Cellufine™ ET clean

Cellufine ET clean is poly(ε-lysine) immobilized cellulose spherical beads. The resin bind and remove endotoxin from your sample solution.

The cationic ligands of the lysine residues of poly(ε-lysine) and the hydrophobic regions of the polymer interact as a mixed mode. (Figure 1） Poly(ε-lysine) is a 20-40 lysine polymer, fermented by *Streptomyces albulus*. Poly(ε-lysine) is produced at JNC. Cellufine ET Clean is stable in washing solutions such as 0.2M NaOH. Table 1 shows the characteristics of Cellufine ET clean.

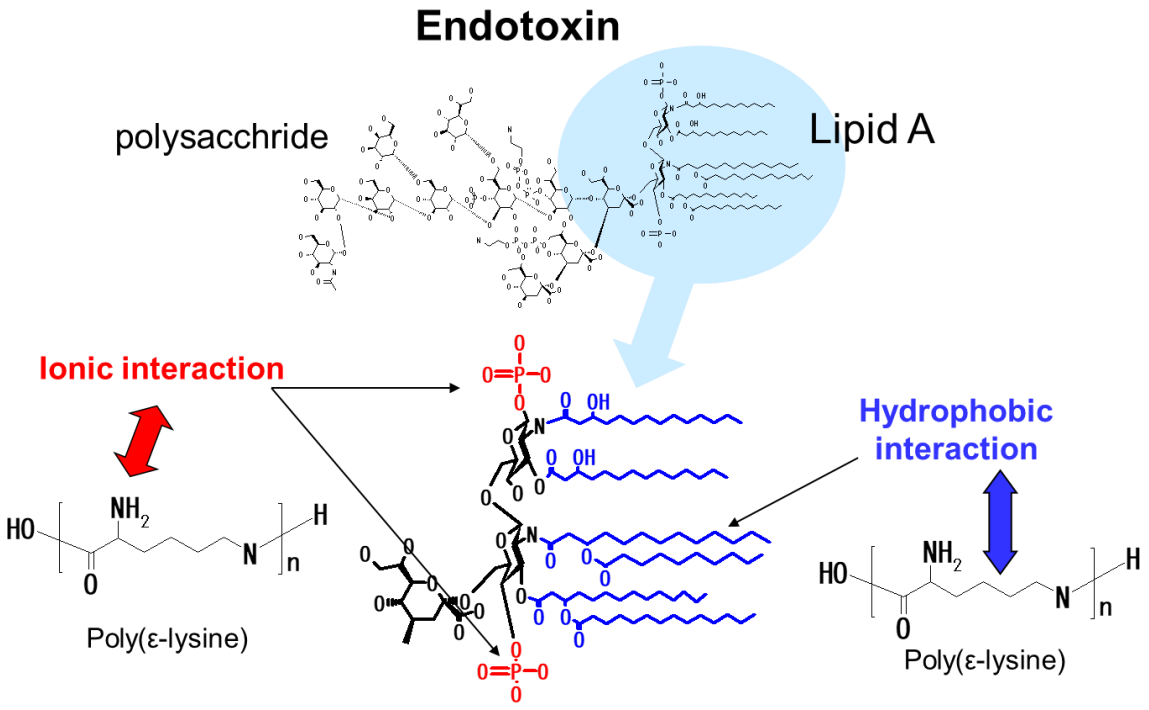
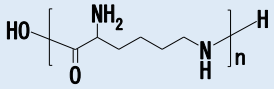


Figure 1　Interaction of endotoxin with poly-ε-lysine

Table 1　Characteristics of Cellufine ET clean

	ET clean　S	ET clean　L
Ligand	 Poly-ε-lysine (n=20-40)　pka7.6	
Matrix	Spherical cellulose particle	
Particle size	40-130 um	
MW exclusion limit	2000	2×10^6
Operating pressure	＜0.30MPa（＜3000 cm/h）	＜0.20MPa（＜900 cm/h）
Recommended CIP solution	0.2mol/l NaOH、0.2mol/l NaOH in 20% EtOH、0.2mol/l NaOH in 95%EtOH	
pH stability range	2 ～ 13	1～13
Storage	2～8 ºC in 20 % ethanol	
Characteristics of Protein Recovery	Almost no entry into pores Low adsorption, high recovery	Possible to enter the pore Low adsorption and high recovery under salt addition conditions
Endotoxin adsorption capacity ※	74×10^4 EU/mL-gel	192×10^4 EU/mL-gel

Values in Table 1 are not specifications.

※The adsorption of LPS was determined using a batchwise method with 0.1mL-gel of ET clean and 4mL of LPS solution.（*E.coli* O111:B4　LPS　100～200μg/mL, pH7.0 0.02M sodium phosphate buffer μ=0.05, Limulus ES-II test *E. coli* UKT-B, 1 endotoxin unit (EU)＝250pg LPS） [1)]

Table2　Endotoxin adsorption and protein recovery from various

Sample solution			ET Clean S		ET Clean L	
Compound	pI	Conc. of endotoxin before treatment (pg/ml)	(0.02M PB,pH7.0,μ=0.05)		(0.02M PB+0.36M NaCl,pH7.0,μ=0.40)	
			Conc. of endotoxin after treatment (pg/ml)	Rec. of protein after treatment (%)	Conc. of endotoxin after treatment (pg/ml)	Rec. of protein after treatment (%)
Ovalbumin	4.6	28,000	81	99	<10	95
BSA	4.9	32,000	45	99	<10	97
Myoglobin	6.8	4,500	18	99	<10	98
γ- Globulin	7.4	5,600	20	99	<10	97
Cytochrome C	10.6	1,500	15	99	<10	98

