⟨130⟩ PROTEIN A QUALITY ATTRIBUTES

INTRODUCTION

Protein A is coupled to a resin support in order to create protein A affinity chromatography media commonly used in the manufacturing of recombinant therapeutic monoclonal antibodies. Natural protein A is derived from *Staphylococcus aureus* and contains five homologous antibody binding regions and a C-terminal region for cell wall attachment. In addition to naturally derived protein A, recombinant material manufactured in *Escherichia coli,* as well as several engineered versions of the protein, also manufactured recombinantly, have entered the market place. When immobilized on a column, protein A provides a highly efficient and robust purification method for purifying antibodies at various scales. However, protein A ligand from the column can co-elute with the antibody during purification, an effect which is often referred to as protein A leaching. This tendency increases as the chromatography medium ages. Engineered versions of protein A may improve the pH tolerance of the medium, but do not eliminate leaching. It is the current regulatory expectation that leached protein A should be cleared during the purification of antibodies for human use, and manufacturing processes should be validated accordingly. Enzyme-Linked Immunosorbent Assay (ELISA)-based residuals testing is generally employed during process development and validation to assure the efficient removal of residual protein A during process steps following protein A affinity chromatography. In addition, the manufacturer should have a clear understanding and documentation of resin and ligand quality through raw materials qualification and column lifetime studies.

General Chapter ⟨130⟩ describes quality attributes of protein A ligands that are used in chromatography media for the manufacture of therapeutic monoclonal antibodies: Protein A; rProtein A; rProtein A, C-Cys; rProtein A, B4, C-Cys.

- **PROTEIN A**

  $C_{1995}H_{3163}N_{597}O_{697}S_3$
  46,760
  N-terminal Sequence AQHDEA
  C-terminal Sequence IAADNK
  Protein A is derived from *Staphylococcus aureus*. The structure is composed of a single polypeptide chain containing four IgG binding domains. With the exception of IgG_3, all other human IgGs bind to protein A. Each molecule of Protein A is capable of binding two IgG molecules. It is manufactured as a bulk solution at a concentration of greater than 20 mg protein A per mL with an IgG-binding potency of greater than 95%. Because Protein A is used as an ancillary material in the manufacture of recombinant therapeutic drugs, regulatory requirements differ from those for therapeutic drug products.
- **PACKAGING AND STORAGE:** Store in closed containers at the temperature indicated on the label.
- **LABELING:** Preserve in sealed containers, and store at a temperature of –20° or below.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Endotoxin RS
  USP Protein A RS
- **IDENTIFICATION**
  A. SDS-PAGE: It meets the requirements of *Identification* test *A* under *rProtein A* using USP Protein A RS.
  B. IgG Binding: It meets the requirements of *Identification* test *B* under *rProtein A* using USP Protein A RS.
- **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩**:** The total aerobic microbial count does not exceed 100 cfu per mL, and the total yeasts and molds count does not exceed 10 cfu per mL.
- **BACTERIAL ENDOTOXINS TEST** ⟨85⟩**:** It contains not more than 1 USP Endotoxin Unit per mg of total protein. [NOTE—The *Bacterial Endotoxins Test* for Protein A is used to describe the quality of this ancillary material. This test does not define the acceptable level of bacterial endotoxin in the preparation of injectable dosage forms in which Protein A is used.]
- **TOTAL PROTEIN**
  (See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)
  Prepare triplicate samples for analysis by diluting Protein A to 3.0 mg per mL in *Water for Injection*. Measure the absorbance of each sample at 275 nm after correcting for the absorbance using *Water for Injection* as the blank. Determine the protein concentration using the equation:

  $$\text{Protein concentration (mg per mL)} = (A275/0.149)$$

  in which A is the absorbance of Protein A at the wavelength of 275 nm and 0.149 is the molar absorptivity. Average the triplicate results, and determine a coefficient of variance (CV): the CV is ≤5%.
- **LIMIT OF COMMON CONTAMINANT PROTEIN AND CORRESPONDING ASSAY**
  Enterotoxin B: Enterotoxin B is determined using a commercially available microstrip enzyme-immunoassay kit.[1] Wells of the microstrips are coated with sheep antibodies to enterotoxin B. Standard curves are made using the ELISA kit control. The negative controls are wells coated with serum from nonimmunized sheep. The level of enterotoxin is determined from the standard curve. The specification for the enterotoxin B level is ≤1 ng per mg of total protein.
- **CHROMATOGRAPHIC PURITY**

[NOTE—The size-exclusion chromatographic purity test resolves Protein A from high molecular weight contaminants.]

