 4.6 mm ID to provide higher sensitivity in sample-limited cases and to cut down on solvent use. It is important to employ an HPLC system that is optimized with regards to extra-column band broadening to take full advantage of the high column efficiency that can be obtained on these columns.

TSKgel BioAssist columns are available within the TSKgel SWXL line. These columns are made of PEEK housing material to reduce sample adsorption to stainless steel or glass. Also available within the TSKgel G2000SWXL and G3000SWXL line are QC-PAK columns. These columns are 15 cm in length with 5 µm particles and offer the same resolution in half the time as the 30 cm, 10 µm TSKgel G2000SW and G3000SW columns.

TSKgel BioAssist DS desalting columns are designed to reduce the concentration of salt and buffer of protein or polynucleotide sample solutions at semi-preparative scale. Packed with 15 µm polyacrylamide beads in PEEK hardware, TSKgel BioAssist DS columns show excellent desalting performance.

Recommendations for TSKgel SW series selection:

- Samples of known molar mass
  - Calibration curves for each TSKgel SW series column are provided in this HPLC Column Product Guide. Each curve represents a series of various standards (protein, PEO, or globular proteins, for example) with known molar masses. The molar mass range of the compound to be analyzed should be within the linear range of the calibration curve and similar to the chemical composition and architecture of the standards used to construct the calibration curve.
- Samples of unknown molar mass
  - Use the TSKgel QC-PAK GFC300 column to develop the method (scouting) and the TSKgel G3000SWXL column to obtain the highest resolution.
  - If the protein of interest elutes near the exclusion volume, then a TSKgel G4000SWXL column is the logical next step. Conversely, if the protein of interest elutes near the end of the chromatogram, try a TSKgel G2000SWXL column.
- Proteins (general)
  - Choose one of the TSKgel SWXL columns using the calibration curves to select the appropriate pore size based on knowledge or estimate of protein molar mass.
- Monoclonal antibodies
  - TSKgel SW mAb columns are ideal for the analysis of monoclonal antibodies. Alternatives include the TSKgel UP-SW3000, G3000SWXL and SuperSW3000 columns when sample is limited or the components of interest are present at very low concentrations.
- Peptides
  - TSKgel G2000SWXL columns are the first selection for the analysis of peptides.
  - TSKgel SuperSW2000 columns are utilized when sample is limited or the components of interest are present at very low concentration.
- Other
  - Use TSKgel SW columns when not sample limited or when larger amounts of sample need to be isolated.

Table 2: Properties and separation ranges of TSKgel UP-SW3000, SW mAb, SW, SWXL, SuperSW, and BioAssist DS columns

			Molar mass of samples (Da)		
TSKgel column	**Particle size**	**Pore size**	**Globular proteins**	**Dextrans**	**Polyethylene glycols & oxides**
G2000SW	10 µm and 13 µm	12.5 nm	5,000 – 1.5×10^5	1,000 – 3×10^4	500 – 1.5×10^4
G3000SW	10 µm and 13 µm	25 nm	1×10^4 – 5×10^5	2,000 – 7×10^4	1,000 – 3.5×10^4
G4000SW	13 µm and 17 µm	45 nm	2×10^4 – 7×10^6	4,000 – 5×10^5	2,000 – 2.5×10^5
G2000SWXL, BioAssist G2SWXL, QC-PAK GFC 200	5 µm	12.5 nm	5,000 – 1.5×10^5	1,000 – 3×10^4	500 – 1.5×10^4
G3000SWXL, BioAssist G3SWXL, QC-PAK GFC 300	5 µm	25 nm	1×10^4 – 5×10^5	2,000 – 7×10^4	100 – 3.5×10^4
G4000SWXL, BioAssist G4SWXL	8 µm	45 nm	2×10^4 – 7×10^6	4,000 – 5×10^5	2,000 – 2.5×10^5
SuperSW2000	4 µm	12.5 nm	5,000 – 1.5×10^5	1,000 – 3×10^4	500 – 1.5×10^4
SuperSW3000	4 µm	25 nm	1×10^4 – 5×10^5	2,000 – 7×10^4	1,000 – 3.5×10^4
BioAssist DS	15 µm	Excludes 2,500 Da PEG	–	–	–
SuperSW mAb HR	4 µm	25 nm	1×10^4 – 5×10^5	–	–
SuperSW mAb HTP	4 µm	25 nm	1×10^4 – 5×10^5	–	–
UltraSW Aggregate	3 µm	30 nm	1×10^4 – 2×10^6	–	–
UP-SW3000	2 µm	25 nm	1×10^4 – 5×10^5	–	–

About: TSKgel UP-SW3000 U/HPLC Size Exclusion Columns

TSKgel UP-SW3000 columns packed with 2 µm silica based particles are the latest addition to the popular TSKgel SW series, the gold standard for QC analysis of antibody therapeutics. These new silica-based UHPLC/HPLC columns are based on the same proven proprietary surface technology of the renowned TSKgel SW series. The surface of the particles has been shielded from interacting with proteins by derivatization with ligands containing diol functional groups.

TSKgel UP-SW3000 columns feature the same pore size as the well-established TSKgel G3000SWXL columns. Hence methods developed using TSKgel G3000SWXL columns can easily be transferred to TSKgel UP-SW3000 columns on conventional HPLC systems as well as on UHPLC systems. TSKgel UP-SW3000 columns are available in 4.6 mm ID with 15 or 30 cm length. The 15 cm column offers a shortened analysis time with improved efficiency versus the TSKgel G3000SWXL column. The 30 cm column delivers dramatically increased peak parameters such as efficiency, asymmetry, and resolution between fragments, monomers, and aggregates compared to the TSKgel G3000SWXL column.

The lifetime of the TSKgel UP-SW3000 columns are superior and can be maintained and further improved when using the corresponding guard columns. A "direct connect" (DC) guard column allows the user to minimize extra column dead volume.

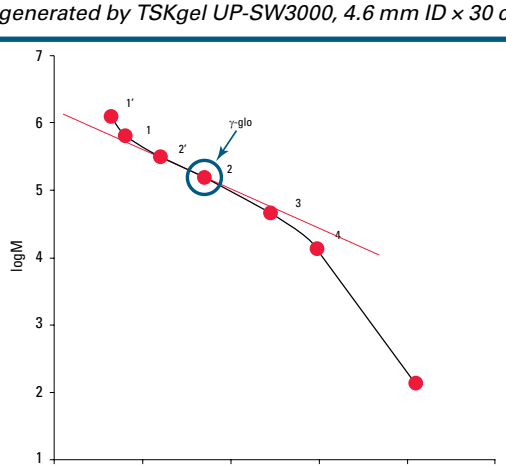
Attributes and Applications

Table 3 lists the product attributes of TSKgel UP-SW3000 columns. Figure 2 shows the protein standard calibration curve data that was generated using the TSKgel UP-SW3000 column. The column was run with a simple aqueous mobile phase (sodium phosphate buffer, pH 6.8) as typically reported in literature for SEC separations. The data demonstrates that the TSKgel UP-SW3000 column has a broad and linear resolving range of molecular weights. The shallow slope around the molecular weight of γ-globulin suggests that the particles of the column have an optimized pore size for the separation of proteins with a molecular weight of approximately 150 kDa.

Table 3: Product attributes

Attribute	Value
Base material	Silica
Particle size (mean)	2 µm
Pore size (mean)	25 nm
Functional group	Diol
pH stability	2.5-7.5
Calibration range (proteins)	10 - 500 kDa

Figure 2: Standard calibration curve of QC protein standard mixture generated by TSKgel UP-SW3000, 4.6 mm ID × 30 cm column

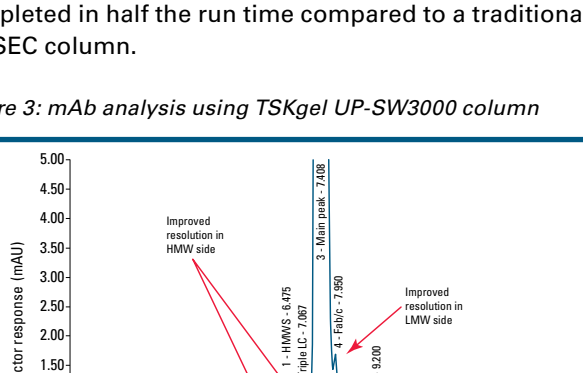


Column: **TSKgel UP-SW3000, 2 µm, 4.6 mm ID × 15 cm**
Mobile phase: 100 mmol/L phosphate buffer, pH 6.7 + 100 mmol/L Na_2SO_4 + 0.05% NaN_3
Flow rate: 0.35 mL/min
Detection: UV @ 280 nm
Temperature: 25 °C
Inj. vol.: 5 µL
Samples: 1'. thyroglobulin dimer, 1. thyroglobulin, 640 kDa
2'. γ-globulin dimer , 2. γ-globulin, 155 kDa
3. ovalbumin, 47 kDa, 4. ribonuclease A, 13,700 Da
5. p-amino benzoic acid, 137 Da

Superior Resolution for mAb Analysis

Figure 3 demonstrates the advantages of the TSKgel UP-SW3000 column for mAb analysis. The TSKgel UP-SW3000 column offers high resolution of both the high molecular weight (HMW) species and the Fab/c on the low molecular weight side. In addition, the analysis was completed in half the run time compared to a traditional 30 cm SEC column.

Figure 3: mAb analysis using TSKgel UP-SW3000 column

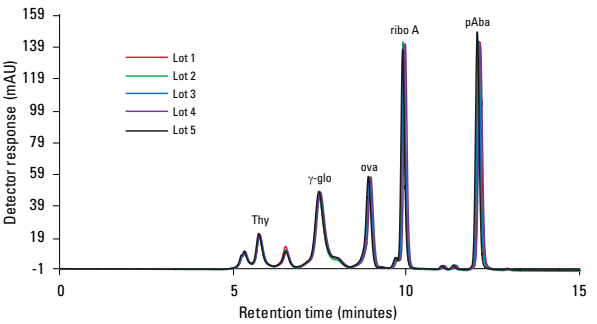


Column: **TSKgel UP-SW3000, 2 µm, 4.6 mm ID × 30 cm**
Instrument: Dionex UltiMate® 3000RS UHPLC System
Mobile phase: 0.2 mol/L potassium phosphate/0.25 mol/L KCl, pH 6.2
Flow rate: 0.35 mL/min
Detection: UV @ 280 nm
Temperature: 40 °C
Injection vol.: 10 µL

Reproducibility

TSKgel UP-SW3000 columns offer superior reproducibility injection-to-injection, from column-to-column within the same lot and from lot-to-lot. A QC standard protein mixture of three consecutive injections was analyzed, yielding low percent relative standard deviation (%RSD) for retention time and theoretical plate count for all peaks, as shown in Figure 4 below.

Figure 4: TSKgel UP-SW3000 lot-to-lot reproducibility

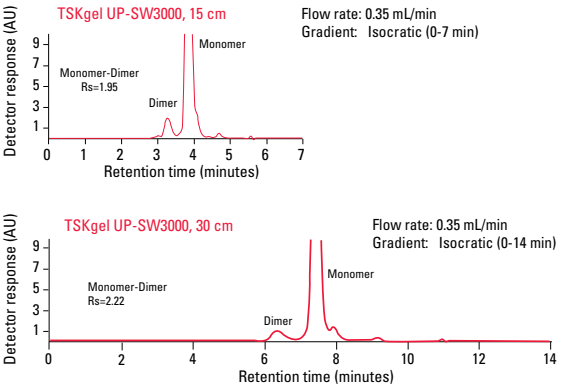


Column:	**TSKgel UP-SW3000, 2 µm, 4.6 mm ID × 30 cm**
Instrument:	Thermo Fisher/Dionex Ultimate 3000 UHPLC System
Mobile phase:	100 mmol/L sodium phosphate buffer, pH 6.7, + 100mmol/L Na_2SO_4 + 0.05% NaN_3
Gradient:	Isocratic
Flow rate:	0.35 mL/min
Detection:	UV @ 280 nm
Temperature:	25 °C
Injection vol.:	5 µL
Samples:	QC standard protein test mixture: thyroglobulin, dimer thyroglobulin, 640 kDa, 0.5 g/L γ-globulin, dimer γ-globulin, 155 kDa, 1 g/L ovalbumin, 47 kDa, 1 g/L ribonuclease A , 13,700 Da, 1.5 g/L p-aminobenzoic acid, 137 Da, 0.01 g/L

Advantages of 15 cm TSKgel UP-SW3000 Column

Figure 5 compares the separation profile of a mAb on a 30 cm and a 15 cm length TSKgel UP-SW3000 column operated under the same mobile phase conditions and flow rate. The results indicate that the 15 cm TSKgel UP-SW3000 column provides a similar profile to the 30 cm column with 50% less run time and 50% lower backpressure at a typical flow rate of 0.35 mL/min (see Figure 6). The resolution between dimer and monomer is slightly less with the 15 cm column but it is still above the resolution guidelines from the USP monogram (1.2 resolution is acceptable). In addition, when the 15 cm column is operated at the typical flow rate of 0.35 mL/min, the backpressure is only 11 MPa. Therefore, these columns can be used with both HPLC and UHPLC systems.