※The adsorption of LPS was determined using a batchwise method with 0.3mL-gel of ET clean and 2mL of protein solution (1mg/mL) containing natural endotoxin. pH7.0 0.02M sodium phosphate buffer + NaCl, Limulus ES-II test (*E. coli* UKT-B, 1 endotoxin unit (EU)＝250pg LPS） [1)]

Column Packing

Materials

- Cellufine ET clean S or L
- Lab scale column, adapter, reservoir
- Pump
- Filtration equipment (Glass filter, Buchner-Roth, aspirator, etc.)
- Graduated cylinder
- Packing solution (water, NaCl solution※, buffer※)
- Mobile phase of packed column evaluation (water, NaCl solution※, buffer※)
- Sample of packed column evaluation (1-2 %(v/v)acetone or 1M NaCl)

※NaCl solution：low salt concentration solution such as 0.1M NaCl solution
※buffer：Adsorption buffer , etc.

Preparation of slurry

1) After the bottle is warmed to room temperature, shake it several times to equalize the slurry in the bottle.
2) Aspirate through a glass filter and wash 3 times with 5 times the volume of the packing solution. Remove 20% ethanol as a preservative. If necessary, the slurry may be washed by decantation.
3) Transfer the resin to a beaker and add packing solution to make a 50-60% (v/v)slurry. Suspend the resin and remove the air under decompression for 30-40 minutes. Slow stirring with a magnetic stirrer can effectively remove the air.

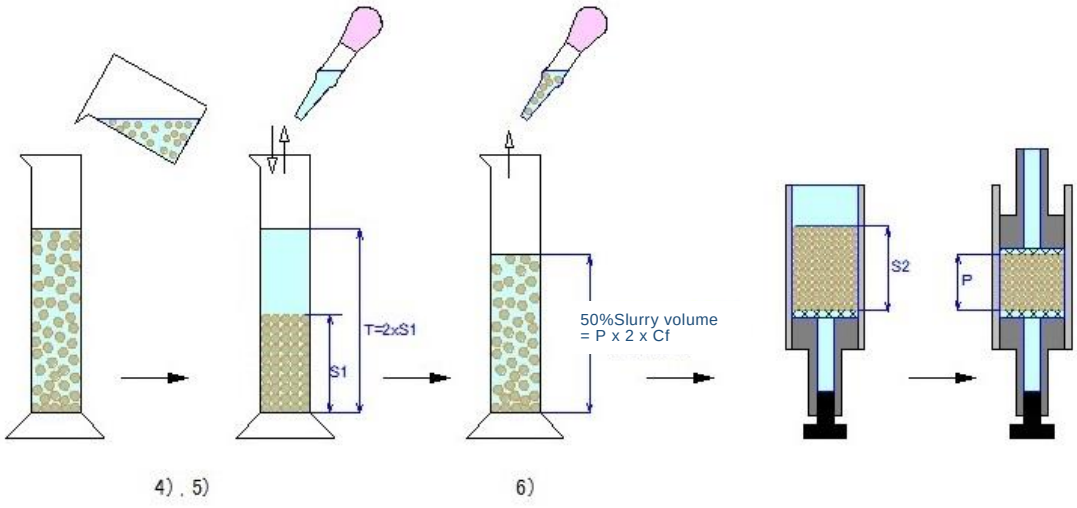


Figure2 Preparation of slurry

4) Pour the slurry into a graduated cylinder and leave it to stand for at least 4 hours. Measure bed height (volume) of a gravity settled bed and calculate the slurry concentration.

Slurry concentration (%) =
Gravity settled bed volume (S1) / Total slurry volume (T)×100

5) Adjust to a 50 % (v/v) slurry concentration of resin with packing solution.
6) Calculate the volume of slurry required to pack the column.

50% slurry volume required to packing = (Target packing volume (P) x 2） x Cf

※Cf = [gravity settled bed volume (S2) / Target packing volume (P)]

Note: *Compression factor (Cf) affects the column packing efficiency (see Appendix 1 for details) and may need to be optimized by adjusting the axial compression on the column. The recommended Cf for Cellufine ET clean is shown below.*

ET clean	Recommended Cf (approximately)
S	1.1
L	1.2

Column packing

1) Set up the column. After opening the column outlet, add packing solution to remove air remaining in the filter. Leave about 1 cm of packing solution from the bottom of the column to prevent air from entering.
2) Close the column outlet and pour the slurry into the column all at once to keep air out.
3) Open the column outlet to allow the resin to settle. As the resin settles, the liquid surface becomes clear. Close the outlet when the packing solution becomes clear to 2-3 cm from the liquid surface.
4) Carefully fill the column to the top with packing solution. Keep the settling resin from floating away.
5) Set the adapter on the column to prevent air from entering the liquid surface. Close the adapter O-ring, lower the adapter, and remove the air inside.

6) Connect the column to the pump and keep the packing solution flowing at a pressure lower than the operating pressure for 30 to 60 minutes. (ET clean S：＜0.3MPa、ET clean L：＜0.2MPa)

Note: *The flow velocity ： Internal pressure at packing ＞ Operating pressure after packing*

7) After the resin height is stabilized, stop the flow. Close the column outlet. Disconnect the inlet piping at the top of the column. Slowly lower the adapter to the surface of the resin. At this time, the packing solution in the column flows out from the column inlet.
8) Connect to the adapter inlet with the piping filled with packing solution to exclude air. Open the column outlet and pump the packing solution. (ET clean S：＜0.3MPa、ET clean L：＜0.2MPa) If the resin compresses and makes space between it and the adapter, lower the adapter to the surface of the resin.
9) Calculate column volume from column height. If the column volume is larger than the target column volume, lower the adapter to the target height. If the column volume is lower than the target column volume, the slurry concentration is low, or the resin may be excessively compressed. Remove the resin from the column and pack again.