Mobile phase: Prepare a solution of 50 mM sodium dihydrogen phosphate, pH 6.5 in the following manner. Add 6.9 ± 0.1 g of sodium dihydrogen phosphate into a 1000-mL beaker. Dilute with water to 900 mL, and adjust with 5 M sodium hydroxide to a pH of 6.50 ± 0.05. Transfer the solution into a 1000-mL volumetric flask, and dilute with water to volume. Pass the solution through a 0.22-µm membrane filter.

Column regeneration solution: Prepare a solution of 0.1 M sodium dihydrogen phosphate, pH 3.0 in the following manner. Add 13.8 ± 0.1 g of sodium dihydrogen phosphate into a 1000-mL beaker. Dilute with water to 900 mL, and adjust with

[1] A suitable enzyme immunoassay kit is available from TECRA International Pty Ltd., Australia (No. SETVIA96).

hydrochloric acid to a pH of 3.0 ± 0.1. Transfer the solution into a 1000-mL volumetric flask, and dilute with water to volume. Pass the solution through a 0.22-µm membrane filter.

Column storage solution: Mix 100 mL of methanol with 900 mL of water.

Test solution: Dilute Protein A to approximately 1 mg per mL with *Mobile phase.*

Calibration standards: Using *Mobile phase,* prepare separate 1 mg per mL solutions of each of the following: thyroglobulin (670 kD), IgG (150 kD), beta lactoglobulin (36 kD), and lysozyme (14 kD).

Standard solution: Prepare a solution containing 1 mg per mL of USP Protein A RS in *Mobile phase.*

Chromatographic system

(See *Chromatography* ⟨621⟩.)

The liquid chromatograph is equipped with a 280-nm detector and a 7.8-mm × 30-cm column that contains packing L33. Equilibrate the column for approximately 30 minutes at 0.5 mL of *Mobile phase* per minute or until a stable baseline is achieved.

Procedure: Separately inject 100 µL of each sample, and run the samples in the following sequence: *Calibration standards,* thyroglobulin, IgG, beta lactoglobulin, and lysozyme; the *Standard solution;* and the *Test solution.* Run the sequence three times isocratically using *Mobile phase* at 0.5 mL per minute for 30 minutes. Absorbance is detected at 280 nm. Analyze the 280-nm peak data, and pick the retention time (RT) with the largest peak area. Using the data from the *Calibration standards,* plot the mean RT versus the log molecular weight to produce the standard curve. The purity should be ≥95% in the main peak. Use the formula from the standard curve to give the log molecular weights of the *Test solutions*. Convert the log molecular weights of the *Test solutions* and the *Standard solutions* to actual molecular weights. The apparent molecular weight of protein A from the *Standard solution* is between 156 and 205 kDa; and the Protein A from the *Test solution* is within the same range.

Column cleaning and storage: Rinse the column with 100 mL of *Column regeneration solution,* and store by flushing with 100 mL of *Column storage solution.*

rPROTEIN A, C-CYS

$C_{1478}H_{2320}N_{432}O_{503}S_4$

34317.5 Da

N-terminal Sequence AQHDEAQQNA

rProtein A, C-Cys is a recombinant Protein A lacking the C-terminal membrane binding part; instead, a C-terminal cysteine has been introduced for directed immobilization purposes. It has five homologous IgG binding domains identical to the native Protein A and is produced using *Escherichia coli* as the host cell followed by purification with conventional chromatography. rProtein A, C-Cys is manufactured as a bulk solution with an IgG-binding potency of greater than 95%. Because rProtein A, C-Cys is used as an ancillary material in the manufacture of recombinant therapeutic drugs, regulatory requirements differ from those for therapeutic drug products.

- **PACKAGING AND STORAGE:** Store in closed containers at the temperature indicated on the label.
- **LABELING:** Preserve in sealed containers, and store at a temperature of –20° or below.
- **USP REFERENCE STANDARDS** ⟨11⟩

  USP Endotoxin RS

  USP rProtein A, C-Cys RS
- **IDENTIFICATION**

  A. SDS-PAGE: It meets the requirements of *Identification* test *A* under *rProtein A* using USP rProtein A, C-Cys RS.

  B. IgG Binding: It meets the requirements of *Identification* test *B* under *rProtein A* using USP rProtein A, C-Cys RS.
- **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩**:** The total aerobic microbial count does not exceed 100 cfu per mL, and the total yeasts and molds count does not exceed 10 cfu per mL.
- **BACTERIAL ENDOTOXINS TEST** ⟨85⟩**:** It contains not more than 1 USP Endotoxin Unit per mg of total protein. [NOTE—The *Bacterial Endotoxins Test* for rProtein A, C-Cys is used to describe the quality of this ancillary material. This test does not define the acceptable level of bacterial endotoxin in the preparation of injectable dosage forms in which rProtein A, C-Cys is used.]
- **TOTAL PROTEIN**

  (See ⟨857⟩.)