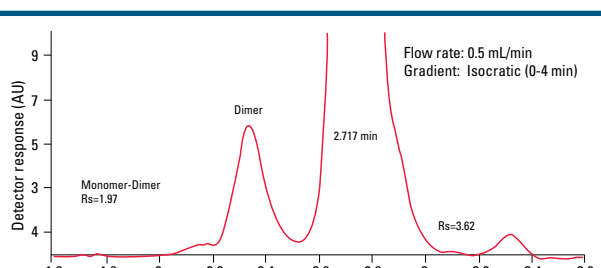
Figure 5: Comparison of mAb aggregates analysis between TSKgel UP-SW3000, 15 cm and 30 cm columns



Columns:	**TSKgel UP-SW3000, 2 µm, 4.6 mm ID × 15 cm** **TSKgel UP-SW3000, 2 µm, 4.6 mm ID × 30 cm**
Mobile phase:	100 mmol/L sodium phosphate buffer, pH 6.8, + 100 mmol/L sodium sulfate + 0.05% sodium azide
Gradient:	Isocratic
Flow rate:	as indicated in each chromatogram
Detection:	UV @ 280 nm
Temperature:	25 °C
Injection vol.:	10 µL
Sample:	mAb (0.4 mg/mL)

Figure 6 demonstrates the rapid aggregate determination of a mAb using a TSKgel UP-SW3000, 4.6 mm ID × 15 cm column operated at 0.5 mL/min. The figure shows that the analysis was completed in only 4 minutes, nearly a 4 times faster run time than the 30 cm length column (compare the run time of Figure 5, bottom panel to this figure) and nearly 8 times faster than a traditional SEC column run time of 30 minutes at 1 mL/min (data not shown). The resolution profile of the aggregates and monomer of mAb (Rs = 1.97) is still maintained at the acceptable range in the USP guideline.

Figure 6: Fast analysis of mAb sample using TSKgel UP-SW3000, 4.6 mm ID × 15 cm column



Columns:	**TSKgel UP-SW3000, 2 µm, 4.6 mm ID × 15 cm**
Mobile phase:	100 mmol/L sodium phosphate buffer, pH 6.8, + 100 mmol/L sodium sulfate + 0.05% sodium azide
Gradient:	Isocratic
Flow rate:	as indicated in each chromatogram
Detection:	UV @ 280 nm
Temperature:	25 °C
Injection vol.:	10 µL
Sample:	mAb (0.4 mg/mL)

Easy Method Transfer: From HPLC to HPLC and UHPLC Systems

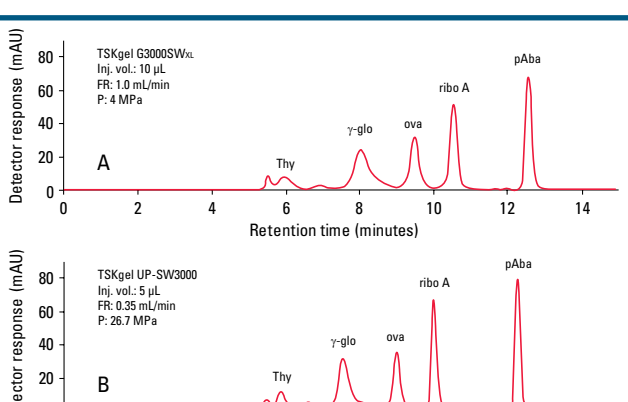
TSKgel UP-SW3000 columns feature the same pore size as the well-established TSKgel G3000SWXL columns. Hence methods developed using TSKgel G3000SWXL columns can easily be transferred to TSKgel UP-SW3000 columns on conventional HPLC systems as well as on UHPLC systems.

The TSKgel UP-SW3000 column offers several advantages versus the TSKgel G3000SWXL column, as shown in Figure 7 comparing the analysis of QC protein standards at the same concentrations. The TSKgel UP-SW3000 column offers higher sensitivity, with better peak shape, higher resolution and slightly shorter retention time. No change in the mobile phase composition is required; only an adjustment to a lower flow rate is necessary.

A method developed on a conventional HPLC system using a TSKgel UP-SW3000, 2 µm column is smoothly transferrable to a UHPLC system. Two separation profiles of a QC protein standard mixture using a TSKgel UP-SW3000 column from a HPLC and a UHPLC system are overlaid and shown in Figure 8. The two overlaid chromatograms clearly show that the method is robust, reproducible and transferrable.

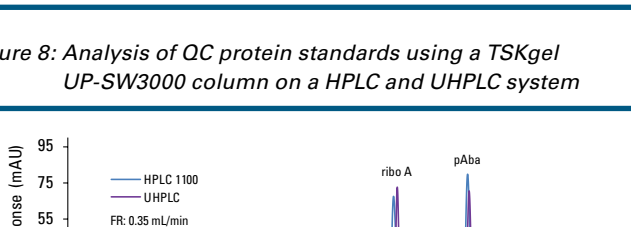
The analysis was carried out under identical chromatographic conditions at a flow rate of 0.35 mL/min. The two sets of three consecutive runs were carried out with the following criteria: two different instruments (Agilent 1100 HPLC and Thermo Fisher/Dionex Ultimate 3000 UHPLC), on two different days, using two columns from two different lots, using two different batches of QC buffer, and two different preparations of the QC protein standard mixture.

Figure 7: Analysis of QC protein standards using TSKgel G3000SWXL and UP-SW3000 columns on a conventional HPLC system



Columns:	**A. TSKgel G3000SWXL 5 µm, 7.8 mm ID × 30 cm** **B. TSKgel UP-SW3000, 2 µm, 4.6 mm ID × 30 cm**
Mobile phase:	100 mmol/L sodium phosphate buffer, pH 6.7 + 100 mmol/L Na_2SO_4 + 0.05% NaN_3
Flow rate:	A. 1.0 mL/min B. 0.35 mL/min
Detection:	UV @ 280 nm
Temperature:	25 °C
Inj. vol.:	A. 10 µL B. 5 µL
Samples:	1'. thyroglobulin, dimer 1. thyroglobulin, 640 kDa 2'. γ-globulin, dimer 2. γ-globulin, 155 kDa 3. ovalbumin, 47 kDa 4. ribonuclease A, 13,700 Da 5. p-amino benzoic acid, 137 Da

Figure 8: Analysis of QC protein standards using a TSKgel UP-SW3000 column on a HPLC and UHPLC system



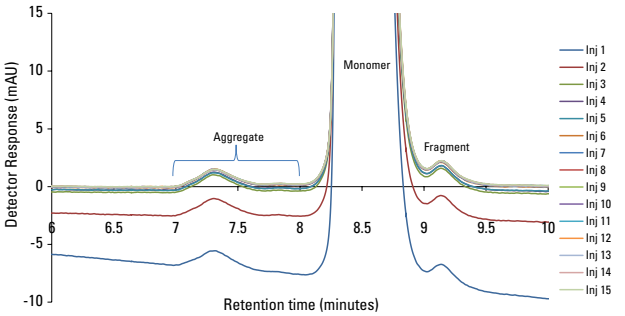
Column:	**TSKgel UP-SW3000, 2 µm, 4.6 mm ID × 30 cm**
Mobile phase:	100 mmol/L sodium phosphate buffer, pH 6.7 + 100 mmol/L Na_2SO_4 + 0.05% NaN_3
Flow rate:	0.35 mL/min
Detection:	UV @ 280 nm
Temperature:	25 °C
Inj. vol.:	5 µL
Samples:	1'. thyroglobulin, dimer 1. thyroglobulin, 640 kDa 2'. γ-globulin, dimer 2. γ-globulin, 155 kDa 3. ovalbumin, 47 kDa 4. ribonuclease A, 13,700 Da 5. p-amino benzoic acid, 137 Da

Usage of Isopropyl Alcohol for mAb Separation

Figure 9 shows the overlay of 15 injections of the USP mAb standard sample onto a TSKgel UP-SW3000 column with the addition of 15% IPA. These injections are performed after the column is subjected to 15 injections of the USP mAb standard sample with sodium phosphate buffer, pH 6.7, without IPA. The baseline of the first injection (as shown in blue) indicates that the column takes only one to two injections to be stabilized. After that all subsequent injections are overlaid perfectly.

At 0.3 mL/min, the pressure of the column is only at 22 MPa with the IPA added. It is still far below the allowance of the maximum pressure of 34 MPa of the column's rating. With this low operating pressure, the TSKgel UP-SW3000 column can be operated with both HPLC and UHPLC systems. As the chromatograms indicate, all runs are completed within 15 minutes.

Figure 9: Separation of USP mAb standard using TSKgel UP-SW3000 column with sodium phosphate buffer, pH 6.7, plus 15% IPA added



Column:	**TSKgel UP-SW3000, 2 µm, 4.6 mm ID × 30 cm**
Instrument:	UltiMate 3000 UHPLC system run by Chromeleon® (ver 7.2)
Mobile phase:	15% IPA in 100 mmol/L KH_2PO_4/ Na_2HPO_4, pH 6.7, 100 mmol/L Na_2SO_4, 0.05% NaN_3
Flow rate:	0.30 mL/min
Detection:	UV @ 280 nm
Temperature:	30 °C
Pressure:	22 MPa (maximum column pressure is 34 MPa)
Injection vol.:	5 µL, 4 mg/mL
Sample:	USP mAb standard

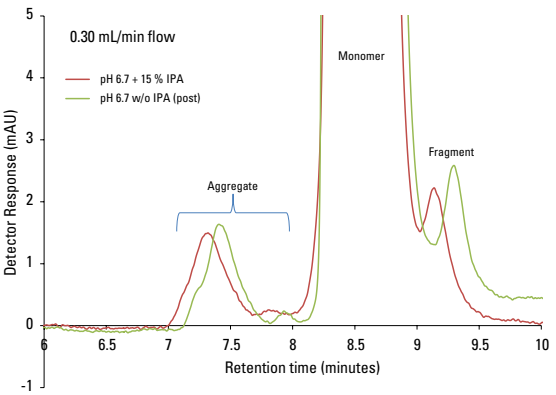
Table 4 lists the calculated data from the monomer and dimer peaks of the 15 injections from Figure 8 with the %RSD of retention time and percent relative area. As shown, the %RSD is below the allowance from the USP monograph guidance.

Table 4: %RSD of monomer and dimer peak of 15 injections: separation of USP mAb standard with sodium phosphate buffer, pH 6.7, plus 15% IPA added

	Monomer peak		Dimer peak	
Injection	Ret. time (min)	Area (mAU*min)	Ret. time (min)	Area (mAU*min)
1	8.340	97.110	7.417	0.470
2	8.340	98.280	7.410	0.460
3	8.340	98.420	7.410	0.470
4	8.340	98.400	7.407	0.490
5	8.340	98.440	7.417	0.470
6	8.340	97.940	7.413	0.500
7	8.337	98.010	7.420	0.470
8	8.337	98.030	7.437	0.470
9	8.337	98.110	7.407	0.470
10	8.337	98.110	7.423	0.470
11	8.337	98.120	7.413	0.460
12	8.337	98.220	7.417	0.470
13	8.337	98.130	7.420	0.480
14	8.337	98.220	7.413	0.460
15	8.337	98.260	7.413	0.450
Average	**8.338**	**98.120**	**7.416**	**0.471**
Std Dev	**0.002**	**0.317**	**0.008**	**0.012**
%RSD	**0.018**	**0.323**	**0.102**	**2.598**

Figure 10 is an overlay of injections with and without IPA added to the mobile phase. The overlay indicates the similarities of peak retention times, peak width and peak height of dimer, monomer, aggregate and fragment peaks between the two different conditions.