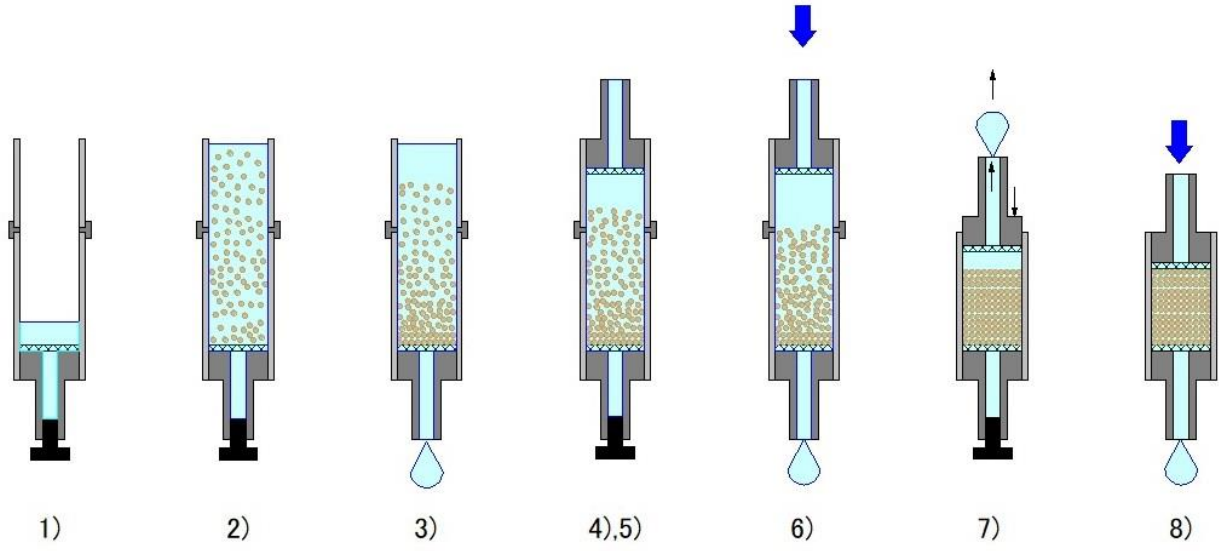


Figure 3 Process of column packing

Evaluation of column packing

Column packing efficiency is evaluated by checking HETP and asymmetry (As).

(Appendix 1)

Column depyrogenation and equilibration

1) Flow 5 column volume (CV) of alkaline solution into the column. Stop to flow the alkaline solution and keep it for the time shown below.

Alkaline solution	Time required for endotoxin removal
0.2 mol/L NaOH	16hr or overnight
0.2 mol/L NaOH in 20 % EtOH	3 - 5 hr
0.2 mol/L NaOH in 95 % EtOH	1 hr

2) Flow 5 CV of pyrogen-free water (PF water). After the eluate becomes neutral pH, measure endotoxin in the eluate by LAL method to confirm the removal of endotoxin.
3) Equilibrate the column by flowing about 5 CV of adsorption buffer with endotoxin removed. Cellufine ET Clean can be used to easily prepare low-endotoxin buffers. (Appendix 3)

Operating Guidelines

How to use

1) Refer to“Column depyrogenation and equilibration”
2) Load the column with sample dissolved in adsorption buffer. (1-20mg/mL)
3) Collect the eluate and measure the endotoxin content.
4) To remove endotoxin in the column, refer to“Column depyrogenation and equilibration”

Recommended buffer

Adsorption buffer:

・PF water + 0.1-0.4 M NaCl,
・0.01 M-0.05 M sodium phosphate + 0.1-0.4 M NaCl,
・Tris-HCl + 0.1-0.4 M NaCl

Setting the buffer pH lower than the isoelectric point(pI) of the target protein reduces protein

Isoelectric point (pI)	Recommended adsorption buffer	Resin
4.0-6.5（acidic protein）	pH5–7、NaCl: 0.1-0.4 M	ET clean S
7.0-10.5 （neutral or basic protein）	pH7-9、NaCl: 0.1-0.4 M	ET clean L

adsorption on ET clean. Acidic proteins with low pI are easily adsorbed on ET Clean, but adsorption can be reduced by increasing the salt concentration or using ET Clean S with a smaller pore size. (Fig. 4) Proteins are difficult to adsorb on ET clean near pI.

Note: Optimal conditions for adsorption of endotoxin with ET Clean can be determined by the tests in Appendix 4. An example of pI and recommended buffer and resin selection is shown below.

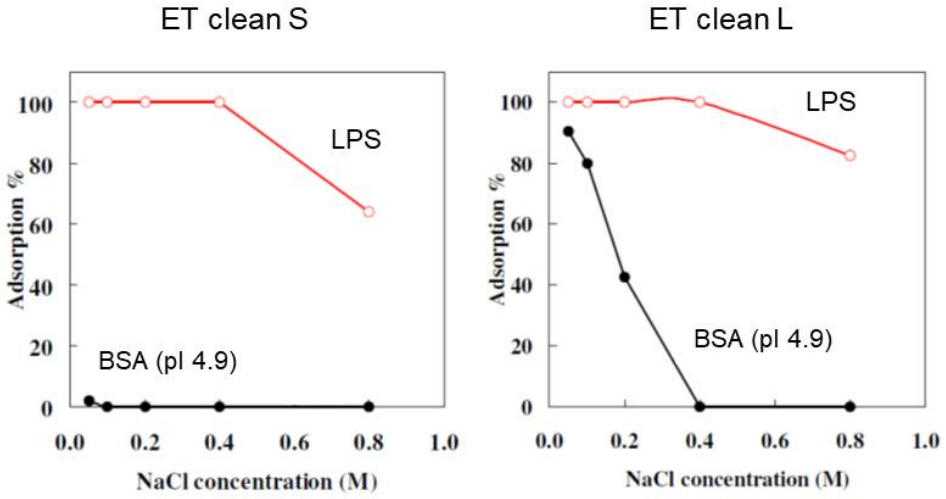


Figure 4 Effect of salt concentration on adsorption of BSA (batchwise method)
0.2g of ET clean and 2mL of LPS solution (BSA: 500 μg/ml, LPS:*E.coli* O111:B4 100 ng/ml, pH 7.0 PB, NaCl:0.05-0.8M)