  Prepare triplicate samples for analysis by diluting the rProtein A, C-Cys to 3.0 mg per mL in *Water for Injection*. Measure the absorbance of each sample at 275 nm after correcting for the absorbance using *Water for Injection* as the blank. Determine the protein concentration using the equation:

  $$\text{Protein concentration (mg per mL)} = (A_{275}/0.22)$$

  in which A is the absorbance of rProtein A, C-Cys, at the wavelength of 275 nm and 0.22 is the molar absorptivity. Average the triplicate results, and determine a coefficient of variance (CV): the CV is ≤2.5%.
- **CHROMATOGRAPHIC PURITY**

  [NOTE—The size-exclusion chromatographic purity test resolves rProtein A, C-Cys from high molecular weight contaminants and low molecular weight contaminants.]

  Mobile phase: Prepare a solution of 0.02 M sodium phosphate, pH 7.2 containing 0.15 M sodium chloride in the following manner. Add 0.96 ± 0.02 g of monobasic sodium phosphate hydrate, 2.32 ± 0.02 g of dibasic sodium phosphate dihydrate, and 8.76 g ± 0.02 g of sodium chloride into a 1000-mL beaker. Dilute with water to 900 mL, and adjust with 1 M sodium hydroxide to a pH of 7.2 ± 0.05. Transfer this solution into a 1000-mL volumetric flask, and dilute with water to volume. Pass the solution through a 0.45-µm membrane filter.

  EDTA solution: Prepare a 20 mM ethylenediaminetetraacetic acid (EDTA) solution by dissolving 0.74 ± 0.02 g of EDTA in 100 mL of *Mobile phase.*

  DTT solution: Prepare a 100 mM DL-dithiothreitol (DTT) solution by dissolving 1.54 ± 0.02 g of DTT in 100 mL of *Mobile phase.*

Pretreatment solution: Prepare a solution containing a mixture of *EDTA solution* and *DTT solution* (1:1, v/v). [NOTE—Prepare fresh just before use.]

Test solution: Dilute rProtein A, C-Cys 1 to 5 in *Pretreatment solution,* and mix gently. Incubate the sample at 40° for 60 minutes.

Chromatographic system

(See *Chromatography* ⟨621⟩.)

The liquid chromatograph is equipped with a 214-nm detector and a 10-mm × 30-cm column that contains packing L54. Equilibrate the column with at least two column volumes of *Mobile phase* at a flow rate of 0.4 mL per minute.

Procedure: Inject 100 µL of *Pretreatment solution,* and allow the chromatography to continue for at least two column volumes. Repeat this twice before injecting 100 µL of the *Test solution*. Absorbance is detected at 214 nm. Integrate the main peak from the *Test solution* run and all other peaks not present in the *Pretreatment solution* runs. Calculate the percentage of impurities in the portion of the rProtein A, C-Cys taken by the formula:

$$100(r_i/r_s)$$

in which r_i is the peak response for each impurity; and r_s is the sum of the responses of all the peaks: the sum of all impurities is not more than 5%; and the *Test solution* shows a major peak at approximately 35 minutes.

rPROTEIN A

$C_{1917}H_{3039}N_{565}O_{658}S_3$

44,618

N-terminal Sequence FLRPVE

Protein A is a component of the cell wall of *Staphylococcus aureus.* Recombinant Protein A (rProtein A) consists of five homologous immunoglobulin (IgG) binding domains (E, D, A, B, C) followed by a partial X domain sequence. It is expressed in *Escherichia coli* and purified via a column chromatography process. IgG columns are not used in the purification process. It is manufactured as a bulk solution with an IgG-binding potency greater than 95%. Release testing methods and specifications are described below. Because rProtein A is used as an ancillary material in the manufacture of recombinant therapeutic drugs, regulatory requirements differ from those of therapeutic drug products.

- **PACKAGING AND STORAGE:** Store in closed containers at the temperature indicated on the label.
- **LABELING:** The labeling states that the material is of recombinant DNA origin along with the lot number, storage conditions, and the statement "Formulated in *Water for Injection*."
- **USP REFERENCE STANDARDS** ⟨11⟩

  USP Endotoxin RS

  USP rProtein A RS
- **IDENTIFICATION**

  A. SDS-PAGE

  Molecular weight marker: Use a suitable molecular weight marker (MWM) containing protein bands between 20 and 200 kD.

  PBS solution: Prepare a solution that contains 8065.0 mg and 200.0 mg of sodium chloride and potassium chloride, respectively, per L of 0.01 M sodium phosphate buffer, pH 7.4.