Figure 10: Separation of USP mAb standard using TSKgel UP-SW3000 column with sodium phosphate buffer, pH 6.7, with and without 15% IPA added



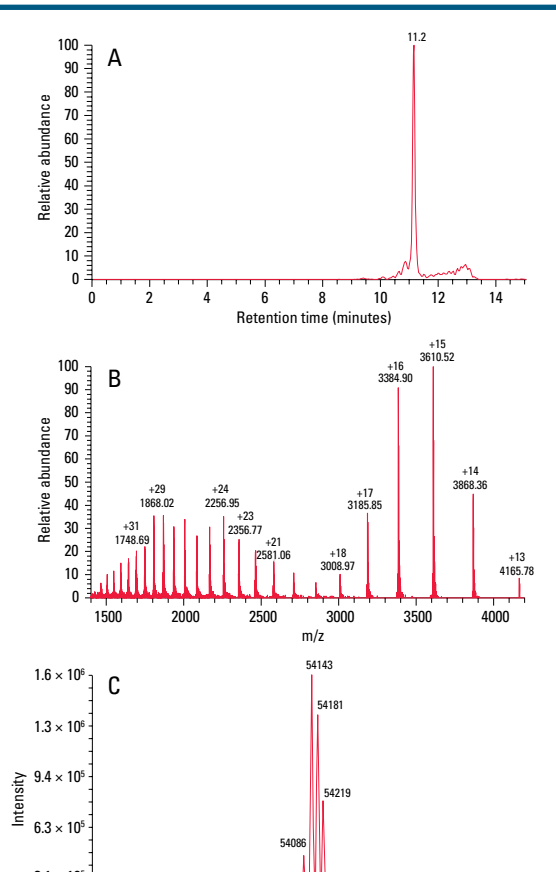
Column:	**TSKgel UP-SW3000, 2 µm, 4.6 mm ID × 30 cm**
Instrument:	UltiMate 3000 UHPLC system run by Chromeleon® (ver 7.2)
Mobile phase:	15% IPA in 100 mmol/L KH_2PO_4/ Na_2HPO_4, pH 6.7, 100 mmol/L Na_2SO_4, 0.05% NaN_3
Flow rate:	0.30 mL/min
Detection:	UV @ 280 nm
Temperature:	30 °C
Pressure:	22 MPa (maximum column pressure is 34 MPa)
Injection vol.:	5 µL, 4 mg/mL
Sample:	USP mAb standard

SEC/MS Analysis of a Bispecific T Cell Engager (BiTE®)

The TSKgel UP-SW3000, 2 µm SEC column can be used for accurate molar mass determination by SEC/MS. A MS compatible mobile phase under non-denaturing condition was successfully used with the TSKgel UP-SW3000 column. No signs of particle shedding or sample carryover, which may interfere with MS signal response, were noted with the TSKgel UP-SW3000 column.

A ~55 kDa BiTE and ~150 kDa parent mAbs (data not shown) were subsequently injected onto a TSKgel UP-SW3000 column coupled to a Q Exactive Plus mass spectrometer for molar mass determination. Figure 11 shows the (*A*) total ion chromatogram, (*B*) mass spectrum and (*C*) deconvoluted mass spectrum of the BiTE. A main peak can be seen at m/z 54,143; adjacent peaks at m/z 54,181, 54,219 and 54,086 correspond to different salt adducts.

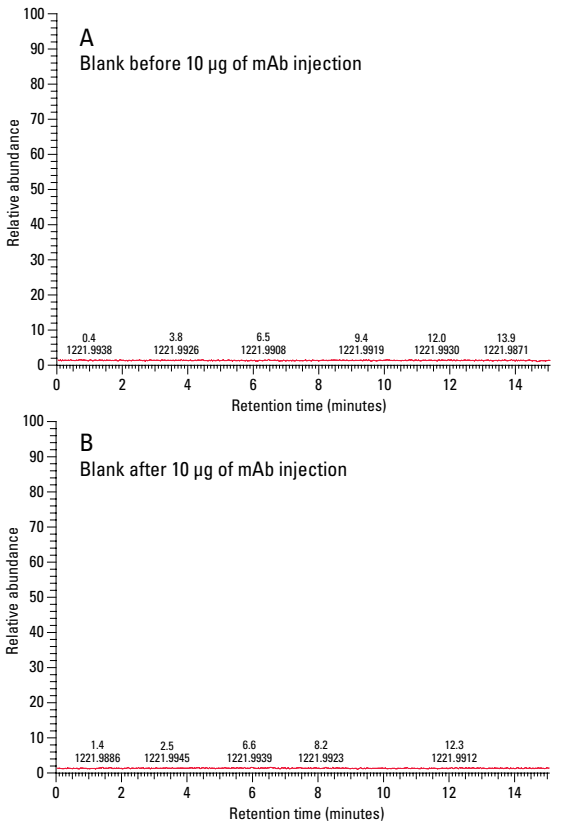
Figure 11: SEC/MS analysis of the BiTE. Accurate molar mass of the BiTE was identified as 54.1 kDa via SEC/MS.



Column:	**TSKgel UP-SW3000, 2 µm, 4.6 mm ID × 30 cm**
HPLC Instrument:	Nexera® XR UHPLC system
MS Instrument:	Q Exactive™ Plus
Mobile phase:	20 mmol/L ammonium acetate, 10 mmol/L ammonium bicarbonate; pH 7.2
Gradient:	isocratic
Flow rate:	0.35 mL/min
Detection:	UV @ 280 nm
Temperature:	30 °C
Injection vol.:	5.0 µL
Samples:	BiTE, 0.3 mg/mL (Creative Biolabs) parent mAb shown, 0.5 mg/mL (Creative Biolabs)
Ionization mode:	Electrospray ionization, positive mode
MS mode:	Scanning, m/z 800-6000

Prior to analysis, a blank injection was run in order to assess column particle shedding. Figure 12A shows the total ion chromatogram of a blank injection that was run on a new TSKgel UP-SW3000 column. MS data indicates that there is no shedding from the TSKgel UP-SW3000 column prior to sample injection. Additionally, a blank injection was run between each of the sample injections in order to monitor sample carryover. Figure 12B shows the total ion chromatogram of a blank injection run between the BiTE and parent mAb. No evidence of carryover can be seen in the run after sample injection. The lack of shedding and carryover indicate that the TSKgel UP-SW3000 column is suitable for use with MS.

Figure 12A and 12B:Column Shedding and Carryover Analysis. No shedding or carryover was observed via MS total ion chromatogram.



About: TSKgel SW mAb Size Exclusion Columns

TSKgel SW mAb columns are the newest innovation in size exclusion technology from Tosoh. This line of columns consists of three specialized columns designed for the separation and analysis of monoclonal antibodies (mAb).

Compared to competitive columns, these new stainless steel, silica-based TSKgel columns offer reduced lot-to-lot variation, long column life, reduction of unspecified adsorption, and superior recovery of aggregates.

These columns are available within the TSKgel SW mAb column line:

- TSKgel SuperSW mAb HR
- TSKgel SuperSW mAb HTP
- TSKgel UltraSW Aggregate

TSKgel SuperSW mAb HR and SuperSW mAb HTP both contain 4 µm particles. The HR designation represents the high resolution analysis of mAb monomer, dimer, and fragments, while the HTP stands for "high throughput" due to the smaller dimensions (4.6 mm ID × 15 cm). The TSKgel SuperSW mAb HTP column is compatible with both HPLC and UHPLC systems. The TSKgel UltraSW Aggregate column is a smaller particle size, 3 µm, and offers high resolution separation of mAb multimers and aggregates.

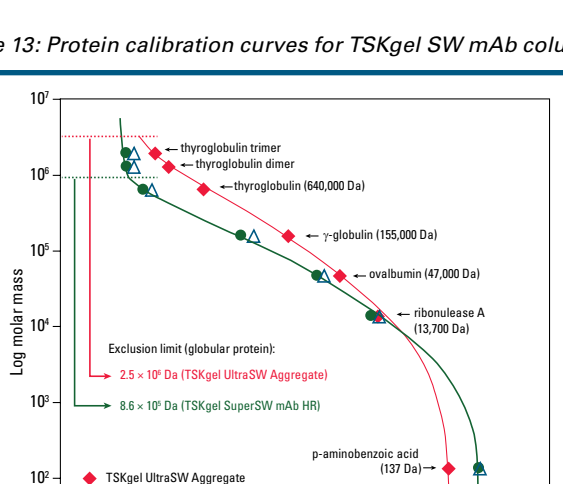
Attributes and Applications

Table 5 shows a summary of the product attributes for the TSKgel SW mAb columns. These columns utilize a unique pore-controlled technology, which produces a shallow calibration curve in the molar mass region of a typical monoclonal antibody. As shown in Figure 13, the calibration curve for the TSKgel SuperSW mAb HR column is similar to that of the TSKgel G3000SW$_{XL}$ column curve and has a shallower slope than the TSKgel UltraSW Aggregate column around the molar mass range of gamma-globulin. This shallow calibration curve produces high resolution separations. The TSKgel UltraSW Aggregrate calibration curve shows a separation range up to around 2 million Da, which implies better resolution of aggregate/multimer of a mAb.

Table 5: Product attributes

TSKgel column	SuperSW mAb HR	SuperSW mAb HTP	UltraSW Aggregate
Base material	Silica		
Particle size (mean)	4 µm	4 µm	3 µm
Pore size (mean)	25 nm	25 nm	30 nm
Functional group	Diol		
pH stability	2.5-7.5		
Calibration range	1×10^4 - 5×10^5 Da (globular proteins)	1×10^4 - 5×10^5 Da (globular proteins)	1×10^4 - 2×10^6 Da (globular proteins)

Figure 13: Protein calibration curves for TSKgel SW mAb columns

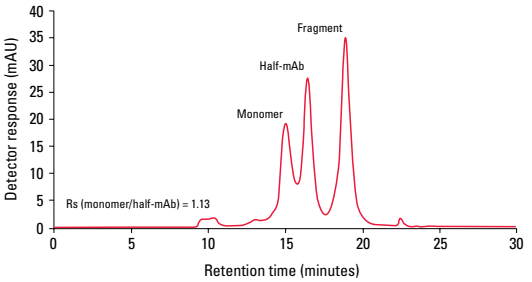


mAb Monomer from its Half-Body

Recent research has shown an interest in mAb half-bodies as therapeutic vectors as they can be further targeted for conjugation, enzyme labeling, or antibody immobilization. Monoclonal antibody half-bodies can be generated through the genetic engineering of cells or by selective reduction of hinge-region disulfide bonds present in the mAb by mild reducing agents, such as TCEP [tris(2carboxyethyl) phosphine]. A mAb half-body was generated through protein reduction using TCEP and subsequently identified by gel electrophoresis.

Figure 14 illustrates the separation of human IgG monomer, half-body (70 kDa) and fragment (1/3 mAb) using a TSKgel SuperSW mAb HR column. High resolution (Rs = 1.13) of the IgG monomer and half-body species was achieved.

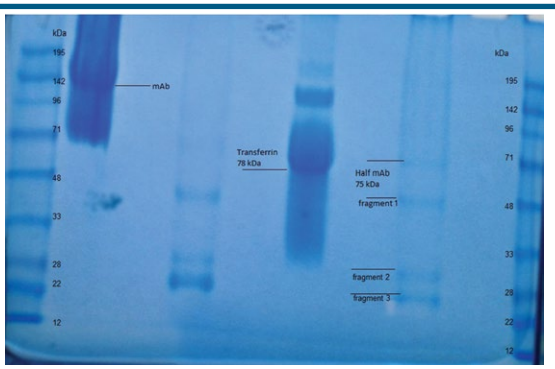
Figure 14: Separation of human IgG monomer, half-body, and fragments using a TSKgel SuperSW mAb HR column



Column:	**TSKgel SuperSW mAb HR, 4 µm, 7.8 mm ID × 30 cm**
Mobile phase:	0.1 mol/L phosphate/0.1 mol/L sulfate buffer + 0.05% NaN_3
Flow rate:	0.5 mL/min
Detection:	UV @ 280 nm
Temperature:	25 °C
Injection vol.:	10 µL
Sample:	human IgG (4.6 g/L) from Sigma Aldrich

SDS-PAGE was used to confirm the identity of the mAb monomer, half-body and fragment collected from the SEC separation on the TSKgel SuperSW mAb HR column. The monoclonal antibody, half mAb and the fragment are clearly identified with the SDS-PAGE molar mass marker as well as transferrin (78 kDa) (Figure 15). This clearly shows that the half mAb could be generated using the TCEP reduction method and separated using the TSKgel SuperSW mAb HR column.

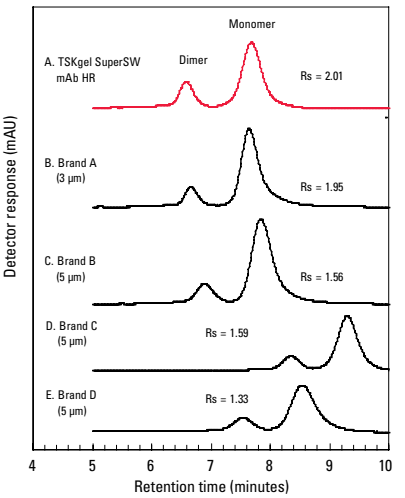
Figure 15: SDS-PAGE gel of human IgG monomer, half-body and fragments separated using a TSKgel SuperSW mAb HR column



mAb Monomer and Dimer

Figure 16 demonstrates the superior resolution of the TSKgel SuperSW mAb HR column compared to four competitive columns in the analysis of a mAb monomer and dimer. TSKgel SuperSW mAb HR shows excellent resolution of gamma-globulin dimer and monomer.