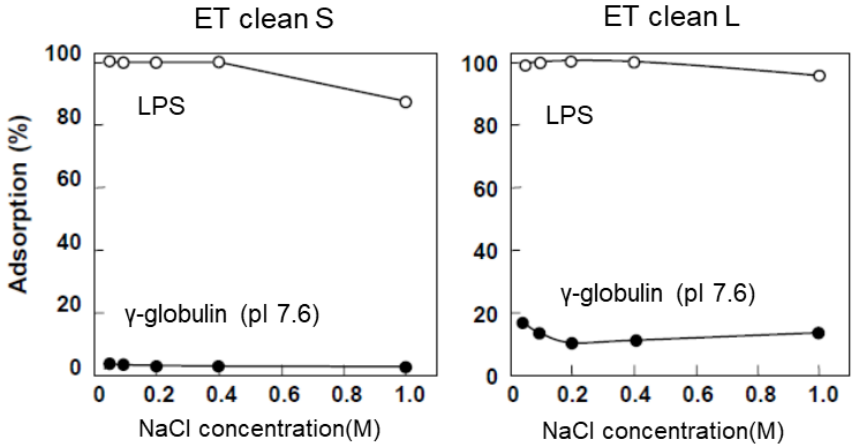


Figure 5　Effect of salt concentration on adsorption of γ-globulin (batchwise method)
0.2mL of ET clean and 2mL of LPS solution（γ-globulin: 500 μg/ml, LPS:*E.coli* UKT-B 100 EU/ml, pH 7.0 PB, NaCl:0.05-1.0M）

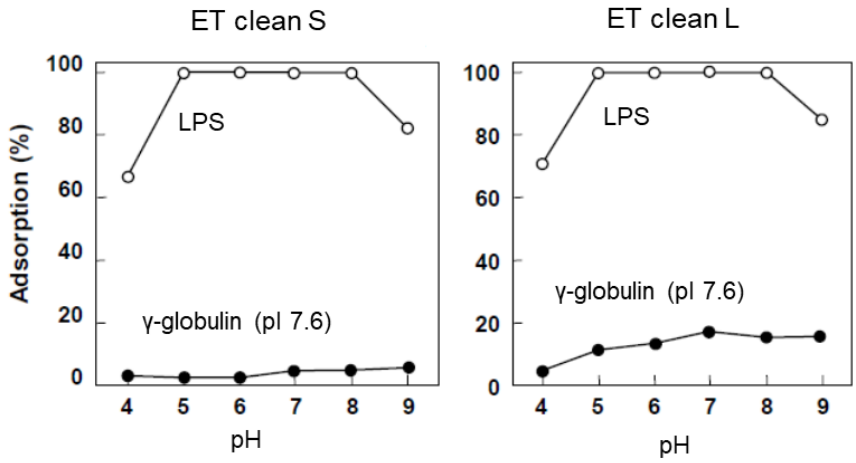


Figure 6　Effect of pH on adsorption of LPS (LPS) (batchwise method)
0.2mL of ET clean and 2mL of LPS solution（γ-globulin: 500 μg/ml, LPS:*E.coli* UKT-B 100 EU/ml, pH 4-9, μ=0.05）

Elution buffer：Add 1-2 M NaCl to the adsorption buffer.

Elute by increasing NaCl concentration up to about 0.5 M using gradient elution (5-10 CV). If the target protein does not elute, flow a high concentration of NaCl or change the pH. Stepwise elution of NaCl concentration, pH gradient, and pH stepwise elution are also available.

Sample preparation and loading

The ionic strength of the sample should be prepared equal to or less than that of the adsorption buffer. A concentration of 1-20 mg/ml of target protein is recommended. Low concentrations or too small a sample volume may not be recovered. Insoluble material is removed by centrifugation or filtering. Buffer may be exchanged with a desalting filter, dialysis, or a desalting column such as Cellufine GH-25.

Recommended flow velocity

10～50 cm/h

Stability

pH range of 2 to 13 and operating temperature of 4 to 25°C are recommended.

Autoclave is not recommended. Regeneration is possible 5 times.

Storage

Unopened resin should be stored at 2-8°C. After opened or packed columns are equilibrated with 20% ethanol and stored at 2-8°C. Do not freeze.