  4X Sample buffer:[2] Dissolve 0.666 g of tris-hydrochloride, 0.682 g of tris base, 0.800 g of lithium dodecyl sulfate (LDS), 0.006 g of ethylenedinitrilotetraacetic acid (EDTA), and 4 g of glycerol in 8 mL of water; add 0.75 mL of 1% Coomassie brilliant blue G-250 (see Coomassie Blue G-250 in *Reagents* under *Reagents, Indicators, and Solutions*) solution and 0.25 mL of 1% phenol red solution. Mix well, and adjust the volume with water to 10 mL.

  2X Sample buffer: Prepare a mixture of *4X Sample buffer* and water (1:1).

  1X Sample buffer: Prepare a mixture of *2X Sample buffer* and water (1:1).

  1 M Dithiothreitol solution: Dissolve 0.154 g of DL-dithiothreitol (DTT) in 1 mL of water.

  2X Reducing sample buffer: Mix 180 µL of *2X Sample buffer* and 20 µL of *1 M Dithiothreitol solution.*

  20X Running buffer:[3] Dissolve 104.6 g of 3-(*N*-morpholino)propanesulfonic acid (MOPS), 60.6 g of tris base, 10 g of sodium dodecyl sulfate (SDS), and 3.0 g of EDTA in 400 mL of water. Mix well, and adjust with water to 500 mL.

  1X Running buffer: Prepare a solution of water and *20X Running buffer* (19:1).

  Gel staining solution: Prepare a solution of Coomassie brilliant blue R-250 (see Coomassie Brilliant Blue R-250 in *Reagents* under *Reagents, Indicators, and Solutions*) having a concentration of 0.5 g per L in a mixture of water, isopropanol, and acetic acid (6.5: 2.5: 1.0). Filter, and store at room temperature. Silver staining is not recommended.

  Destaining solution: Mix 100 mL of acetic acid with 900 mL of water.

  Standard preparation: Dilute USP rProtein A RS to 0.4 mg per mL with *PBS solution.* Further dilute this solution 1:1 with *2X Reducing sample buffer,* and incubate in a closed tube for 5 minutes at 90°. Mix, and quick spin prior to loading.

  Test preparation: Dilute rProtein A with *PBS solution* to 0.4 mg per mL. Proceed as directed under *Standard preparation* beginning with "Further dilute."

  Comix solution: Dilute rProtein A and USP rProtein A RS with *PBS solution* to 0.8 mg per mL. This solution contains 0.4 mg per mL of each protein. Proceed as directed under *Standard preparation* beginning with "Further dilute."

  SDS-PAGE gel and apparatus set-up: Assemble gel apparatus following the manufacturer's instructions. Lock the gel tension wedge in place, and fill approximately 200 mL of *1X Running buffer* into the inside chamber. If there are no leaks, pour 600 mL of *1X Running buffer* into the outer chamber. Gently pull the comb out of the cassette to immerse the wells in *1X Running buffer.* Load 10 µL of each preparation as directed below under *Gel loading* onto a 10% Bis-Tris SDS-PAGE gel.[4]

[2] 4X NuPAGE LDS sample buffer is available from Invitogen (No. NP0007).

[3] 20X NuPAGE MOPS SDS Running Buffer is available from Invitrogen (No. NP0001).

[4] 10% Bis-Tris SDS-PAGE gel is available from Invitrogen (No. NP0301).

Gel loading: Use the following gel loading scheme when running one *Test preparation* (see *Table 1*). Each *Test preparation* is run by itself and as part of the *Comix solution* that contains the rProtein A and USP rProtein A RS.

Table 1

Lane	Sample	Load Volume (µL)	Load Amount (µg)
1	*1X Sample buffer*	10	N/A
2	MWM	20	N/A
3	*Test preparation* #1	10	2
4	*Comix solution* #1	10	4 (total)
5	*Test preparation* #1	10	2
6	*1X Sample buffer*	10	N/A
7	*Standard preparation*	10	2
8	MWM	20	N/A
9	—	—	—
10	—	—	—

Running the gel: Set the voltage to 125 volts, and run at a constant voltage. Run the gels until the bromophenol blue band is approximately 5 mm from the bottom of the gel (approximately 120 to 140 minutes).

Gel staining: Pour approximately 100 mL of *Gel staining solution* into the staining container. Place the gel into the staining container, and allow the stain to completely cover the gel. Cook the gel and container in a microwave for 30 seconds. Place the staining container on an orbital shaker, and stain the gel for 1 hour with gentle shaking.

Destaining: Drain the *Gel staining solution,* and add enough *Destaining solution* to the container to cover the gel. Place the container on an orbital shaker, and shake at low speed. Change the *Destaining solution* as necessary until a clear background is obtained. After destaining, rinse the gel thoroughly with water, and leave the gel in water for 10 minutes before scanning.

