Appendix

Tosoh Corporation closely monitors all stages of the manufacturing process for chromatographic media that are used to pack TSKgel columns. Packing materials are produced in large batches which must pass stringent quality control specifications for particle size distribution, pore size distribution, pore volume, and surface area. After producing the particles, each lot is then used to prepare multiple batches of bonded phase by attaching the appropriate ligand. Each bonded lot is again tested to ensure that it meets the specifications for parameters such as ligand density, retention, selectivity, etc.

TSKgel columns are designed for general purpose HPLC or FPLC applications. They are not guaranteed to work for specific customer applications. Suitability of a column has to be determined by the end user. Good Laboratory Practice (GLP) demands that a rugged method must be developed by testing at least three different gel lots to enable consistency in retention time and selectivity irrespective of the use of different lots of the same column type.

Tosoh Bioscience recommends that shipments are inspected for the presence of the Inspection Data Sheet, Operating Conditions and Specifications (OCS) Sheet, and column appearance. After review of the shipping contents, the column should be tested within 30 days according to the conditions listed in the Inspection Data Sheet to confirm that the column meets the specifications listed in the OCS Sheet.

Guard Product Options

GLP procedures often specify that the separation column be protected by a guard column. The guard column is installed between the injector and the analytical column. It is designed to protect the analytical column from unwanted materials, such as highly retained or irreversibly adsorbed compounds and particulate matter. Tosoh Bioscience supplies an assortment of packed guard columns, guardgel kits, guard cartridges, and guardfilters.

Guardgel kits contain the hardware and the gel packing material to fill a guard column using an aspirator. Detailed instructions on how to pack a stainless steel and a glass guardgel column are available from the Tosoh Bioscience website in the Literature section, under the heading “Instruction Manuals”. The manual is entitled “Instruction Manual -- Guardgel Kits for TSKgel HPLC Columns” (IM04). In addition, step-by-step instructions are available on the Tosoh Bioscience YouTube channel (www.youtube.com/tosohbioscience). Figure 1 is an example of a guardgel kit, in this case for a TSKgel DEAE-5PW column.

Guard cartridges (Figure 2) are pre-packed, small replaceable columns easily inserted into a hand-tight guard cartridge holder (Figure 3).

Guardfilters (Figure 4) are pre-packed, small replaceable columns easily inserted into a hand-tight guardfilter holder (Figure 5).

For those columns where a guard product is not available, Tosoh Bioscience recommends the use of an in-line filter with a 0.5 µm cutoff to avoid frequent plugging of the 1.0 µm pores in the column frit of TSKgel ODS-140HTP, Super-ODS, Super-Octyl, and Super-Phenyl columns. A pre-injector membrane filter is also recommended to prevent particles generated by pump seal wear from reaching the column.

Troubleshooting Column Problems

Listed below are the five most common causes of poor column performance and the precautions that must be taken to prevent these problems:

1. Void or dead space at the column inlet or channeling of the packing

   Sudden pressure surges and higher than recommended flow rates can compress the column packing, which can result in a void or a channel, especially with large pore size columns such as TSKgel G4000SW and TSKgel G4000SW XL. We recommend using an injector that ensures continuous flow onto the column during injection, i.e., no pressure pulse due to interrupted flow, and installation of a pulse dampener to suppress the sudden pressure surges encountered with quick-return pumps.
Bulk packing material is available to refill voids in some of the analytical and semi-preparative columns. We highly recommend the use of a guard column to protect your analytical column from pressure surges and to prevent irreversibly binding impurities from reaching the analytical column. The pH of the sample (if different from the mobile phase pH) will be adjusted to the pH of the mobile phase before entering into the analytical column provided a guard column is used. This is particularly important in the silica-based SW columns because this silica-type is not stable at a pH higher than 7.5.