References

1) Masayo Sakata, PhD, Yoshihisa Yamaguchi, Chuichi Hirayama, PhD, Ivars Bemberis, Masami Todokoro, PhD, Masashi Kunitake, PhD, Minoru Nakayama, BioPharm International, 2005, Volume 18, Issue 1

Product Ordering Information

Description	Quantity	Catalogue No.
Cellufine ET clean L	5 x 1 mL (Mini-column)	20051
	1 x 5 mL (Mini-column)	20015
	10 mL	681 984 324
	50 mL	681 984 326
	500 mL	681 984 328
Cellufine ET clean S	5 x 1 mL (Mini-column)	20151
	1 x 5 mL (Mini-column)	20115
	10 mL	682 985 324
	50 mL	682 985 326
	500 mL	682 985 328

Purchase/Technical Support

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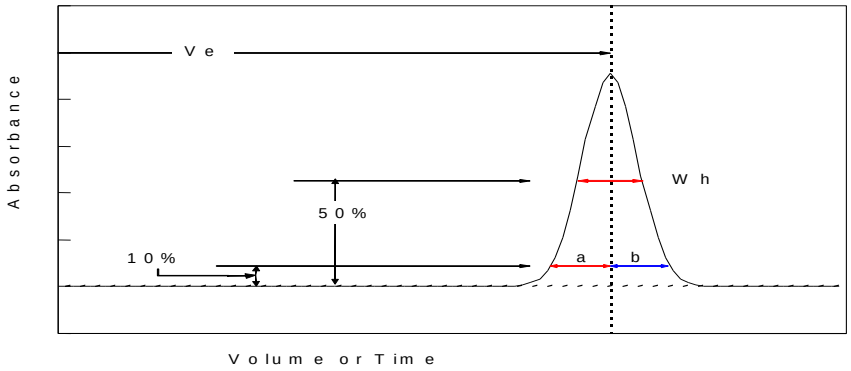
E-mail: cellufine@jnc-corp.co.jp

Appendix 1: Evaluation of column packing

The packing condition of the column is evaluated using indices such as theoretical number of stage plates (N), theoretical stage equivalent height (HETP), and asymmetry (As). These indices are affected by the measurement conditions. For example, these indices vary with column diameter/height differences, piping, solvent sample volume, flow rate, temperature, etc. Therefore, it is necessary to use the same measurement conditions each time. A flow velocity of 30 cm/h is recommended, but higher velocities are possible. However, the higher the flow velocity, the smaller the theoretical number of plates (N). The same conditions (flow rate, column size, mobile phase, and sample) must be used each time to evaluate the column.

Parameter	Condition
Sample volume	1 -2.5% of column volume (CV)
Sample concentration	1-2 %(v/v) acetone (mobile phase：water or adsorption buffer)
	1M NaCl （mobile phase： 0.1-0.2M NaCl aq）
Flow rate (cm/h)	30 cm/h
Detector	UV, Conductivity

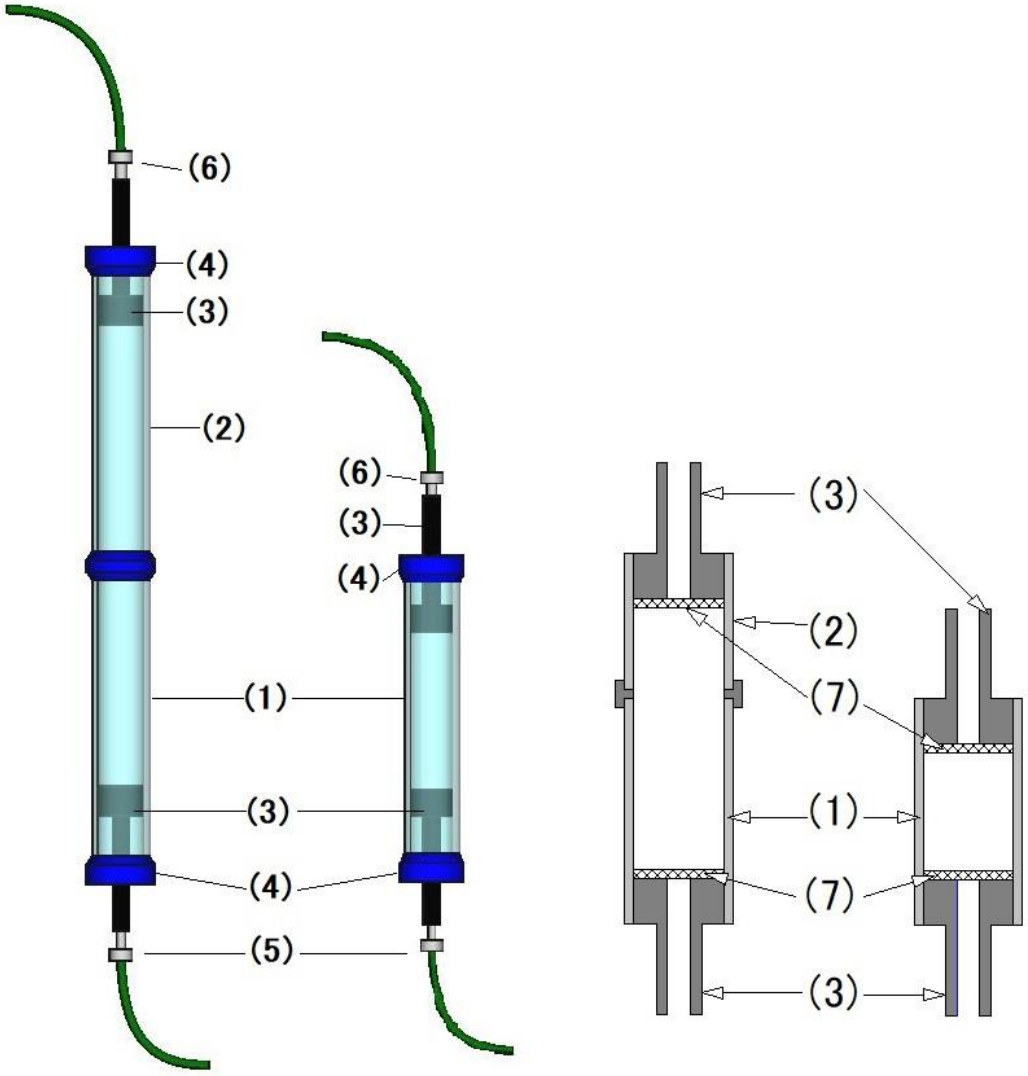


L	Column length [cm or m]
V_e	Elution time or volume
W_h	Half of width of peak
a, b	Peak width of 10% peak hight (a) front (b) rear
Note	Ve,Wh and a, b should have same dimensional units

HETP = L/N	**$N = 5.54 \times (Ve/Wh)^2$**	**As = b/a**

Generally, number of (theoretical) plates (N) is good if it is over 3000. Acceptable asymmetry factor (As) values range from 0.7-1.5.