Gel scanning: Apply some water to the glass plate of the scanner, and place the gels on a wetted glass plate. Eliminate any bubbles. Using appropriate settings, scan the gels.

Data analysis: Choose a band between the 20 kD and 30 kD bands of the MWM to calculate the percentage of the retention factor. Draw a line in one lane (lane containing *1X Sample buffer*) from the well to the apex (region of greatest intensity) of the chosen band.

The length of this line is denoted as the total distance (D_T). For the lanes containing samples draw a line from the well to the apex of each band. For each band the length of this distance is the migration distance (D_M) in mm. Record the D_T and D_M on the report sheet for each peak or band. The total distance should be the same for each lane on a gel. Calculate the percentage of the retention factor (R_F) of each major peak or band, and document on the report sheet using the following equation:

$$\%R_F = D_M/D_T \times 100$$

Also for each gel, record the number of bands and approximate molecular weight of each band in each sample.

System suitability: All bands between 20 kD and 70 kD are present. The lane containing *1X Sample buffer* does not contain any bands.

Specificity: The rProtein A has one major band and a similar molecular weight that corresponds to those of the USP rProtein A RS. The *Comix solution* also shows a single major band.

B. IgG Binding: [NOTE—The IgG binding assay is a functional method for determining the percentage of rProtein A capable of binding to immobilized human polyclonal immunoglobulin. Since the percent of functional rProtein A in each lot is not less than 95%, the assay measures unbound protein versus total protein injected. This is done by comparing the absorbance in the flow-through to absorbance from an injection bypassing the column.]

Sample pretreatment (desalting): In order to remove any buffer components that may contribute to absorbance in the "unbound" IgG column fraction, samples are desalted with *Solution A.* Desalting may be performed using a suitable desalting column[5] depending on the volumes required.

IgG column: A 1-mL Sepharose column[6] with immobilized human polyclonal IgG (hIgG) is required to perform this assay. [NOTE—The IgG column requires washing when it is new, when it has performed several analysis cycles, or after system suitability failure. Column washing procedure is not required for each sample injection.]

Column washing solution A: Prepare a solution of 0.5 M acetic acid, pH 3.4 by adding 28.6 mL of acetic acid into a 1000-mL beaker, diluting to 900 mL with water, and adjusting with ammonium acetate to a pH of 3.4. Transfer the solution into a 1000-mL volumetric flask, and dilute with water to volume. Pass the solution through a 0.45-µm membrane filter.

Column washing solution B: Prepare a solution of 50 mM Tris, pH 7.6, 150 mM sodium chloride, and 0.05% Tween 20 by the following procedure. Add 6.06 ± 0.01 g of Tris and 8.77 ± 0.01 g of sodium chloride into a 1000-mL beaker.

[5] Zeba columns are available from Pierce; Nap-10 columns are available from GE Healthcare.
[6] HiTrap IgG Sepharose 6 FF column is available from GE Healthcare (No. 90-1003-97).

Dilute with water to 900 mL, and adjust with 0.5 M sodium hydroxide to a pH of 7.60 ± 0.05. Transfer the solution into a 1000-mL volumetric flask, and dilute with water to volume. Pass the solution through a 0.45-µm membrane filter (buffer solution). Add 0.5 mL of Tween 20 into 1 L of the buffer solution and mix thoroughly.

Solution A: Prepare a solution of 20 mM monobasic sodium phosphate and 150 mM sodium chloride, pH 7.6 by the following procedure. Add 2.76 ± 0.01 g monobasic sodium phosphate hydrate and 8.77 ± 0.01 g sodium chloride into a 1000-mL beaker. Dilute with water to 900 mL, and adjust with 5 M sodium hydroxide to a pH of 7.60 ± 0.05. Transfer the solution into a 1000-mL volumetric flask, and dilute with water to volume. Pass the solution through a 0.45-µm membrane filter.

Solution B: Prepare a solution of 100 mM phosphoric acid pH 2.8 by the following procedure. Add 6.8 mL of phosphoric acid into a 1000-mL beaker. Dilute with water to 900 mL, and adjust with 2 M potassium hydroxide to a pH of 2.80 ± 0.05. Transfer the solution into a 1000-mL volumetric flask, and dilute with water to volume.

Mobile phase: Use variable mixtures of *Solution A* and *Solution B* as directed for *Chromatographic system.* Make adjustments if necessary (see *System Suitability* under *Chromatography* ⟨621⟩).

Standard preparation: Thaw USP rProtein A RS, and use directly.