2. **Air in Column**
The column should be tightly capped when not in use to prevent air from entering it. Air can be removed by sparging with helium, mobile phase filtration or other degassing procedures. This is particularly important for polymer-based columns. If air does enter the column, follow the rehydration procedure described later in this appendix.

3. **Column contamination or incomplete sample recovery**
   Cleaning conditions for all column types are provided on the Tosoh Bioscience website in the resource section of the technical support channel. Cleaning solvents are discussed in the cleaning section below.

4. **Frit plugging and high pressure**
   Solvents and samples should be filtered through at least a 0.45 µm filter to prevent clogging the column frits. If the frit becomes partially plugged, the result may be split peaks or high pressure. The entire end-fitting can be removed and sonicated in 6 mol/L nitric acid. Rinse the end-fitting thoroughly after cleaning. (Be careful not to disturb the packing.) Alternatively, this end-fitting can be replaced. Installing a membrane filter prior to the injector is recommended to prevent particles created by pump seal wear from reaching the analytical column. Consult the Replacement Parts chapter within this Product Guide for these and other hardware products.

5. **Peak splitting**
   Column overload, whether in volume or concentration, can cause peak splitting and poor resolution. The appropriate concentration and volume of analyte will vary on the loading capacity of the column.

### Cleaning Solutions

**Size Exclusion:** TSKgel SW and SWxL columns
1. Turn the column in reverse flow direction and run at half the maximum flow rate.
2. Clean with 5 column volumes (CV) of 1 mol/L NaCl, pH 7.0
3. Clean with 5 CV of ultra-pure water.
4. Clean with 5 CV of 20% acetonitrile.
5. Clean with 5 CV of ultra-pure water.
6. Turn column in normal flow direction and equilibrate in mobile phase for at least 45 minutes.

**Size Exclusion:** TSKgel PW and PWxL columns
1. Turn the column in reverse flow direction and run at half the maximum flow rate.
2. Clean with 5 column volumes (CV) of 1 mol/L NaCl, pH 7.0
3. Clean with 5 CV of ultra-pure water.
4. Clean with 5 CV of 20% acetonitrile.
5. Clean with 5 CV of ultra-pure water.
6. Turn column in normal flow direction and equilibrate in mobile phase for at least 45 minutes.

**Ion Exchange, TSKgel SW-type columns**
1. High concentration salt (e.g. 0.5 mol/L - 1.0 mol/L Na2SO4) in aqueous buffer
2. Buffered solutions at low pH (e.g. 2 - 3)
3. Water soluble organic (MeOH, ACN, EtOH, 10% - 20%) in aqueous buffer
4. Urea* (8 mol/L) or non-ionic surfactant in buffer solution. *Note: caution for chaotropic agents.

### Cleaning

Columns should be cleaned at regular intervals. The frequency depends on the purity of the samples. Occasionally, samples are run which adsorb onto the packing material. If one of the performance characteristics (asymmetry factor, retention time, theoretical plates, or resolution) changes by 10% or more, it is prudent to clean the column.

An Inspection Data Sheet and an Operating Conditions and Specifications (OCS) Sheet accompanies all TSKgel columns. The Data Inspection Sheet identifies the testing method that was used to verify the column's performance. The column's specifications are listed on the OCS Sheet. However, a well resolved sample component could be used to monitor the column. Establish that the column is performing properly using the standard test probes listed on the Inspection Data Sheet. Calculate the asymmetry factor, theoretical plates and resolution of one or more of the sample components. Note the retention time. This becomes the baseline test mix which provides a basis for comparison.

**Basic rules for cleaning TSKgel columns - all types**
1. Clean the column in the reverse flow direction.
2. During cleaning, do not connect the column to the detector.
3. Run the column at half the maximum flow rate making sure to monitor the pressure.
4. If cleaning with a high or low pH solution, make certain that the rest of the chromatographic system (pump, pump seals, injector, etc.) is compatible.
5. Use at least 5 column volumes (CV) of each cleaning solution and rinse with 5 CV of ultra-pure water between each cleaning step.
6. Equilibrate with 5 CV of the mobile phase for the method.