Appendix 2 : Figure of columns



The Operating Instructions use a simple cross-sectional figure of the column, as shown on the right.

(1)	Column tube	(4)	Column end
(2)	Reservoir	(5)	Column outlet
(3)	Adapter	(6)	Column inlet
(7)	Filter		

Appendix 3：Preparation of endotoxin-free buffer using ET Clean

ET Clean L easily removes endotoxin from buffers. Reduces endotoxin to lower concentrations more easily than polymyxin-immobilized resins or the anion exchanger Cellufine A-500.

Materials

・Column：ET Clean L, polymyxin-immobilized agarose gel, Cellufine A-500（9mm I.D. x 100mm）
・Pump：Peristaltic pump with silicon tube
・Buffer ：1M sodium phosphate buffer、pH 7.0 (spiked 2.6 EU/ml LPS)
・dry-heat sterilization glass tubes
・LAL (rate assay, EndospecyES-50M Set; SEIKAGAKU CORPORATION

Pre-washing

Refer to " Column depyrogenation and equilibration"

Removal of endotoxin

1) Buffer flows through the column at a flow rate of 30 ml/h. (Residence time 12.8min, linear velocity 47cm/h)
2) Collect eluate 1 CV at a time.（CV=column volume）
3) Measure the concentration of endotoxin in the eluate using LAL reagent.

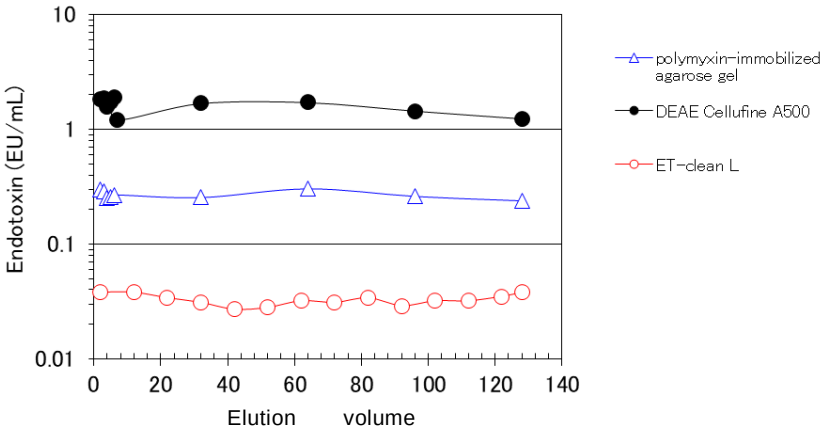


Figure 1 Comparison of the LPS removal capability from 1M phosphate buffer of ET-clean L, and polymyxin-immobilized agarose gel and Cellufine A-500.

Appendix 4： Removal of endotoxin by batch method

Materials

- ET clean S or L
- Filtration equipment (Glass filter, aspirator, etc.)
- medicine spoon（metal）
- pipette or Pasteur pipette（grass）
- glass beaker or grass bottle
- Storage containers（Glass bottles or pyrogen-free plastic containers）
- aluminum foil
- triangular flask（10mL～20mL）
- Disposable syringe（1～2.5mL）
- membrane filter（Syringe connection type and pyrogen-free recommended）
- LAL reagents
- Pyrogen-free water： If water produced by ultrapure water production equipment is used, it must be confirmed that the endotoxin concentration is low. Distilled water for injection will not be contaminated with endotoxin.

Depyrogenation of Materials

・Equipment [1]

1) Glassware should be cleaned and dried. The openings of the glassware are covered with aluminum foil and sterilized by dry heat at 250°C for 3 hours.
2) Metal instruments (such as medicine spoon) should be cleaned and dried. They are covered with aluminum foil and sterilized by dry heat at 250°C for 3 hours.
3) Aluminum foil is cut into 5 cm squares and placed in a glass or metal petri dish or other container and sterilized by dry heat at 250°C for 3 hours.

・ET clean

1) Shake the bottle to slurry the resin. Put the resin into a glass filter and filter by suction. Aspirate the slurry using a glass filter and wash off the 20% ethanol with water.

[1] Dry heat sterilized instruments should be removed from the autoclave after the temperature has cooled.

2) Prepare 0.2 mol/L NaOH/20% EtOH in laboratory ultrapure water.[2)]
3) Add 0.2 mol/L NaOH/20% EtOH until the rinsed resin is soaked. Mix gently with a medicine spoon and filter by suction. This process is repeated three times.
4) Remove the alkaline solution until almost no liquid comes out of the glass filter outlet. Put the resin into a beaker or bottle, add 0.2 mol/L NaOH/20% EtOH (twice the volume of the resin), and keep at 4°C to 25°C overnight (approximately 3 hours or more).
5) Resin is filtered and washed (3 times) with 0.2 mol/L NaOH 20% EtOH using a glass filter, then filtered and washed with PF water until neutral pH. Use a clean booth or clean bench for suction filtration.
6) Wash the resin with adsorption buffer and equilibrate until the pH at the filter outlet is the same as its buffer.
7) After equilibration, measure the endotoxin in the filter solution to confirm that it does not contain endotoxin. The final filtration takes 10 to 15 minutes to remove buffer. The wet weight of the resin at this step is expressed in g-wet.
8) Endotoxin-free resin should be weighed immediately and used for batch method or stored in a dry heat sterilized container and sealed. For long-term storage, add PF water or equilibration buffer and store tightly (about 4°C).