Test preparation: Prepare a 4.0 to 6.0 mg per mL rProtein A solution in *Solution A.*

Chromatographic system: The liquid chromatograph is equipped with a 280-nm detector and a 1-mL column with immobilized hIgG. The chromatograph is equipped with a bypass valve to allow flow to be diverted from the column. Each analysis consists of a series of two injections, one where the sample is injected onto the column and one where the sample bypasses the column and flows directly into the detector. Perform three replicate analyses. The chromatograph is programmed as follows (see *Table 2*).

Table 2

Flow Rate (mL per minute)	Time (minutes)	Solution A (%)	Solution B (%)	Valve Position	Elution
1.0	0–6	100	0	Column	Re-equilibration
1.0	6–12	100→0	0→100	Column	Re-equilibration
1.0	12–22	100	0	Column	Equilibration
0.4	22–25	100	0	Column	Equilibration
0.4 (sample injected)	25–35	100	0	Column	Isocratic
1.0	35–49	100→0	0→100	Column	Regeneration
1.0	49–63	0→100	100→0	Column	Re-equilibration
1	63–65	100	0	Bypass	Equilibration
0.4	65–68	100	0	Bypass	Equilibration
0.4 (sample injected)	68–75	100	0	Bypass	Isocratic

Chromatograph the *Standard preparation,* record the peak responses, and calculate the percentage of hIgG binding as directed for *Procedure:* the percentage of hIgG binding ≥ 95% and the relative standard deviation for replicate analysis is not more than 1%.

Procedure: Inject a volume (about 100 µL) of the *Test preparation.* Record the chromatogram, and measure the peak responses. Calculate the percentage of hIgG binding activity by the following formula:

$$100 - 100(r_C/r_B)$$

in which r_C is the unbound material peak response from the column injection and r_B is the bypass peak response from the bypass injection. Each replicate analysis of the *Test preparation* is not less than 95% of hIgG binding. Report the average value from three replicate analyses.

- **Microbial Enumeration Tests** ⟨61⟩ and **Tests for Specified Microorganisms** ⟨62⟩**:** The total aerobic microbial count does not exceed 100 cfu per mL, and the total yeasts and molds count does not exceed 10 cfu per mL.
- **Bacterial Endotoxins Test** ⟨85⟩**:** It contains not more than 0.5 USP Endotoxin Unit per mg of total protein. [Note—The *Bacterial Endotoxins Test* for rProtein A is used to describe the quality of this ancillary material. This test does not define the acceptable level of bacterial endotoxin in the preparation of injectable dosage forms in which rProtein A is used.]
- **Total Protein**

  (See ⟨857⟩.)

  Prepare triplicate samples for analysis by diluting the rProtein A to 3.0 mg per mL in *Water for Injection*. Measure the absorbance of each sample at 275 nm after correcting for the absorbance using *Water for Injection* as the blank. Determine the protein concentration using the equation:

$$\text{Protein concentration (mg per mL)} = (A_{275}/0.165)$$

  in which A is the absorbance of rProtein A at the wavelength of 275 nm and 0.165 is the molar absorptivity. Average the triplicate results, and determine a coefficient of variance (CV): the CV is ≤5%.
- **UV Spectral Analysis:** Dilute rProtein A to 1 mg per mL in *Water for Injection*. Using a scanning UV spectrophotometer and *Water for Injection* as the blank, obtain spectral scans over the range of 240 to 360 nm. From the resulting data, calculate

the absorbance value at 270 nm and the ratio of absorbance at 270 to 250 nm (i.e., E270/E250): the absorbance at 270 nm of a 1 mg per mL solution of rProtein A in *Water for Injection* is within the range 0.14–0.20.

• **CHROMATOGRAPHIC PURITY**

[NOTE—The size-exclusion chromatographic purity test resolves rProtein A from high molecular weight contaminants.]

Mobile phase: A solution of 0.3 M sodium phosphate, pH 7 is prepared by mixing monobasic and dibasic phosphate solutions in the following manner. Weigh 21.3 ± 0.1 g of dibasic anhydrous sodium phosphate, and dissolve in 500 mL of water to obtain a 0.3 M dibasic sodium phosphate solution *(Solution 1).* Into a separate container, weigh 18.0 ± 0.1 g monobasic anhydrous sodium phosphate, and dissolve in 500 mL of water to obtain a 0.3 M monobasic sodium phosphate solution *(Solution 2).* Calibrate a pH meter using pH calibrators at a pH of 7 and 10. Add 400 mL of *Solution 1* to a 1-L beaker. Transfer the pH probe to the beaker. Slowly add *Solution 2* to the solution until the pH is 7.0 ± 0.1. Pass the solution through a 0.45-µm membrane filter.