Figure 16: Comparison of resolution of mAb monomer and dimer

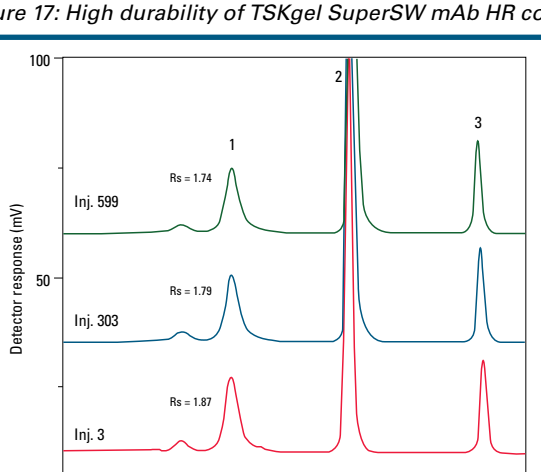


Columns:	**A. TSKgel SuperSW mAb HR, 4 µm, 7.8 mm ID × 30 cm**
	B. Brand A, 3 µm, 7.8 mm ID × 30 cm
	C. Brand B, 5 µm, 7.8 mm ID × 30 cm
	D. Brand C, 5 µm, 8.0 mm ID × 30 cm
	E. Brand D, 5 µm, 8.0 mm ID × 30 cm
Mobile phase:	200 mmol/L phosphate buffer, pH 6.7 + 0.05% NaN_3
Flow rate:	1.0 mL/min
Detection:	UV @ 280 nm
Temperature:	25 °C
Injection vol.:	10 µL
Sample:	IgG (human polyclonal), 1.0 g/L

Durability

Figure 17 demonstrates the good durability of the TSKgel SuperSW mAb HR column through the reproducibility of Rs for a γ-globulin sample injection.

Figure 17: High durability of TSKgel SuperSW mAb HR column

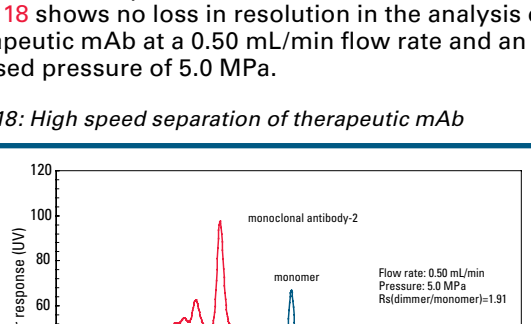


Column:	**TSKgel SuperSW mAb HR, 4 µm, 7.8 mm ID × 30 cm**
Mobile phase:	0.2 mol/L phosphate buffer, pH 6.7 + 0.05% NaN_3
Flow rate:	0.8 mL/min
Detection:	UV @ 280 nm
Injection vol.:	10 µL
Samples:	1. γ-Globulin 2. Cytochrome C 3. DNP-L-Alanine

Therapeutic mAb

A shorter column length allows the TSKgel SuperSW mAb HTP column to provide fast and efficient run times in the high resolution separation of a mAb monomer and dimer. Figure 18 shows no loss in resolution in the analysis of a therapeutic mAb at a 0.50 mL/min flow rate and an increased pressure of 5.0 MPa.

Figure 18: High speed separation of therapeutic mAb



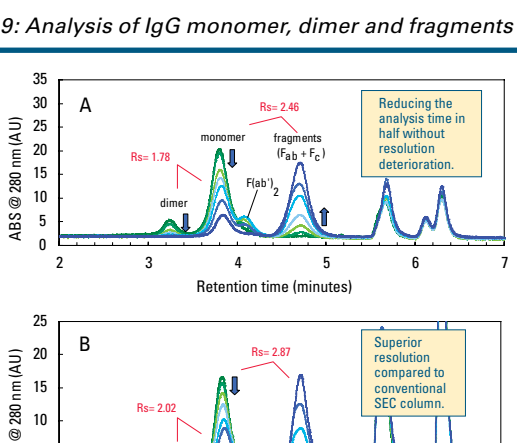
Column:	**TSKgel SuperSW mAb HTP, 4 µm, 4.6 mm ID × 15 cm**
Mobile phase:	0.2 mol/L phosphate buffer, pH 6.7 + 0.05% NaN_3
Flow rate:	0.50 mL/min, 0.35 mL/min
Detection:	UV @ 280 nm
Temperature:	25 °C
Sample:	monoclonal antibody-2 (mouse-human chimeric IgG, Erbitux®), 5 µL

Papain Digested IgG

IgG monomer, dimer, and fragments from IgG digested by papain over a 24 hour period were analyzed using the TSKgel SuperSW mAb HR and SuperSW mAb HTP columns (Figure 19). The results exhibit the superior resolving power of these columns for monomer/fragment and monomer/dimer separation. The TSKgel SuperSW mAb HTP column shows no deterioration in resolution while decreasing the analysis time in half.

The results also show that the TSKgel SuperSW mAb HR column has superior performance of mAb separation in comparison to the TSKgel G3000SWXL column. While TSKgel G3000SWXL has set the standard for the separation of general proteins for more than 25 years, the new TSKgel SuperSW mAb HR column is more specifically suited for the analysis of mAb, as seen in the results of the analysis of IgG.

Figure 19: Analysis of IgG monomer, dimer and fragments

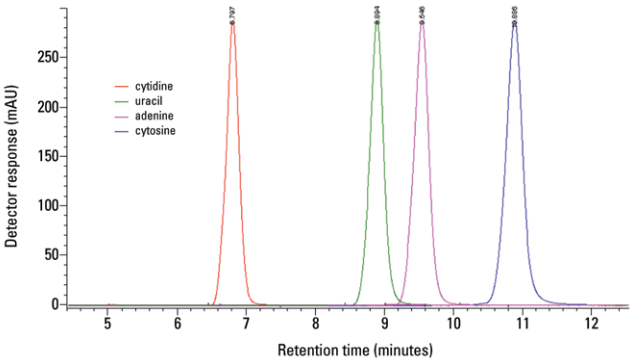


Columns:	**A. TSKgel SuperSW mAb HTP, 4 µm, 4.6 mm ID × 15 cm** **B. TSKgel SuperSW mAb HR, 4 µm, 7.8 mm ID × 30 cm**
Mobile phase:	200 mmol/L phosphate buffer + 0.05% NaN_3, pH 6.7
Flow rate:	A: 0.35 mL/min; B: 1.0 mL/min
Detection:	UV @ 280 nm
Temperature:	25 °C
Injection vol.:	A: 5 µL; B: 10 µL
Sample:	10 g/L IgG digested with papain for 0-24 hr

Nucleobases Analyzed in HILIC Mode

Figure 20 illustrates the separation of 4 nucleobases using the TSKgel SuperSW mAb HTP column in HILIC mode with CH_3CN as mobile phase A, and 15 mmol/L ammonium bicarbonate, pH 7.4, as mobile phase B. It is important to note that the order of elution of the analytes does not correlate with their molecular mass (as in SEC separations), but instead is based on their relative hydrophilicity.

Figure 20: Separation of four nucleobases using TSKgel SuperSW mAb HTP column in HILIC mode at pH 7.4

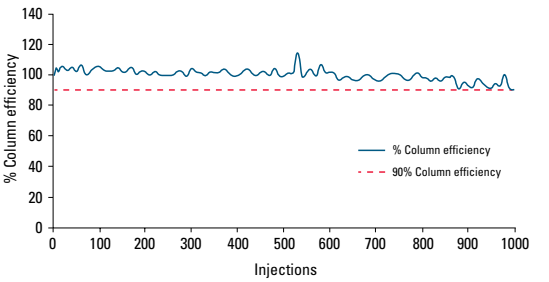


Column:	**TSKgel SuperSW mAb HTP, 4 µm, 4.6 mm ID × 15 cm**
Mobile phase:	A: acetonitrile B: 15 mmol/L ammonium bicarbonate, pH 7.4
Gradient:	isocratic
Flow rate:	0.4 mL/min
Detection:	UV @ 280 nm
Injection vol.:	1 µL
Temperature:	ambient
Samples:	uracil (1.5 g/L), adenine (1.5 g/L), cytosine (1.5 g/L), cytidine (1.5 g/L) from Sigma Aldrich

Column Lifetime

The TSKgel SuperSW mAb HTP column demonstrates highly reproducible performance over a significant number of injections of protein standard, as shown in Figure 21. The column yielded less than a 10% loss in column efficiency over 1,000 consecutive injections. Additionally, the packing integrity of the column is extremely high since even in the reverse flow orientation a nearly identical chromatographic trace to that of normal flow orientation was obtained (data not shown). During this study a guard column was not used, the analytical column was not cleaned/back flushed, and the mobile phase and sample were not filtered, to give additional stress to the analytical column. Implementation of such protective measures can be expected to yield extended column lifetime.

Figure 21: Performance stability of the TSKgel SuperSW mAb HTP column over 1000 consecutive injections of protein standard



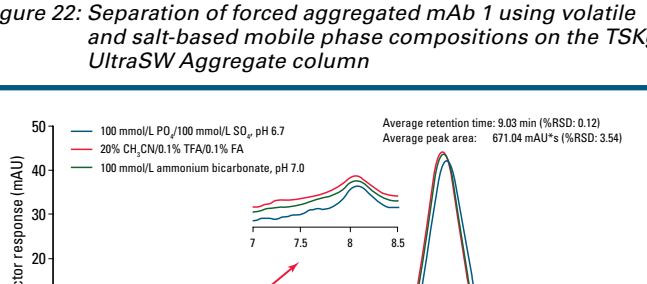
Column:	**TSKgel SuperSW mAb HTP, 4 µm, 4.6 mm ID × 15 cm**
Mobile phase:	100 mmol/L phosphate/100 mmol/L sodium sulfate, pH 6.7 + 0.05 % NaN_3
Gradient:	isocratic
Flow rate:	0.35 mL/min
Detection:	UV @ 280 nm
Temperature:	ambient
Injection vol.:	5 µL (21.1 µg total protein load)
Samples:	protein standard: thyroglobulin, 0.58 g/L, γ-globulin, 1.02 g/L, ovalbumin, 1.08 g/L, ribonuclease, 1.53 g/L, PABA, 0.01 g/L

Monoclonal Antibody Aggregate Analysis using MS-Friendly Mobile Phases

Conventional SEC separations make use of relatively high ionic strength mobile phase compositions in an effort to minimize ionic interactions between the analyte and stationary phase. Due to the substantial amount of salt present in the mobile phase, on-line interfacing with mass spectrometry is not feasible due to the inevitable contamination of the MS ion source by the mobile phase salts. In order to make SEC-MS an applicable technique, volatile, MS-friendly mobile phase compositions must be implemented to avoid damage to the MS system.

A mAb 1 antibody was subjected to thermal stress for forced aggregation to evaluate various mobile phase compositions – volatile and salt-based. As shown in Figure 22, aggregates of mAb 1 are clearly separated from the monomeric species using all three mobile phase compositions. Results for critical peak parameters of the mAb 1 monomer are highly reproducible regardless of the mobile phase composition.

Figure 22: Separation of forced aggregated mAb 1 using volatile and salt-based mobile phase compositions on the TSKgel UltraSW Aggregate column

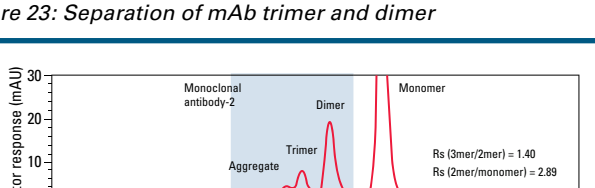


Column:	**TSKgel UltraSW Aggregate, 3 µm, 7.8 mm ID × 30 cm**
Mobile phase:	100 mmol/L PO_4/100 mmol/L SO_4, pH 6.7 20% CH_3CN/0.1% TFA/0.1% FA 100 mmol/L ammonium bicarbonate, pH 7.0
Gradient:	isocratic
Flow rate:	1 mL/min
Detection:	UV @ 280 nm
Temperature:	25 °C
Injection vol.:	10 µL
Sample:	TBL mAb 1 (4.0 g/L)

Mouse-Human Chimeric IgG

Figure 23 shows the analysis of a mouse-human chimeric IgG using the TSKgel UltraSW Aggregate column. Superior resolution of the mAb trimer and dimer is obtained. The smaller particle size (3 µm) and higher molar mass exclusion limit (2,500 kDa, globular proteins) of the TSKgel UltraSW Aggregate column, compared to the TSKgel SuperSW mAb HR and HTP columns, allows for high resolution separation of mAb multimers and aggregates.