Each type of TSKgel column has a recommended set of cleaning solutions specific to the column, as described below and on the Tosoh Bioscience website in the resource section of the technical support channel. Choose a cleaning solution based upon the column and sample type. In general low pH salt solution will remove basic proteins, and organics will remove hydrophobic proteins. Chaotropic agents will remove strongly adsorbed materials (e.g. hydrogen bonded).

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Call customer service: 866-527-3587, technical service: 800-366-4875
Ion Exchange, TSKgel PW-type columns
1. Inject up to 1 CV in 250 µL increments of 0.1 mol/L - 0.2 mol/L NaOH on analytical columns. Inject proportionally larger volumes on semi-preparative columns.
2. Inject up to 1 CV in 250 µL increments of 20% - 40% aqueous acetic acid on analytical columns. (Since acid can precipitate protein it should be used after other cleaning methods.) Inject proportionally larger volumes on semi-preparative columns.
3. Water soluble organic (MeOH, ACN, EtOH, 10% - 20%) in aqueous buffer
4. Urea* (8 mol/L) or non-ionic surfactant in buffer solution. *Note: caution for chaotropic agents.

Note: Rinse ion exchange columns with 5 CV of the appropriate solution to restore the correct counter-ion before equilibrating with loading buffer.

Hydrophobic Interaction columns
1. Inject up to 1 CV in 250 µL increments of 0.1 mol/L - 0.2 mol/L NaOH on analytical columns. Inject proportionally larger volumes on semi-preparative columns.
2. Inject up to 1 CV in 250 µL increments of 20% - 40% aqueous acetic acid on analytical columns. (Since acid can precipitate protein it should be used after other cleaning methods.) Inject proportionally larger volumes on semi-preparative columns.

Reversed Phase, silica-based columns
1. 100% acetonitrile or methanol
2. Gradient from 10%- 100% acetonitrile in 0.05% trifluoroacetic acid

Reversed Phase, polymer-based columns
1. 100% acetonitrile or methanol
2. Inject up to 1 CV in 250 µL increments of 0.1 mol/L - 0.2 mol/L NaOH on analytical columns. Inject proportionally larger volumes on semi-preparative columns.
3. Inject up to 1 CV in 250 µL increments of 20% - 40% aqueous acetic acid on analytical columns. (Since acid can precipitate protein it should be used after other cleaning methods.) Inject proportionally larger volumes on semi-preparative columns.

HILIC columns
1. Water
2. 45% acetonitrile or acetone
3. 0.1% triethylamine in at least 75% acetonitrile
4. 50 mmol/L phosphate buffer, pH 6.0, in 50% acetonitrile

Affinity columns
Consult the OCS Sheet of the specific column type for cleaning directions.

Rehydration
Dehydration of TSKgel liquid chromatography columns can occur during long term storage or from improper use. Dehydration can also occur if the plugs are not tightened or if air inadvertently is pumped into the column during use. It is easier to detect dehydration in glass columns because the dry packing will appear to pull away from the column walls. This condition can be remedied by using the following procedure:

1. Connect the column to your LC system in the reverse flow direction.
2. Do not connect the column to the detector.
3. Pump a filtered mobile phase of 20% methanol in ultrapure water over the column at half of the recommended maximum flow rate. Note: reversed phase columns require 60% methanol.
4. Continue this procedure until the column has been rehydrated. Rehydration can take several hours, depending on the column size.
5. Connect the column to the LC system in the proper flow direction.
6. Rinse with 3 column volumes (CV) of ultrapure water to remove the organic if it is not part of the normal mobile phase.
7. Equilibrate with loading buffer (usually 3-5 CV).
8. Perform the recommended QC tests to ensure that the column is performing properly.