Standard solution: Dilute USP rProtein A RS to 1 mg per mL in *Mobile phase.*

Test solution: Dilute rProtein A to 1 mg per mL in *Mobile phase.*

Chromatographic system

(See *Chromatography* ⟨621⟩.)

The liquid chromatograph is equipped with a 214-nm and 280-nm detector and a 9.4-mm × 25-cm column that contains packing L35. The flow rate is 1 mL per minute. Chromatograph the *Standard solution* as directed for *Procedure:* rProtein A shows a single major peak at approximately 9 minutes and the area percentage is ≥98% at 214 nm and ≥95% at 280 nm.

Procedure: Inject 100 µL of the *Test solution* into the chromatograph, run isocratically for 15 minutes, and record the chromatogram. The values for the rProtein A from the *Test solution* correspond to the specifications of the USP rProtein A RS from the *Standard solution.*

• **ISOFORMS**

Standard solution: Thaw USP rProtein A RS, and use directly.

Test solution: Dilute rProtein A to 4 mg per mL in *Water for Injection.*

pI Markers: Use a suitable marker set containing markers between 3 and 10.[7]

IEF gel: Use a suitable gel with the range of between 3 and 10 and a size of 100 × 125 mm.[8]

Procedure: Apply 5-µL aliquots of the *pI Markers, Test solution,* and the *Standard solution* to the *IEF gel*, and run under 1W of power for approximately 10 minutes. Remove the sample mask, and apply power with concurrent cooling between 5° to 10° of the focusing chamber for 40 minutes at a setting of 1000V, 20 mA, 25W. Fix the *IEF gel* for 1 hour in 20% trichloroacetic acid, then stain using a suitable stain for IEF gels.[9] Finally, wash and dry the gel: the correlation coefficient of the best fit line for the *pI Markers* versus their migration in cm is ≥0.990, and the rProtein A from the *Standard solution* shows a single major band within the pI range of 4.6 to 5.2. A single band is seen in the *Test solution* that corresponds to the pI range of the *Standard solution*.

• **LIMIT OF TRITON X-100**

Mobile phase: Prepare a filtered and degassed mixture of water and acetonitrile (60:40).

Test solution: Dilute rProtein A to 5 mg per mL in *Water for Injection*.

Triton X-100 spike solution: Combine 5 mg per mL of USP rProtein A RS and 0.15% Triton X-100 (9:1) to obtain a solution having known concentrations of rProtein A and 0.015% Triton X-100.

Chromatographic system

(See *Chromatography* ⟨621⟩.)

The liquid chromatograph is equipped with a 214-nm and a 280-nm detector and a 4.6-mm × 25-cm column that contains 5-µm packing L11. The flow rate is 1 mL per minute. Chromatograph the *Triton X-100 spike solution* as directed for *Procedure:* Triton X-100 has a single major peak at approximately 9 minutes, and the rProtein A shows a smaller or undetectable peak at the same retention time.

Procedure: Inject about 100 µL of the *Test solution* into the chromatograph, run isocratically for 35 minutes, and record the chromatogram. The absorbance is detected at 223 nm: the Triton X-100 peak is not more than 0.015% (equivalent to the *Triton X-100 spike solution*).

rPROTEIN A, B_4, C-CYS

$C_{1177}H_{1854}N_{326}O_{384}S_1$

26747.6 Da

N-terminal Sequence AQGTVDAKFD

rProtein A, B_4, C-Cys is a recombinant protein derived from the B-domain of Protein A. The Protein A domain has been alkali-stabilized by site-specific mutagenesis and multimerized to a tetramer with a C-terminal cysteine for directed immobilization purposes. rProtein A, B_4, C-Cys is produced using *Escherichia coli* as the host cell followed by purification with conventional chromatography. rProtein A, B_4, C-Cys is manufactured as a bulk solution with an IgG-binding potency of greater than 95%. Because rProtein A, B_4, C-Cys is used as an ancillary material in the manufacture of recombinant therapeutic drugs, regulatory requirements differ from those for therapeutic drug products.

• **PACKAGING AND STORAGE:** Store in closed containers at the temperature indicated on the label.

• **LABELING:** Preserve in sealed containers, and store at a temperature of –20° or below.

• **USP REFERENCE STANDARDS** ⟨11⟩

USP Endotoxin RS

USP rProtein A, B4, C-Cys RS

• **IDENTIFICATION**

A. SDS-PAGE: It meets the requirements of *Identification* test *A* under *rProtein A* using USP rProtein A, B4, C-Cys RS.

[7] pI markers in the 3–10 range are available from BioRad (No. 161-0310).

[8] IEF gels in the 3–10 range are available from Cambrex (No. 56015).

[9] ISS Pro-Blue is available from Integrated Separation Systems.