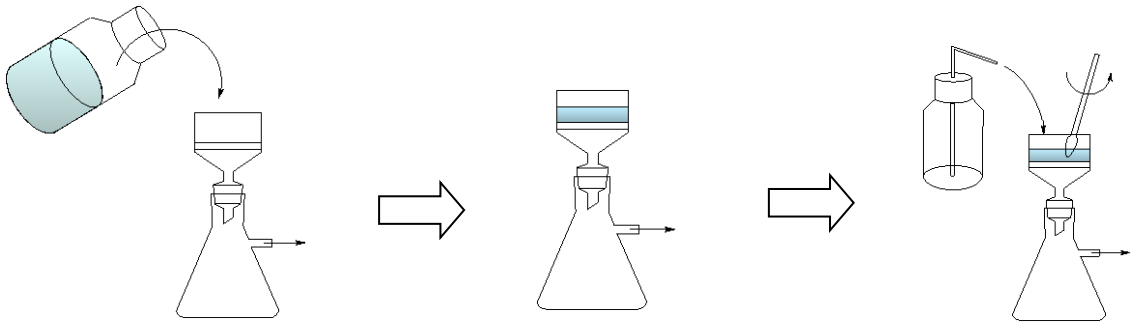


Figure1 Suction Filtration and Washing of resin

[2] Wear protective goggles as the NaOH solution may cause blindness if it gets into the eyes. When 20% EtOH is changed to 95% EtOH, leaving time to endotoxin free is more than 1 hour.

Batch adsorption method

Determine the amount of protein and endotoxin adsorption at different salt concentrations and set the optimum salt concentration.

Table1 Example of sample preparation（Sample dilution rate：75%）

Final NaCl conc.	sample	4M NaCl	PF water
mol/L	mL	mL	mL
0.00	1.5	0.000	0.50
0.05	1.5	0.025	0.48
0.10	1.5	0.050	0.45
0.20	1.5	0.100	0.40
0.30	1.5	0.150	0.35
0.40	1.5	0.200	0.30
0.50	1.5	0.250	0.25
0.60	1.5	0.300	0.20
0.70	1.5	0.350	0.15
0.80	1.5	0.400	0.10
0.90	1.5	0.450	0.05
1.00	1.5	0.500	0.00

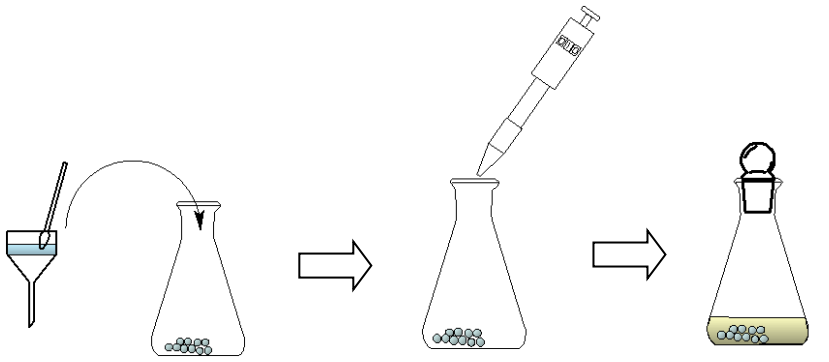
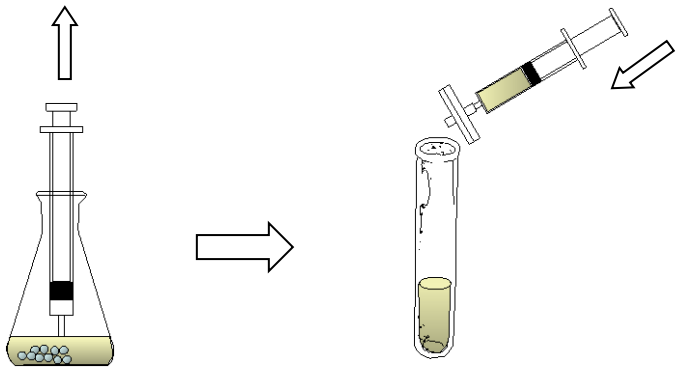


Figure2 Operation of batch method

1) Weigh 0.2 g of resin into a 10-20 mL triangular flask.
2) Add 2 mL of the sample prepared in Table 1 to the weighed gel.
3) Cover the opening with a stopper or sterile aluminum foil. Stir at 25°C for 2 hours. If the sample is sensitive to heat, the temperature should be kept low.
4) Aspirate the sample using a disposable syringe. The aspirated sample is filtered through a membrane filter attached to the syringe.

5) Samples are first determined for endotoxin, and the remaining solution is used for protein determinations.