Column Storage
When the column will be used the next day, allow it to run overnight at a low flow rate in a buffer that does not contain a halide salt. When the column will not be used for more than a day, clean it first, then flush salt from the column and store in 0.05% sodium azide or 20% ethanol. Seal tightly to prevent the column from drying out.

Beware of extra-column band broadening
It is well known that when the column diameter decreases, peak volumes decrease by the square of the ratio of column diameter. In contrast, a decrease in column length results in a proportional decrease in peak volume. Thus, when changing column dimensions from 7.8 mm ID × 30 cm to 6 mm ID × 15 cm, this results in a reduction of peak volume by a factor of (7.8/6)²(30/15) = 3.4. Similarly, the reductions in peak volume are 5.8 when going from 7.8 mm ID × 30 cm to 4.6 mm ID × 15 cm, and 21.1 when replacing a 4.6 mm ID × 30 cm column by one that is 1 mm ID × 30 cm. Such large reductions in peak volume require that the HPLC system is optimized with respect to external factors that contribute to the sample band broadening that takes place inside the column.

Main contributors to extra-column band broadening are capillary tubing that connect the column to the injector and the detector, injection volume, detector cell volume, detector time constant, and others. Neglecting to optimize the HPLC system can seriously detract from the true column efficiency, which ultimately can result in unacceptable analysis results.
United States Pharmacopeia (USP) specifications and corresponding Tosoh Bioscience columns

