# Peginterferon Alfa-2b (Genetical Recombination)

ペグインターフェロン　アルファー2b (遺伝子組換え)

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CDLPQTHSLG SRRTLMLLAG MRRISLFSCL KDRHDFGFPQ EEFGNQFQKA
ETIPVLHEMI QQIFNLFSTK DSSAAWDETL LDKFYTELYQ QLNDLEACVI
QGVGVTETPL MKEDSILAVR KYFQRITLYL KKKKYSPCAW EVVRAEIMRS
FSLSTNLQES LRSKE
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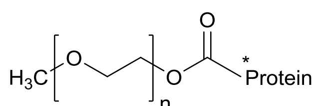
C1, H7, K31, H34, K49, K83, K112, K121, Y129, K131, K133, K134, S163 and K164: PEGylation site

Binding mode of polyethylene glycol




* $\alpha$-amino group of N-terminal cysteine residue, $\varepsilon$-amino group of lysine residue, imidazole group of histidine residue, hydroxyl groups of tyrosine and serine residues

$C_{858}H_{1349}N_{229}O_{252}S_9$: 19192.86 (Protein moiety)
[*215647-85-1*]

The desired product of Peginterferon Alfa-2b (Genetical Recombination) is a pegylated (polyethylene glycolated) recombinant interferon alfa-2b (molecular mass: 38,000 to 44,000) in which one methoxy polyethylene glycol molecule (average molecular mass: about 12,000) is covalently bound to one of amino residues (Cys-1, His-7, Lys-31, His-34, Lys-49, Lys-83, Lys-112, Lys-121, Tyr-129, Lys-131, Lys-133, Lys-134, Ser-163 and Lys-164) through the carbonyl group. Interferon Alfa-2b (Genetical Recombination) is one of the variants of human interferon alfa, and is a protein consisting of 165 amino acids where 23rd and 34th amino acid residues are substituted with arginine and histidine residues, respectively. It is an aqueous solution.

It contains not less than 3.68 mg and not more than 5.82 mg of protein per mL, and not less than $4.8 \times 10^7$ units and not more than $8.7 \times 10^7$ units per mg of protein.

**Manufacture** Regard interferon alfa-2b (Genetical Recombination) as an important intermediate and prescribe test methods and pass/fail acceptance criteria. Tests for interferon alfa-2b (genetical recombination) contain peptide mapping as the identification test and tests for desamido substance, N-terminal methionylated substance, host cell proteins, tetracycline and isoform as the purity tests. Interferon alfa-2b (Genetical Recombination) is purified by the method which is verified to be able to reduce the remained amount of DNA in a drug substance to not more than the standard value. Methoxy polyethylene glycol succinimidyl carbonate is used as a reagent in the PEGylation process and tests for substitutional rate, endotoxin, residual solvent and triethylamine are prescribed. In the purification process after PEGylation, select eluted fractions so that the content of dipeginterferon is not more than the standard value.

**Description** Peginterferon Alfa-2b (Genetical Recombination) occurs as a colorless to pale yellow, clear or opalescent liquid.

**Identification** **(1)** Proceed as directed in the Assay (2): the color of the liquid in the twelfth column is not more colored than that of the liquid in the first column.

**(2)** Compare the chromatogram of the sample solution obtained in the Assay (1) with that of the standard solution: the form and the retention time of the principal peaks obtained from the sample solution and the standard solution are the same.

**Molecular mass** Proceed as directed in the Purity (1). Prepare a calibration curve from the migration distances of the molecular mass markers (proteins having molecular mass of 14,400, 20,100, 30,000, 43,000 and 67,000) obtained from the molecular mass standard solution by plotting the logarithm of the molecular mass on the vertical axis and the migration distance on the horizontal axis. Determine the migration distance of the main band obtained from the sample solution, and calculate the molecular mass using the calibration curve: the molecular mass is between 38,000 and 44,000.