Figure 23: Separation of mAb trimer and dimer

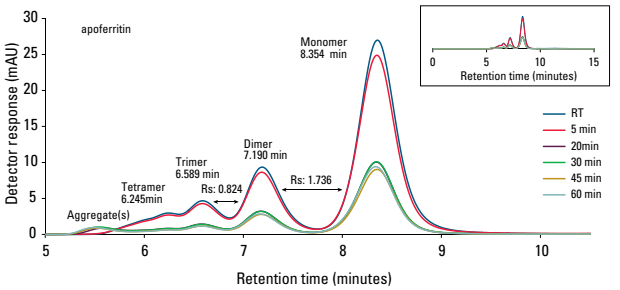


Column:	**TSKgel UltraSW Aggregate, 3 µm, 7.8 mm ID × 30 cm**
Mobile phase:	0.2 mol/L phosphate buffer, pH 6.7 + 0.05% NaN_3
Flow rate:	0.8 mL/min
Detection:	UV @ 280 nm
Temperature:	25 °C
Sample:	monoclonal antibody-2 (mouse-human chimeric IgG, Erbitux), 10 µL

Metalloprotein

The analysis of a heat denatured, large hydrophobic metalloprotein, apoferritin, is shown in Figure 24. A set of six, 0.3 mL HPLC vials each containing 100 µL stock solution of apoferritin was used for protein thermal denaturation. Thermal denaturation was carried out at 60 °C using an electric heating block. Individual sample vials were tightly capped and exposed to the heat for 5, 20, 30, 45, and 60 minutes. Samples were analyzed using a TSKgel UltraSW Aggregate column at the end of each incubation time period. The TSKgel Ultra SW Aggregate column yielded high resolution between the monomer and dimer. The trimer, tetramer and higher order aggregates of apoferritin were well separated.

Figure 24: Analysis of heat induced forced denatured, large hydrophobic metalloprotein, apoferritin



Protein	Molecular weight (kDa)			
	Monomer	Dimer	Trimer	Tetramer
ferritin and apoferritin	450	900	1350	1800

Column:	**TSKgel UltraSW Aggregate, 3 µm, 7.8 mm ID × 30 cm**
Mobile phase:	50 mmol/L potassium phosphate (monobasic), 50 mmol/L sodium phosphate (dibasic), 100 mmol/L sodium sulfate, 0.05% NaN_3, pH 6.7
Flow rate:	1.0 mL/min
Detection:	UV @ 280 nm
Temperature:	30 °C
Injection vol.:	10 µL
Samples:	ferritin – Sigma, 4.7 g/L, in saline (0.9% NaCl in water) solution, stored at 2-8 °C apoferritin – Sigma, 5.0 g/L, in 50% glycerol and 0.075 mol/L sodium chloride, stored at -20 °C

About: TSKgel SW Size Exclusion Columns

TSKgel SW columns, introduced in 1977, were the first of a long line of high performance Gel Filtration columns that have become synonymous with isolating proteins and analyzing protein molar masses in the emerging field of biotechnology.

TSKgel SW columns are based on highly porous silica particles, the surface of which has been shielded from interacting with proteins by derivatization with ligands containing diol functional groups. TSKgel SW columns stand out from other silica- or polymer-based high performance size exclusion columns by virtue of their large pore volumes.

Particles having three different pore sizes are available packed as:

- TSKgel G2000SW
- TSKgel G3000SW
- TSKgel G4000SW

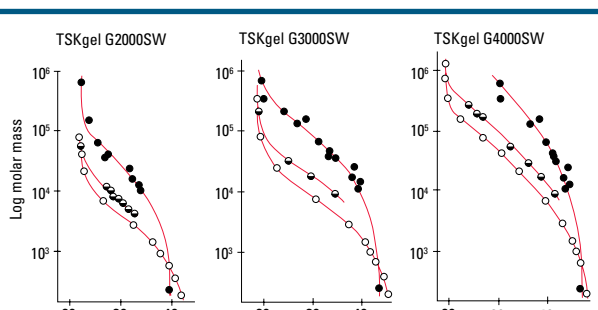
Attributes and Applications

Table 6 shows a summary of the product attributes for each of the TSKgel SW columns. The TSKgel G2000SW column provides excellent separation of peptides and proteins with molar masses up to 1.0×10^5 Da. TSKgel G3000SW columns are the best choice for separation of proteins and other biomolecules with molar masses up to 5.0×10^5 Da, while TSKgel G4000SW columns are preferred for proteins and other biomolecules of even higher molar masses. Figure 25 shows the calibration curves for globular proteins, polyethylene oxides and dextrans for each of the three TSKgel SW columns.

Table 6: Product attributes

TSKgel column	G2000SW	G3000SW	G4000SW
Base material	Silica		
Particle size (mean)	10 µm and 13 µm	10 µm and 13 µm	13 µm and 17 µm
Pore size (mean)	12.5 nm	25 nm	45 nm
Functional group	Diol		
pH stability	2.5-7.5		
Calibration range	5,000 - 1.0×10^5 Da (globular proteins)	1.0×10^4 - 5.0×10^5 Da (globular proteins)	2.0×10^4 - 7.0×10^6 Da (globular proteins)

Figure 25: Calibration curves for globular proteins, polyethylene oxides and dextrans for TSKgel SW columns

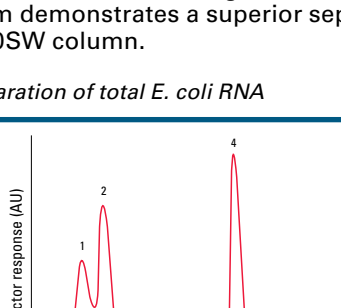


Column:	**TSKgel SW columns, 7.5 mm ID × 60 cm × 2**
Mobile phase:	dextrans and polyethylene oxides: distilled water; proteins: 0.3 mol/L NaCl in 0.1 mol/L phosphate buffer, pH 7.0
Flow rate:	1.0 mL/min
Detection:	UV @ 220 nm and RI
Samples:	● proteins, ○ polyethylene oxides, ◒ dextrans

Separation of *E. coli* RNA

Separation of four *E. coli* RNAs, shown in Figure 26, confirms the high performance of a TSKgel G4000SW column for samples with a wide molar mass range. The sample consists of 4S tRNA (2.5×10^4 Da), 5S rRNA (3.9×10^4 Da), 16S rRNA (5.6×10^5 Da), and 23S rRNA (1.1×10^6 Da). All four polynucleotides are within the molar mass range recommended for this TSKgel SW column. The chromatogram demonstrates a superior separation with the TSKgel G4000SW column.

Figure 26: Separation of total E. coli RNA

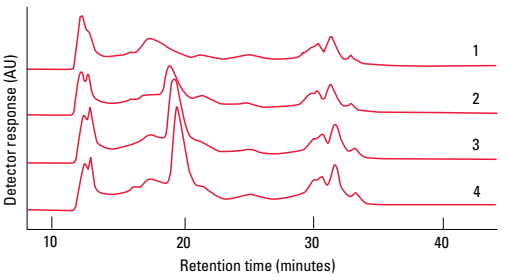


Columns:	**TSKgel G4000SW, 13 µm, 7.5 mm ID × 30 cm × 2** **TSKgel G4000SW, 17µm, 7.5 mm ID × 30 cm × 2**
Mobile phase:	0.13 mol/L NaCl in 0.1 mol/L phosphate buffer, pH 7.0, + 1 mmol/L EDTA
Flow rate:	1.0 mL/min
Detection:	UV @ 260 nm
Injection vol.:	5 µg
Sample:	0.1 mL of 1:10 diluted solution of total *E. coli* RNA: 1. 23s rRNA (1.1×10^6 Da) 2. 16s rRNA (5.6×10^5 Da) 3. 5s rRNA (3.9×10^4 Da) 4. 4s rRNA (2.5×10^4 Da)

Membrane Protein

Surfactants are routinely used for the isolation of proteins from membranes. Although this is an efficient method for solubilization, the presence of detergents affects the performance of chromatographic separations. A TSKgel G3000SW column was used to study the effect of different concentrations of the non-ionic surfactant octaethyleneglycol dodecylether on the analysis of membrane proteins from a crude extract from rat liver microsome. Figure 27 demonstrates that as the concentration of the surfactant increases to 0.05%, the main peak becomes sharper and recovery increases (chromatogram #4). Caution: we recommend that columns that have been used with a surfactant-containing mobile phase are dedicated for that particular use.

Figure 27: Analysis of membrane protein with differing surfactant concentrations in the mobile phase

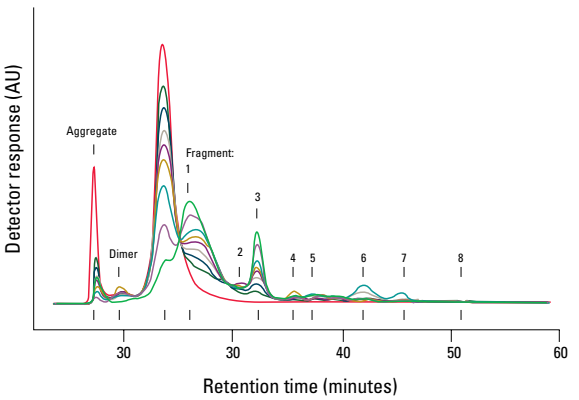


Column:	**TSKgel G3000SW, 10 µm, 7.5 mm ID × 60 cm**
Mobile phase:	(0.2 mol/L sodium chloride + 20% glycerol + octaethylene glycol dodecylether) in 50 mmol/L phosphate buffer, pH 7.0 Note: concentration of surfactant: 1. 0.005% 2. 0.01% 3. 0.025% 4. 0.05%
Flow rate:	1.0 mL/min
Detection:	UV @ 280 nm
Sample:	membrane protein from a crude extract from rat liver microsome

Degradation Products of IgG

High speed is important when analyzing the rate of chemical alteration of proteins (denaturation, condensation, degradation, etc.). Tomono et al[1] tracked the course of enzyme digestion of commercial IgG by pepsin using a TSKgel G3000SW column (Figure 28).

Figure 28: Tracking changes over time



Column:	**TSKgel G3000SW, 10 µm, 7.5 mm ID × 60 cm**
Mobile phase:	0.1 mol/L acetate buffer, pH 5.0 + 0.1 mol/L sodium sulfate
Samples*:	100 µL solutions produced by digestion of IgG (20 g/L) by pepsin after 0, 2, 4, 6, 8, 10, 15, 30 and 60 minutes

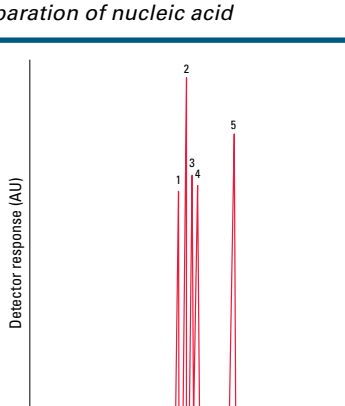
*Courtesy of Tsugikazu Tomono, Director of Japanese Red Cross Central Blood Center

1. a) T. Tomono, T. Suzuki, and E. Tokunaga, Anal, Biochem., 123, 394 (1982)
b) T. Tomono, T.Suzuki, and E. Tokunaga, Bio, Bio. Phys. Acta., 660, 186 (1981)

Nucleic Acid

Figure 29 shows the separation of nucleic acid bases and nucleosides using a TSKgel G2000SW column.

Figure 29: Separation of nucleic acid

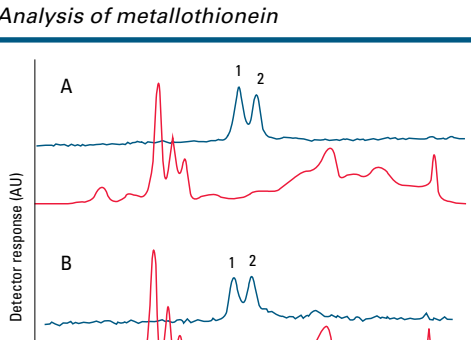


Column:	**TSKgel G2000SW, 10 µm, 7.5 mm ID × 60 cm**
Mobile phase:	acetic acid/triethylamine/H_2O = 3/3/94
Flow rate:	0.74 mL/min
Detection:	UV @ 260 nm
Samples:	1. uridine
	2. uracil
	3. thymine
	4. adenosine
	5. adenine

Metallothionein

Suzuki et al have conducted detailed studies involving the quantitative analysis of metallothionein. In these studies, the liver and kidney of cadmium-administered rats were used as samples, and the SEC columns were directly coupled to an atomic absorption detector. Metallothionein was separated into two isozymes. Presumably, the cation exchange capacity of residual silanol groups on the TSKgel SW packing material played a role in this isozyme separation. Representative chromatograms are shown in Figure 30.