4) Aspiration and filtration

Figure3 Operation of batch method

Note

- For blank test, add 0.2 mL of pyrogen-free water instead of resin.
- The ionic strength of the buffer solution is not included in the final salt concentrations in Table 1. Buffer concentrations should be prepared to 0.01-0.05 mol/L.
- To prepare 100 mL of 4 mol/L NaCl, weigh 23.4 g of NaCl and meth up to 100 mL with pyrogen-free water. To prepare a solution with a lower endotoxin concentration, weigh 23.4 g NaCl into a 100-mL female flask, dry heat sterilized at 250°C for 3 hours, and dissolve in 100 mL of pyrogen-free water.
- For efficient stirring, a swirling stirrer or rotary stirrer can be used. Teflon-coated rotors can also be dry heat sterilized at 250°C for 3 hours, and can be stirred with a magnetic stirrer.
- Protein concentration is determined from absorbance at 280 nm and by various colorimetric determination methods. If 2 mL of test solution is insufficient, proportionally double the volume of solution in Table 1. In that case, increase the amount of resin as well.
- In this experiment, the ratio of sample volume to resin volume was set to 10 times, but may be changed as needed depending on the endotoxin concentration.
- In experiments to examine the effect of pH, resins washed with PF water should be used. Samples should be replaced in advance with buffer of the respective pH.

Appendix 5：Removal of endotoxin by column method

Case 1

Bovine serum albumin (BSA: isoelectric point 4.9) solution containing endotoxin was loaded into a 1mL column. Eluate was collected in test tubes and endotoxin and BSA concentrations were determined. BSA was almost recovered without adsorption on ET clean. Although BSA is an acidic protein with low pI, the addition of NaCl to the buffer reduced adsorption to the column. Endotoxin was adsorbed on ET clean and the concentration in the buffer was reduced to 1/1000. Endotoxin was selectively adsorbed on ET clean and BSA solution with low endotoxin concentration was recovered.

Materials

- Column：ET Clean L (11mm I.D. x 10mm ⇒1mL)
- Pump
- Buffer ：pH 7.0 50 mM sodium phosphate + 0.15M NaCl
- Dry-heat sterilization glass tubes
- Sample：150mL (BSA 1mg/mL, endotoxin 100EU/mL)
- LAL reagents (rate assay, EndospecyES-50M Set; SEIKAGAKU CORPORATION)

Depyrogenation and equilibration

Refer to " Column depyrogenation and equilibration"

Flow

- 0.17mL/min (10cm/h)

Assay

- BSA：Abs. 280 nm
- Endotoxin：LAL reagents

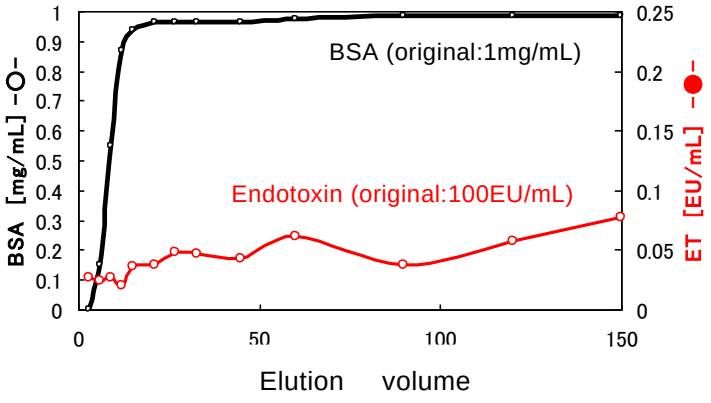


Figure 1 Endotoxin removal from BSA solution by column method

Case 2

Lysozyme (isoelectric point 11) solution containing endotoxin was loaded into a 9.6mL column. After washing with adsorption buffer, the samples were eluted by gradient with NaCl. The eluate was collected in test tubes and endotoxin and lysozyme concentrations were determined. Lysozyme eluted without adsorption to the column, while endotoxin adsorbed to the column and was recovered by a gradient of increasing NaCl concentration. Endotoxin was selectively adsorbed on ET clean and Lysozyme solution with low endotoxin concentration was recovered.

Materials

- Column：ET Clean L (9 mm I.D. x 100mm ⇒9.6mL)
- Pump
- Adsorption buffer：pH 7.3　1 mM Tris-HCl
- Elution buffer：pH 7.3　1 mM Tris-HCl + 1.0M NaCl
- Elution gradient：0→1M NaCl
- Dry-heat sterilization glass tubes
- Sample：1 mL　(Lysozyme 14mg/mL, endotoxin contained)
- LAL reagents (rate assay, EndospecyES-50M Set; SEIKAGAKU CORPORATION)

Depyrogenation and equilibration

Refer to " Column depyrogenation and equilibration"

Flow

- 0.5 mL/min (47m/h)

Assay

- Lysozyme：Abs. 280 nm
- Endotoxin：LAL reagents

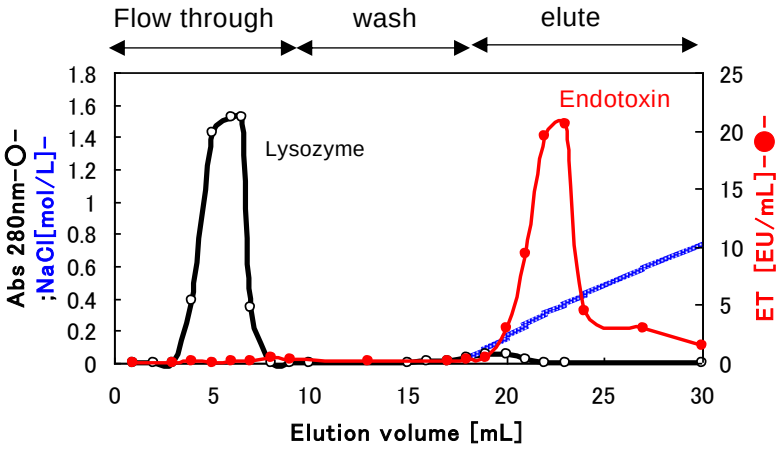


Figure 2　Endotoxin removal from lysozyme solution by column method