B. IgG Binding: It meets the requirements of *Identification* test *B* under *rProtein A* using USP rProtein A, B4, C-Cys RS.

- **MICROBIAL ENUMERATION TESTS ⟨61⟩ and TESTS FOR SPECIFIED MICROORGANISMS ⟨62⟩:** The total aerobic microbial count does not exceed 100 cfu per mL, and the total yeasts and molds count does not exceed 10 cfu per mL.
- **BACTERIAL ENDOTOXINS TEST ⟨85⟩:** It contains not more than 1 USP Endotoxin Unit per mg of total protein. [NOTE—The *Bacterial Endotoxins Test* for rProtein A, B_4, C-Cys is used to describe the quality of this ancillary material. This test does not define the acceptable level of bacterial endotoxin in the preparation of injectable dosage forms in which rProtein A, B_4, C-Cys is used.]
- **TOTAL PROTEIN**

(See ⟨857⟩.)

Formulation buffer solution: Prepare a solution of 0.02 M potassium phosphate, pH 7.0, containing 0.15 M potassium chloride and 2 mM of ethylenediaminetetraacetic acid (EDTA) in the following manner. Add 2.72 ± 0.01 g of monobasic potassium phosphate anhydrous, 11.18 g ± 0.22 g of potassium chloride, and 0.744 ± 0.02 g of EDTA into a 1000-mL beaker. Dilute with water to 900 mL, and adjust with 1 M sodium hydroxide to a pH of 7.00 ± 0.05. Transfer the solution into a 1000-mL volumetric flask, and dilute with water to volume. Pass the solution through a 0.45-µm membrane filter.

Test preparation: Dilute the rProtein A, B_4, C-Cys to 3.0 mg per mL with *Formulation buffer solution.*

Procedure: Prepare triplicate samples for analysis. Measure the absorbance of each *Test preparation* at 275 nm after correcting for the absorbance using the *Formulation buffer solution* as the blank. Determine the protein concentration using the equation:

$$\text{Protein concentration (mg per mL)} = (A275/0.22)$$

in which A is the absorbance of rProtein A, B_4, C-Cys, at the wavelength of 275 nm and 0.22 is the molar absorptivity. Average the triplicate results, and determine a coefficient of variance (CV): the CV ≤2.5%.

- **CHROMATOGRAPHIC PURITY**

[NOTE—The size-exclusion chromatographic purity test resolves rProtein A, B_4, C-Cys from high molecular weight contaminants and low molecular weight contaminants.]

Mobile phase: Prepare a solution of 0.02 M sodium phosphate, pH 7.2 containing 0.15 M sodium chloride in the following manner. Add 0.96 ± 0.02 g of monobasic sodium phosphate hydrate, 2.32 ± 0.02 g of dibasic sodium phosphate dihydrate, and 8.76 g ± 0.02 g of sodium chloride into a 1000-mL beaker. Dilute with water to 900 mL, and adjust with 1 M sodium hydroxide to a pH of 7.2 ± 0.05. Transfer this solution into a 1000-mL volumetric flask, and dilute with water to volume. Pass the solution through a 0.45-µm membrane filter.

EDTA solution: Prepare a 20 mM EDTA solution by dissolving 0.74 ± 0.02 g of EDTA in 100 mL of *Mobile phase.*

DTT solution: Prepare a 100 mM DL-dithiothreitol (DTT) solution by dissolving 1.54 ± 0.02 g of DTT in 100 mL of *Mobile phase.*

Pretreatment solution: Prepare a solution containing a mixture of *EDTA solution* and *DTT solution* (1:1, v/v). [NOTE—Prepare fresh just before use.]

Test solution: Dilute rProtein A, B_4, C-Cys 1 to 5 in *Pretreatment solution,* and mix gently. Incubate the sample at 40° for 60 minutes.

Chromatographic system

(See *Chromatography* ⟨621⟩.)

The liquid chromatograph is equipped with a 214-nm detector and a 10-mm × 30-cm column that contains packing L54. Equilibrate the column with at least two column volumes of *Mobile phase* at a flow rate of 0.4 mL per minute.

Procedure: Inject 100 µL of *Pretreatment solution,* and allow the chromatography to continue for at least two column volumes. Repeat this twice before injecting 100 µL of the *Test solution.* Absorbance is detected at 214 nm. Integrate the main peak from the *Test solution* run and all other peaks not present in the *Pretreatment solution* runs. Calculate the percentage of impurities in the portion of rProtein A, B_4, C-Cys taken by the formula:

$$100(r_i/r_s)$$

in which r_i is the peak response for each impurity; and r_s is the sum of all the responses of all the peaks: the sum of all impurities is not more than 5%; and the *Test solution* shows a major peak at approximately 37 minutes.