Figure 30: Analysis of metallothionein



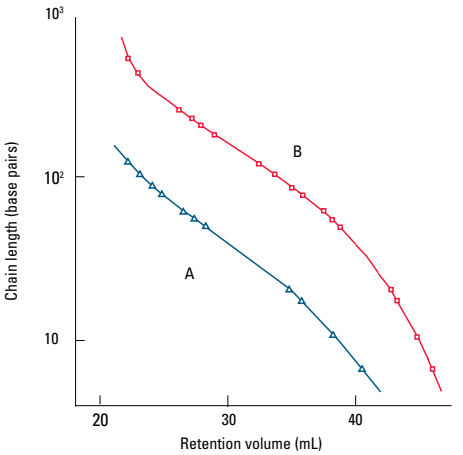
Column:	**TSKgel G3000SW, 13 µm, 21.5 mm ID × 60 cm**
Mobile phase:	50 mmol/L Tris-HCl buffer
Detection:	atomic absorption (Cd, Zn) + UV @ 280 nm
	A: Cd
	B: Zn
Samples*:	rat liver supernatant
	1.metallothionein I
	2.metallothionein II

*Courtesy of Professor Kazuo Suzuki of the National Institute for Environmental Studies

DNA Fragments

DNA fragments smaller than 300 bases have been separated into discrete peaks using the TSKgel G3000SW and G4000SW columns. Recovery of the fragments from these columns was greater than 90%. A plot of chain length versus elution volume for double-stranded DNA is shown in Figure 31.

Figure 31: Double stranded DNA fragments



Columns:	**A: TSKgel G3000SW, 10 µm, 7.5 mm ID × 60 cm × 2** **B: TSKgel G4000SW, 13 µm, 7.5 mm ID × 60 cm × 2**
Mobile phase:	0.05 mol/L Tris-HCl, 0.2 mol/L NaCl, 1 mmol/L EDTA, pH 7.5
Flow rate:	A: 1 mL/min, B: 0.33 mL/min
Detection:	UV @ 260 nm
Temperature:	25 °C
Sample:	Hae III-cleaved pBR322 DNA
Sample load:	13 µg in 50 µL

About: TSKgel SWXL Size Exclusion Columns

TSKgel SWXL columns, introduced in 1987, are packed with 5 or 8 µm particles to improve sample resolution or to reduce analysis time (over TSKgel SW columns). Like the TSKgel SW columns, TSKgel SWXL columns feature highly porous silica particles, the surface of which has been shielded from interacting with proteins by derivatization with ligands containing diol functional groups. TSKgel SWXL columns stand out from other silica- or polymer-based high performance size exclusion columns by virtue of their large pore volumes.

These columns are available within the TSKgel SWXL column line:

- TSKgel G2000SWXL
- TSKgel G3000SWXL
- TSKgel G4000SWXL
- TSKgel BioAssist G2SWXL
- TSKgel BioAssist G3SWXL
- TSKgel BioAssist G4SWXL
- TSKgel QC-PAK GFC 200
- TSKgel QC-PAK GFC 300

The TSKgel BioAssist columns are made of PEEK housing material to reduce sample adsorption to stainless steel or glass. QC-PAK columns are 15 cm in length with 5 µm particles and offer the same resolution in half the time as the 30 cm, 10 µm TSKgel G2000SW and G3000SW columns.

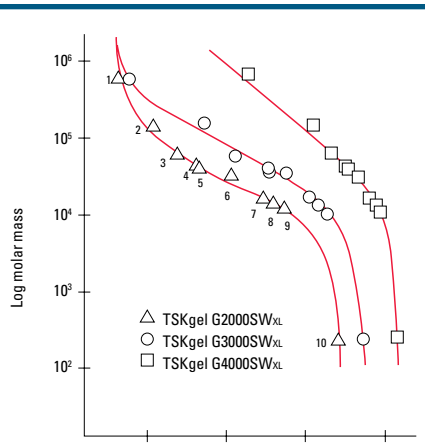
Attributes and Applications

Table 7 shows a summary of the product attributes for each of the TSKgel SWXL columns. TSKgel SWXL columns are commonly used in the quality control of monoclonal antibodies and other biopharmaceutical products. TSKgel G2000SWXL columns are an excellent choice for small proteins and peptide separations. Proteins and large peptides are separated well on TSKgel 3000SWXL columns, while TSKgel G4000SWXL provides the largest exclusion limit and the widest fractionation range. It is an excellent choice for pegylated proteins or glycosylated biomolecules. Figure 32 shows the calibration curves for globular proteins, polyethylene oxides, and dextrans for each of the three TSKgel SWXL columns.

Table 7: Product attributes

TSKgel column	G2000SWXL	G3000SWXL	G4000SWXL
Base material	Silica		
Particle size (mean)	5 µm	5 µm	8 µm
Pore size (mean)	12.5 nm	25 nm	45 nm
Functional group	Diol		
pH stability	2.5-7.5		
Calibration range	5,000 - 1.5 × 10^5 Da (globular proteins)	1.0 × 10^4 - 5.0 × 10^5 Da (globular proteins)	2.0 × 10^4 - 7.0 × 10^6 Da (globular proteins)

Figure 32: Calibration curves for proteins for TSKgel SWXL columns



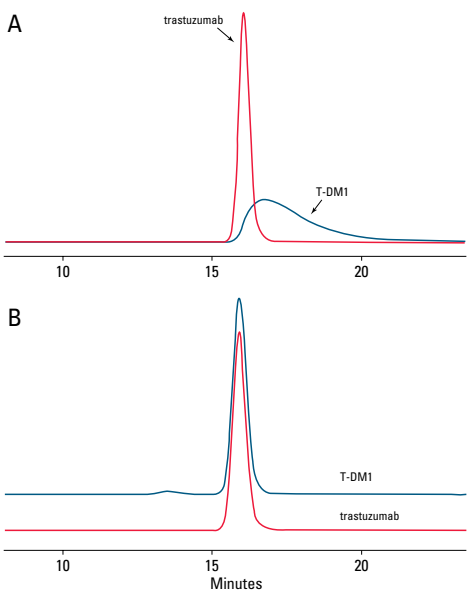
Column: **TSKgel SWXL columns, 7.8 mm ID × 30 cm**
Mobile phase: 0.3 mol/L NaCl in 0.1 mol/L sodium phosphate buffer, pH 7.0
Detection: UV @ 220 nm
Samples:
1. thyroglobulin (6.6 × 10^5 Da)
2. IgG (1.56 × 10^5 Da)
3. bovine serum albumin (6.7 × 10^4 Da)
4. ovalbumin (4.3 × 10^4 Da)
5. peroxidase (4.02 × 10^4 Da)
6. β-lactoglobulin (3.5 × 10^4 Da)
7. myoglobin (1.69 × 10^4 Da)
8. ribonuclease A (1.37 × 10^4 Da)
9. cytochrome C (1.24 × 10^4 Da)
10. glycine tetramer (246 Da)

Size Variant Analysis of Conjugates

A sample of both conjugated (T-DM1) and unconjugated (Trastuzumab) monoclonal antibody was analyzed on a TSKgel G3000SWXL column eluted isocratically with a phosphate-buffered saline mobile phase. The use of an inorganic mobile phase for unconjugated mAb analysis showed no change in the expected results. With the analysis of the conjugated mAb (ADC) in an inorganic mobile phase, poor peak shape (greatly increased tailing) and incomplete resolution of aggre¬gates from the monomeric conjugated antibody were observed (see A in Figure 33).

Addition of an organic modifier to the mobile phase, in this case 15% 2-propanol, restored peak shape and resolution of the conjugated mAb analyzed on a TSKgel G3000SWXL column (B in Figure 33). These results indicate that the attached hydrophobic drugs lead to non-specific interaction between the ADC and the column stationary phase. The addition of organic solvents to the mobile phase can be used to overcome non-specific interactions between the ADC and the column stationary phase.

Figure 33: Size variant analysis of conjugates using a TSKgel G3000SWXL column with mobile phase 0.2 mol/L KPi and 0.25 mol/L KCl (A) and 85% KPi/KCl, 15% 2-propanol (B)

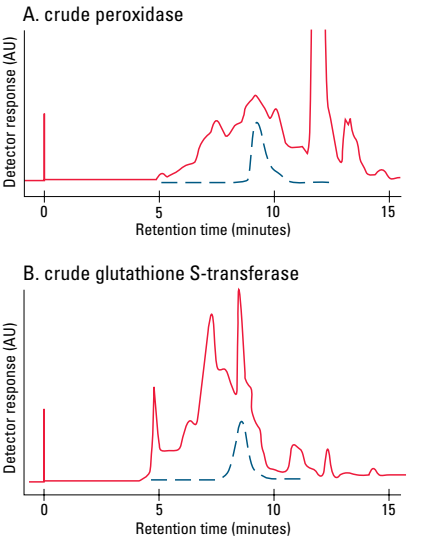


Column:	**TSKgel G3000SWXL, 7.8 mm ID × 30 cm**
Mobile phase:	A: 0.2 mol/L KPi and 0.25 mol/L KCl, pH 6.95 B: 85% KPi/KCl + 15% 2-propanol
Flow rate:	0.5 mL/min
Detection:	UV @ 280 nm

Enzymes

Mobile phase conditions in GFC are optimized to ensure little or no interaction of the sample with the packing material. This gentle technique allows for high recovery of enzymatic activity. For example, crude samples of peroxidase (Figure 34A) and glutathione S-transferase (Figure 34B) were separated in only 15 minutes on a TSKgel G3000SWXL column and activity recovery was 98% and 89%, respectively. The elution profiles of the separations show that all of the activity eluted in a narrow band of about 1.5 mL.

Figure 34A and 34B: Analysis of crude protein samples

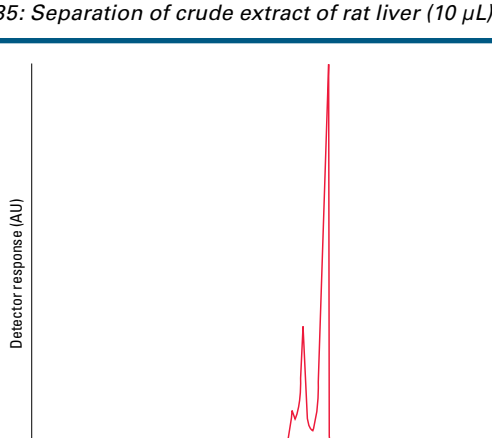


Column:	**TSKgel G3000SWXL, 5 µm, 7.8 mm ID × 30 cm**
Mobile phase:	0.3 mol/L NaCl in 0.05 mol/L phosphate buffer, pH 7.0
Flow rate:	1.0 mL/min
Detection:	UV @ 220 nm (solid line) and enzyme assay tests (dashed line)
Recovery:	enzymatic activity recovered was 98% in A and 89% in B
Samples:	A. crude peroxidase from Japanese radish, 0.15 mg in 0.1 mL B. crude glutathione S-transferase from guinea pig liver extract, 0.7 mg in 0.1 mL

Rat Liver Extract

The separation of a crude extract of rat liver using a TSKgel G2000SWXL column is displayed in Figure 35.

Figure 35: Separation of crude extract of rat liver (10 µL)

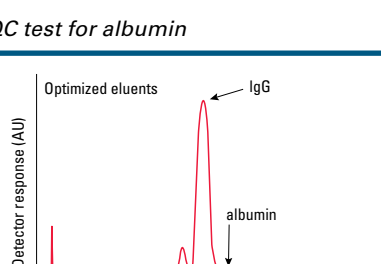


Column:	**TSKgel G2000SWXL, 5 µm, 7.8 mm ID × 30 cm**
Mobile phase:	0.05 mol/L phosphate buffer, pH 7.0 + 0.3 mol/L NaCl
Flow rate:	1 mL/min
Detection:	UV @ 220 nm
Temperature:	25 °C

IgG

A therapeutic solution of intravenous IgG may contain albumin as a stabilizer, and both proteins must be quantified following manufacture. Although literature reports describe the separation of these two proteins by many other chromatographic methods, long analysis times and complex gradient elutions are required. A method developed on a TSKgel G3000SWXL column provides quantitative separation of the two proteins in 15 minutes with a simple, isocratic elution system. As shown in Figure 36, human albumin can be separated from a 20-fold excess of IgG and quantified using an optimized elution buffer. This simple separation method can be applied to the isolation of other IgGs, such as monoclonal antibodies in ascites fluid.