<table>
<thead>
<tr>
<th>USP number</th>
<th>USP Description</th>
<th>Recommended columns</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1</td>
<td>Octadecyl silane chemically bonded to porous or non-porous silica or ceramic micro-particles, 1.5 to 10 µm in diameter, or a monolithic rod.</td>
<td>TSKgel ODS-100V, ODS-100Z, Super-ODS, ODS-80T, ODS-80T5, ODS-120U, ODS-120T, ODS-140HTP, OligoDNA-RP, ODS-100S</td>
</tr>
<tr>
<td>L3</td>
<td>Porous silica particles, 1.5 to 10 µm in diameter, or a monolithic silica rod.</td>
<td>TSKgel Silica-60</td>
</tr>
<tr>
<td>L7</td>
<td>Octylsilane chemically bonded to totally or superficially porous silica particles, 1.5 to 10 µm in diameter, or a monolithic silica rod.</td>
<td>TSKgel Super-Octyl, Octyl-80T5</td>
</tr>
<tr>
<td>L8</td>
<td>An essentially monomolecular layer of aminopropylsilane chemically bonded to totally porous silica gel support, 1.5 to 10 µm in diameter.</td>
<td>TSKgel NH₂-100, TSKgel NH₂-100 DC</td>
</tr>
<tr>
<td>L9</td>
<td>Irregular or spherical, totally porous silica gel having a chemically bonded, strongly acidic cation exchange coating, 3 to 10 µm in diameter.</td>
<td>TSKgel SP-2SW</td>
</tr>
<tr>
<td>L10</td>
<td>Nitrile groups chemically bonded to porous silica particles, 2 to 10 µm in diameter.</td>
<td>TSKgel CN-80T5</td>
</tr>
<tr>
<td>L11</td>
<td>Phenyl groups chemically bonded to porous silica particles, 1.5 to 10 µm in diameter.</td>
<td>TSKgel Super-Phenyl</td>
</tr>
<tr>
<td>L13</td>
<td>Trimethylsilane chemically bonded to porous silica particles, 3 to 10 µm in diameter.</td>
<td>TSKgel TMS-250</td>
</tr>
<tr>
<td>L14</td>
<td>Silica gel having a chemically bonded, strongly basic quaternary ammonium anion exchange coating, 5 to 10 µm in diameter.</td>
<td>TSKgel QAE-2SW</td>
</tr>
<tr>
<td>L20</td>
<td>Di(hydroxypropano) groups chemically bonded to porous silica or hybrid particles, 1.5 to 10 µm in diameter.</td>
<td>TSKgel QC-PAK GFC, SWXL, SW, SuperSW, and SW mAb series</td>
</tr>
<tr>
<td>L21</td>
<td>A rigid, spherical styrene-divinylbenzene copolymer, 3 to 30 µm in diameter.</td>
<td>TSKgel HXl, HMa, SuperH, SuperHZ, and SuperMultiporeHZ series</td>
</tr>
<tr>
<td>L22</td>
<td>A cation exchange resin made of porous polystyrene gel with sulfonic acid groups, about 10 µm in size.</td>
<td>TSKgel SCX</td>
</tr>
<tr>
<td>L23</td>
<td>An anion exchange resin made of porous polymethacrylate or polyacrylate gel with quaternary ammonium groups, about 10 µm in size.</td>
<td>TSKgel SuperQ-5PW, BioAssist Q, Q-STAT, DNA-STAT, IC-Anion-PW</td>
</tr>
<tr>
<td>L25</td>
<td>Packing having the capacity to separate compounds with a molecular weight range from 100-5000 Da (as determined by polyethylene oxide), applied to neutral, anionic, and cationic water-soluble polymers.</td>
<td>TSKgel G1000PW, G2000PW, G2500PW, G2500PWXL</td>
</tr>
<tr>
<td>L26</td>
<td>Butyl silane chemically bonded to totally porous silica particles, 1.5 to 10 µm in diameter.</td>
<td>TSKgel Protein C-300</td>
</tr>
<tr>
<td>L33</td>
<td>Packing having the capacity to separate dextrins by molecular size over a range of 4000 to 5.0 \times 10^8 Da. It is spherical, silica-based, and processed to provide pH stability.</td>
<td>TSKgel SuperSW, SWXL, QC-PAK GFC, SW, and SW mAb series</td>
</tr>
<tr>
<td>L37</td>
<td>Packing having the capacity to separate proteins by molecular size over a range of 2000 to 4.0 \times 10^8 Da. It is a polymethylacrylate gel. TSKgel G3000PWXL, G3000PW, G3000PWXL-CP</td>
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<tr>
<td>L38</td>
<td>A methacrylate-based size exclusion packing for water-soluble samples.</td>
<td>TSKgel PWXL, PW, PWXL-CP, Alpha, SuperAW series, and SuperMultiporePW</td>
</tr>
<tr>
<td>L39</td>
<td>A hydrophilic polyhydroxymethacrylate gel of totally porous spherical resin.</td>
<td>TSKgel PWXL, PW, PWXL-CP, Alpha, and SuperAW, SuperMultipore series</td>
</tr>
<tr>
<td>L52</td>
<td>A strong cation exchange resin made of porous silica with sulfopropyl groups, 5 to 10 µm in diameter.</td>
<td>TSKgel SP-2SW, IC-Cation SW</td>
</tr>
<tr>
<td>L58</td>
<td>Strong cation exchange resin consisting of sulfonated cross-linked styrene-divinylbenzene copolymer in the sodium form, about 6 to 30 µm diameter.</td>
<td>TSKgel SCX (Na⁺)</td>
</tr>
<tr>
<td>L59</td>
<td>Packing for the size exclusion separations of proteins (separation by molecular weight) over the range of 5 to 7,000 kDa. The packing is spherical, 1.5 - 10 µm, silica or hybrid packing with a hydrophilic coating.</td>
<td>TSKgel SuperSW, SWXL, SW, and SW mAb series</td>
</tr>
<tr>
<td>L68</td>
<td>Spherical, porous silica, 10 µm or less in diameter, the surface of which has been covalently modified with alkyl amide groups and not endcapped</td>
<td>TSKgel Amide-80</td>
</tr>
<tr>
<td>L89</td>
<td>Packing having the capacity to separate compounds with a molecular weight range from 100 - 3000 as determined by polyethylene oxide), applied to neutral and anionic water-soluble polymers.</td>
<td>TSKgel G-Oligo-PW, SuperOligoPW</td>
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