Figure 36: QC test for albumin

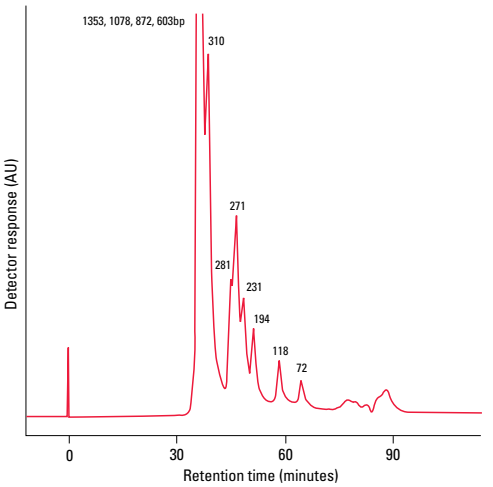


Column:	**TSKgel G3000SWXL, 5 µm, 7.8 mm ID × 30 cm**
Mobile phase:	0.1 mol/L Na_2SO_4 in 0.05 mol/L sodium phosphate buffer, pH 5.0
Flow rate:	1.0 mL/min
Detection:	UV @ 280 nm
Sample:	5 µL of venilon, containing 237.5 mg of IgG and 12.5 mg of albumin

DNA Digest

Figure 37 shows the separation of øX174 RF DNA-Hae III digest using a TSKgel G4000SWXL column.

Figure 37: Separation of ϕX174 RF DNA-Hae III digest (4.5 µg/50 µL)

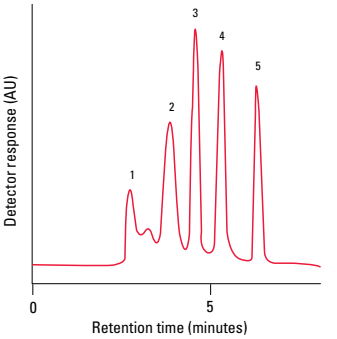


Column:	**TSKgel G4000SWXL, 8 µm, 7.8 mm ID × 30 cm**
Mobile phase:	0.05 mol/L phosphate buffer, pH 7.0 + 0.3 mol/L NaCl + 1 mmol/L EDTA
Flow rate:	0.15 mL/min
Detection:	UV @ 260 nm
Temperature:	25 °C

Reduced Analysis Times

For preliminary research or reducing quality control testing time, the 15 cm long TSKgel QC-PAK columns provide analysis times half as long as those on standard 30 cm columns, while retaining baseline resolution of protein mixtures (Figure 38).

Figure 38: Analysis of various proteins



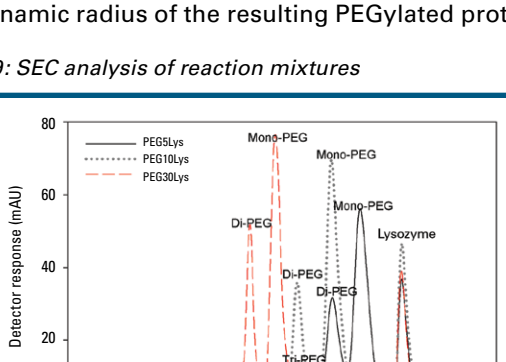
Column:	**TSKgel QC-PAK GFC 300, 5 µm, 8 mm ID × 15 cm**
Mobile phase:	0.1 mol/L Na_2SO_4 in 0.1 mol/L phosphate buffer, pH 7.0 and 0.05% NaN_3
Flow rate:	1.0 mL/min
Detection:	UV @ 220 nm
Samples:	1. thyroglobulin 2. IgG 3. ovalbumin 4. ribonuclease 5. p-aminobenzoic acid

Characterization Studies of PEGylated Lysozyme

Chemical modification of therapeutic proteins in order to enhance their biological activity is of increasing interest. One of the most frequently used protein modification methods, PEGylation, changes the biochemical and physicochemical properties of the protein, which can result in several important benefits, among them more effective target delivery, slower in vivo clearance, and reduced toxicity and immunogenicity of therapeutic proteins. After PEGylation reaction the mixture has to be purified in order to remove non-reacted protein and undesired reaction products.

A TSKgel G3000SWXL column was used for the characterization of PEGylated lysozyme, as shown in Figure 39. A random PEGylation of lysozyme using methoxy PEG aldehyde of sizes 5 kDa, 10 kDa and 30 kDa was performed. The retention volumes of PEGylated lysozymes were used to assign the peaks based on a standard calibration curve. As a result of PEGylation, a large increase in the size of lysozyme by size exclusion chromatography was observed. The SEC elution position of lysozyme modified with a 30 kDa PEG was equivalent to that of a 450 kDa globular protein. There was a linear correlation between the theoretical molar mass of PEGylated protein and the molar mass calculated from SEC. This result illustrates the strong effect that PEG has on the hydrodynamic radius of the resulting PEGylated protein.

Figure 39: SEC analysis of reaction mixtures

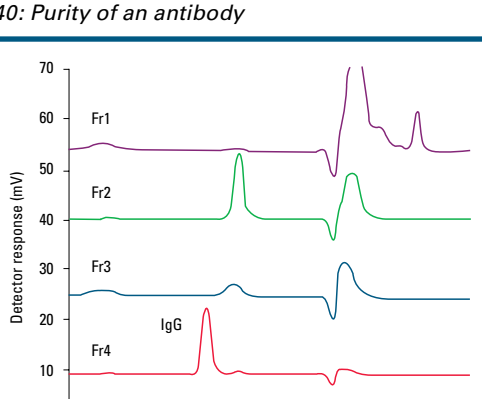


Column:	**TSKgel G3000SWXL, 5 µm, 7.8 mm ID × 30 cm**
Mobile phase:	0.1 mol/L phosphate buffer, 0.1 mol/L Na_2SO_4, pH 6.7
Flow rate:	1.0 mL/min
Detection:	UV @ 280 nm
Injection vol.:	20 µL
Sample:	5, 10, 30 kDa methoxy PEG aldehyde

Purity of an Antibody

When the analysis of proteins needs to be performed in a metal free environment, the TSKgel BioAssist columns can be used. These columns offer TSKgel SWXL packings in PEEK housings featuring the same performance as with stainless steel columns. Figure 40 demonstrates the purity of an antibody from a cell culture supernatant (Anti TSH). The chromatograms represent the fractions collected from a HIC purification step.

Figure 40: Purity of an antibody



Column:	**TSKgel BioAssist G3SWXL, 5 µm, 7.8 mm ID × 30 cm**
Mobile phase:	0.3 mol/L phosphate buffer, pH 7.0
Flow rate:	1.0 mL/min
Injection vol.:	50 µL

About: TSKgel SuperSW Size Exclusion Columns

TSKgel SuperSW columns, introduced in 1997, contain smaller particles than TSKgel SWXL columns; 4 µm versus 5 µm. In addition, the column internal diameter has been reduced from 7.8 mm ID to 4.6 mm ID to provide higher sensitivity in sample-limited cases and to cut down on solvent use.

It is important to employ an HPLC system that is optimized with regards to extra-column band broadening to take full advantage of the high column efficiency that can be obtained on TSKgel SuperSW columns. See Table 9 for recommendations on minimizing the dead volume in the HPLC system.

The following two columns are available within the TSKgel SuperSW column line:

- TSKgel SuperSW2000
- TSKgel SuperSW3000

Attributes and Applications

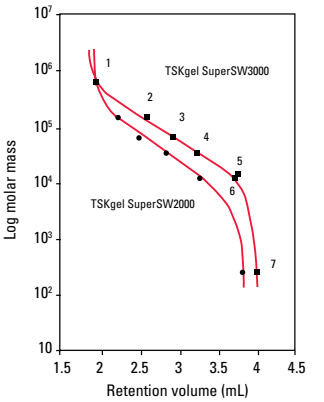
Table 8 shows a summary of the product attributes for each of the TSKgel SuperSW columns. The 12.5 nm pore size of the TSKgel SuperSW2000 columns results in a fractionation range up to 1.5×10^5 Da for globular proteins. The TSKgel SuperSW3000 columns have a fractionation range up to 5.0×10^5 Da for globular proteins due to its 25 nm pore size. Since both columns have a 4.6 mm inner diameter, they are ideal for sample-limited applications. Figure 41A and 41B show the calibration curves for protein, polyethylene oxides and glycols for the TSKgel SuperSW columns.

Table 8: Product attributes

TSKgel column	SuperSW2000	SuperSW3000
Base material	Silica	
Particle size (mean)	4 µm	4 µm
Pore size (mean)	12.5 nm	25 nm
Functional group	Diol	
pH stability	2.5-7.5	
Calibration range	5,000 - 1.5×10^5 Da (globular proteins)	1.0×10^4 - 5.0×10^5 Da (globular proteins)

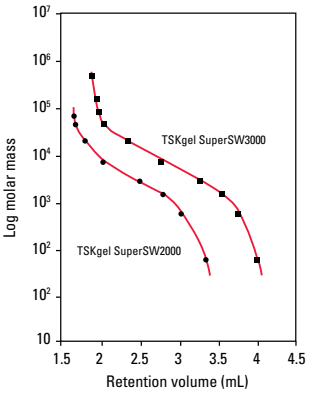
Figure 41A and 41B: Calibration curves for proteins and polyethylene oxides and glycols for TSKgel SuperSW columns

A



Column: **TSKgel SuperSW columns, 4.6 mm ID × 30 cm**
Mobile phase: 0.2 mol/L phosphate buffer, pH 6.7
Flow rate: 0.35 mL/min
Detection: UV @ 280 nm
Samples: standard proteins (5 µL, 0.1 g/L each)
1. thyroglobulin
2. γ-globulin
3. bovine serum albumin
4. β-lactoglobulin
5. lysozyme
6. cytochrome C
7. glycine tetramer

B



Column: **TSKgel SuperSW columns, 4.6 mm ID × 30 cm**
Mobile phase: 0.05% sodium azide aqueous solution
Flow rate: 0.35 mL/min
Detection: RI
Temperature: 25 °C
Samples: polyethylene oxides (PEO) standards
polyethylene glycols (PEG) standards, (5 µL)

Table 9: Operating instructions when using TSKgel SuperSW columns

In general:

- Suppress peak broadening in connecting tubing between injector, guard column, analytical column, and detector.
- Prevent the sample volume from causing extra-column band broadening due to volume overloading. You can test this by injecting half the sample volume and measuring peak efficiency.

Tubing:

- Use 0.004" or 0.005" ID (0.100 mm or 0.125 mm) tubing, when available, and as short a length as is practical.
- Sections requiring 0.004" or 0.005" ID tubing
  - o Between injection valve and guard column, and between guard column outlet and column
  - o Between column outlet and detector inlet

Pumping system:

- The pump(s) should work well at low flow rates as the recommended flow rate range is 0.1-0.35 mL/min.

Injector:

- A low dispersion injector (such as Rheodyne 8125) is recommended.

Guard column:

- We recommend that you install a guard column (part no. 18762) to protect your TSKgel SuperSW column.

Detector:

- When working with a UV detector, install a micro flow cell or a low dead volume-type cell. Low dead volume-type cells are effective in high-sensitivity analysis. (Use of a standard cell is also possible. However, theoretical plates will be approximately 80% of those obtained with a micro flow cell.)

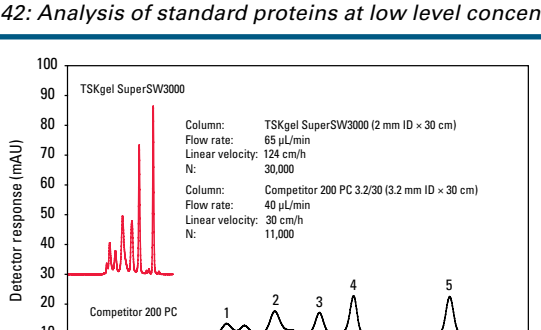
Sample:

- Sample injection volume should be 1-10 µL. Sample load should be 100 µg or smaller.

Trace Levels of Proteins

Figure 42 shows a comparative separation of several standard proteins at low level concentrations on a 2 mm ID TSKgel SuperSW3000 column and on a competitive GFC column. As the results reveal, the TSKgel SuperSW3000 column is an excellent choice for the rapid analysis of proteins at trace levels, showing improved peak shape and superior resolution.

Figure 42: Analysis of standard proteins at low level concentrations



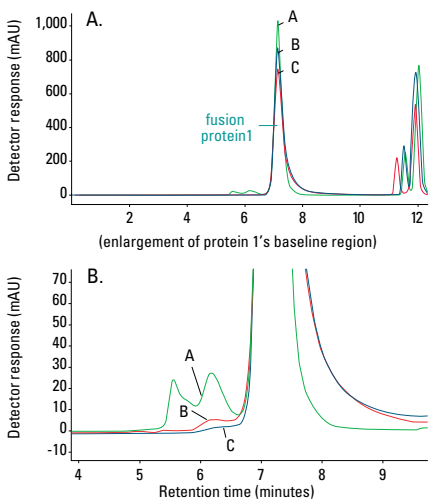
Columns:	**TSKgel SuperSW3000, 4 µm, 2 mm ID × 30 cm** Competitor 200 PC 3.2/30, 13 µm, 3.2 mm ID × 30 cm
Mobile phase:	0.1 mol/L phosphate buffer + 0.1 mol/L Na_2SO_4 + 0.05% NaN_3, pH 6.7
Detection:	UV @ 280 nm
Temperature:	25 °C
Injection vol.:	0.2 µL
Samples:	1. thyroglobulin (1.0 g/L) 2. β-globulin (2.0 g/L) 3. ovalbumin (2.0 g/L) 4. ribonuclease A (3.0 g/L) 5. p-aminobenzoic acid (0.02 g/L)

Antibody-Based Fusion Protein and Aggregates

During method development, many variables are examined to ensure method robustness. Factors such as elution profile, peak shape, and recovery are required to be consistent by GMP/GLP protocols. During a method re-qualification at Lexigen Pharmaceuticals, several variables were investigated to eliminate non-specific binding and increase the robustness of an established antibody separation method using a TSKgel SuperSW3000 column.

As shown in Figure 43A, excessive peak tailing of "fusion protein 1" is evident with the use of 0.2 mol/L NaCl (chromatogram c in the figure). Additionally, the expected protein dimer and trimer aggregates are not visible in the chromatogram. By switching from 0.2 mol/L sodium chloride to 0.2 mol/L of the more chaotropic sodium perchlorate salt, together with a two-fold reduction in the buffer concentration, less peak tailing and distinct peaks for the dimer and trimer species of mAb1 resulted (chromatogram b in the figure). Doubling the perchlorate concentration to 0.4 mol/L provided further improvement in the peak shape of fusion protein 1 and associated aggregate species (chromatogram a in the figure). Figure 43B is an enlargement of fusion protein 1's baseline region, showing an improved peak shape of the dimer and trimer aggregates with the use of 0.4 mol/L $NaClO_4$.

Figure 43A and 43B: Overlays of monoclonal antibody separation

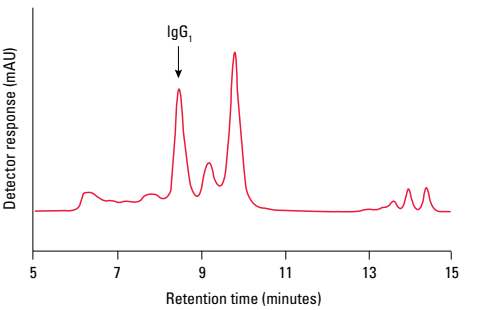


Column:	**TSKgel SuperSW3000, 4 µm, 4.6 mm ID × 30 cm**
Mobile phase:	A: 0.4 mol/L $NaClO_4$, 0.05 mol/L NaH_2PO_4, B: 0.2 mol/L $NaClO_4$, 0.05 mol/L NaH_2PO_4, C: 0.2 mol/L NaCl, 0.1 mol/L NaH_2PO_4
Flow rate:	0.35 mL/min
Detection:	UV @ 214 nm
Injection vol.:	5 µL
Samples:	monoclonal antibodies

IgG_1

The TSKgel Super SW3000 provides an excellent high resolution separation of IgG_1 from mouse ascites fluid as can be seen in Figure 44.

Figure 44: Separation of monoclonal antibody

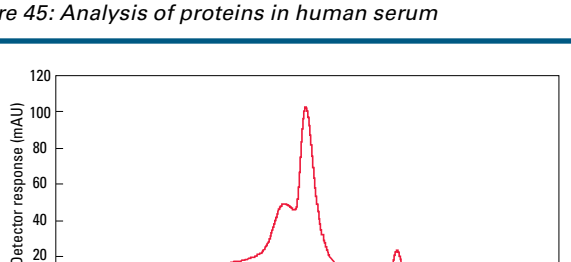


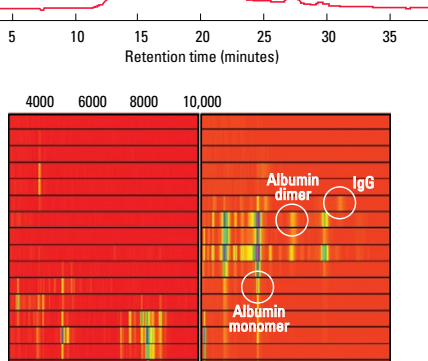
Column:	**TSKgel SuperSW3000, 4 µm, 4.6 mm ID × 30 cm**
Mobile phase:	0.2 mol/L phosphate buffer, pH 6.7
Flow rate:	0.35 mL/min
Detection:	UV @ 280 nm, micro flow cell
Sample:	mouse ascites (5 µL)

Human Serum Proteins

A 1 mm ID TSKgel SuperSW3000 column was used to analyze proteins in human serum. A fraction of interest was then analyzed by off-line SELDI/TOF/MS to establish the presence of BSA aggregates and IgG. Figure 45 demonstrates the applicability of TSKgel SuperSW3000 columns for the trace analysis of biological components by LC/MS analysis.

Figure 45: Analysis of proteins in human serum





Fraction of interest analyzed by off-line SELDI/TOF/MS to establish presence of BSA aggregates and IgG.

Column:	**TSKgel SuperSW3000, 4 µm, 1 mm ID × 30 cm**
Mobile phase:	50 mmol/L NaH_2PO_4 + 0.5 mol/L NaCl, pH 7.0
Flow rate:	8 µL/min
Detection:	UV @ 280 nm
Temperature:	ambient
Sample:	serum (x 10), 1 µL

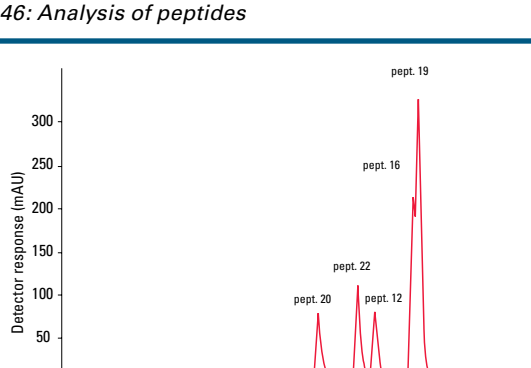
Fraction (1 mL) was directly loaded to SELDI chip H50.
The chip was washed and desalted then applied to MS.

This data is courtesy of Dr. Majima, Protenova.

Peptide Mixture

Figure 46 demonstrates that very small molecules can be separated efficiently on a TSKgel SuperSW2000 column under non-SEC conditions. Although the peptides 16 and 19 do not elute according to their molar mass, a separation was possible with only one amino acid difference (based on different interaction with the gel surface).

Figure 46: Analysis of peptides



Column:	**TSKgel SuperSW2000, 4 µm, 4.6 mm ID × 30 cm**
Mobile phase:	0.1% TFA in 45% aq. ACN
Flow rate:	0.35 mL/min
Detection:	UV @ 210 nm
Injection vol:	3 µL
Samples:	Peptide P12: Tyr-Gly-Gly-Phe-Met-Arg-Gly-Leu Peptide P16: Trp-Gly-Gly-Tyr Peptide P19: Gly-Trp-Gly Peptide P20: H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val- Gly-Lys-Gly-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro-OH Peptide P22: H-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys-OH

About: TSKgel BioAssist DS Size Exclusion Columns

TSKgel BioAssist DS columns are designed for the desalting and buffer exchange of proteins and polynucleotides at analytical and semi-preparative scale. Packed with 15 µm polyacrylamide beads in PEEK hardware, TSKgel BioAssist DS columns show excellent desalting performance.

The novel* hydrophilic highly cross-linked polyacrylamide beads exhibit superior mechanical strength compared with conventional hydrophilic polyacrylamide beads and cross-linked dextran beads. This increase in strength is what allows the use of the small spherical 15 µm beads.

*US patent number 7,659,348

Attributes and Applications

Table 10 summarizes the product attributes of the TSKgel BioAssist DS columns. TSKgel BioAssist DS columns can be operated in standard HPLC systems to quickly and efficiently reduce salt and/or buffer concentrations of collected protein or nucleic acid fractions.

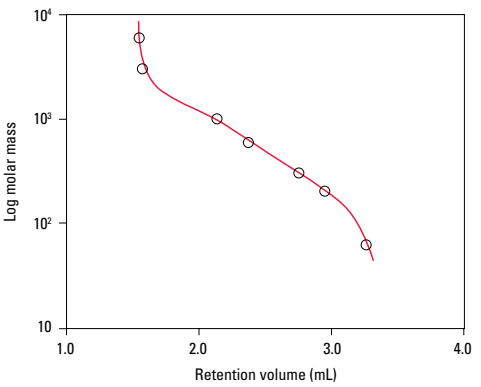
Table 10: Product attributes

Attribute	Value
Base material	urea cross-linked polyacrylamide
Particle size	15 µm
Pore size	excludes 2,500 Da PEG
Particle porosity	ca. 60%
Mechanical strength	<4 MPa

Calibration Curve

Figure 47 shows the calibration curve of a 6 mm ID × 15 cm TSKgel BioAssist DS column using polyethylene glycol standards and a water mobile phase. As is desirable in SEC, the pore volume of BioAssist DS columns is larger than the volume in between the particles. The molar mass cut-off (exclusion limit) for PEGs is about 2,500 Da. Results similar to those shown in Figure 47 can be obtained on the commercially available 4.6 mm ID × 15 cm and 10 mm ID × 15 cm TSKgel BioAssist DS columns.

Figure 47: Calibration curve of TSKgel BioAssist DS desalting columns



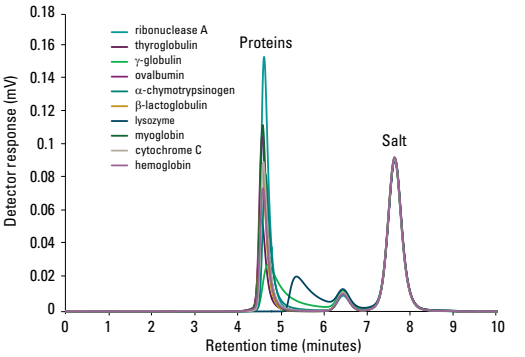
Column: **Custom TSKgel BioAssist DS column, 6 mm ID × 15 cm**
Mobile phase: distilled H_2O
Flow rate: 0.5 mL/min
Sample: ethylene glycol, PEGs

Desalting of Large Protein Sample Loads

Figure 48 demonstrates the rapid and reproducible desalting of a large number of proteins (see Table 11) at semi-preparative scale using a TSKgel BioAssist DS, 10 mm ID × 15 cm column. In this application, the salt concentration of the proteins was reduced 10-fold from 0.1 to 0.01 mol/L. The reproducibility of the separation was determined by measuring the plate number of the ribonuclease A peak for four injections of various sample loads. The % RSD value (n=4) was less than 5% for a 1.5 mg injection. At this load, the resolution between ribonuclease A and the salt peak was larger than 6. At 1.95 mg load of ribonuclease A, the resolution between the protein and salt peak was 4.3. Note that the analysis is complete within 10 minutes.

In a similar study performed on a 4.6 mm ID × 15 cm TSKgel BioAssist DS column, the resolution for a 1.95 mg load of ribonuclease A was larger than 2 at the high flow rate of 0.8 mL/min.

Figure 48: Desalting of proteins



Column: **TSKgel BioAssist DS, 15 µm, 10 mm ID × 15 cm**
Mobile phase: 0.1 mol/L KH_2PO_4/K_2HPO_4, pH 6.7, 0.1 mol/L Na_2SO_4 + 0.005% NaN_3
Proteins in 0.1 mol/L phosphate buffer, pH 6.7
Flow rate: 0.8 mL/min (4.6 mm ID) and 1.0 mL/min (10 mm ID)
Detection: UV @ 80 nm and RI
Temperature: 25 °C
Injection vol.: 10 µL

Table 11: Proteins

Protein	*MM* (kDa)	Concentration* (g/L approx.)
ribonuclease A	14.7	19.5
thyroglobulin	670	11.3
γ-globulin	150	14.5
ovalbumin	45	13.1
α-chymotrypsinogen	25.6	13.1
β-lactoglobulin	18.4	10.8
lysozyme	14.7	11.6
myoglobin	16.7	14.5
cytochrome C	12.3	11.0
hemoglobin	68	11.9

*in 100 mmol/L phosphate buffer, pH 6.7