**Isoelectric point** To a suitable amount of Peginterferon Alfa-2b (Genetical Recombination) add the diluting solution to make a solution so that each mL contains 0.2 mg of protein, and use this solution as the sample solution. Separately, to a suitable amount of Peginterferon Alfa-2b RS for Identification add the diluting solution to make a solution so that each mL contains 0.2 mg of protein, and use this solution as the standard solution. Separately, to a suitable amount of Interferon Alfa-2b RS for Identification add the diluting solution to make a solution so that each mL contains 40 $\mu$g of protein, and use this solution as the control solution. Separately, to a suitable amount of the isoelectric point marker for peginterferon alfa-2b add the diluting solution to make a solution so that each mL contains 2 mg of protein, and use this solution as the isoelectric point standard solution. Perform the test with 25 $\mu$L each of the sample solution, standard solution, control solution and isoelectric point standard solution by the isoelectric focusing method according to the following

conditions, and determine the migration distance of each band from the cathode. Plot the isoelectric points of the isoelectric point markers on the logarithmic axis and their migration distances on the other axis on a semi logarithmic graph, and connect each point by a straight line. Calculate the isoelectric point from the migration distances of the stained bands obtained from the sample solution, the standard solution and the control solution: the isoelectric point of the main band obtained from the sample solution is 5.7 to 6.1.

Diluting solution: To 30 mL of glycerin add 0.08 mol/L lysine solution to make 100 mL.

*Operating conditions*—

Equipment: Vertical type isoelectric focusing electrophoresis device.

Slab gel: 5% polyacrylamide slab gel for isoelectric focusing (size: 8 cm × 8 cm, thick: 1.0 mm, range of isoelectric point: pH 3 to 7).

Electrophoresis: Pour more than 150 mL of the cathode solution into the upper vessel of the equipment, introduce the sample solution, the standard solution, the control solution and the isoelectric point standard solution to each well of the slab gel, and pour more than 300 mL of the anode solution into the lower vessel. Run at about 2 W per a gel for about 2.5 hours.

Cathode solution: 0.004 mol/L lysine solution (pH 10.1).

Anode solution: 0.0002 mol/L phosphoric acid solution (pH 2.4).

Fixing and staining: Remove the slab gel from the equipment, and immerse in a suitable volume of a mixture of a solution of trichloroacetic acid (23 in 100) and a solution of 5-sulfosalicylic acid dihydrate (69 in 1000) (1:1) for more than 1 hour. Remove the fixing solution, immerse in colloidal coomassie blue TS overnight, and decolorize the gel with water until a band appears against a transparent background.

*System suitability*—

(1) The bands of isoelectric point markers can be confirmed at 3 to 7 of isoelectric point, and are distributed in the whole lanes of the gel.

(2) The isoelectric point of the main band from the standard solution is between 5.7 and 6.1.

(3) The isoelectric point of the main band from the control solution is between 6.0 and 6.3.

**Protein profile** Perform the test with 25 $\mu$L of the sample solution obtained in the Assay (1) as directed under Liquid Chromatography *<2.01>* according to the following conditions. Determine each peak area by the automatic integration method, and calculate their amounts by the area percentage method: the amount of monopeginterferon alfa-2b is not less than 90.6%, the amount of dipeginterferon alfa-2b is not more than 7.5%, the amount of free interferon alfa-2b is not more than 2.4%, and the amount of the other protein is not more than 0.5%. Furthermore, the total amount of the other proteins is not more than 1.0%.

*Operating conditions*—

Detector, column, column temperature, mobile phases and flow rate: Proceed as directed in the operating conditions in the Assay (1).

Time span of measurement: For 27 minutes.

*System suitability*—

Proceed as directed in the system suitability in the Assay (1).

**pH** *<2.54>* 6.8 – 7.1.

**Positional isomer** To a suitable amount of Peginterferon Alfa-2b (Genetical Recombination) add the mobile phase A to make a solution so that each mL contains 0.2 mg of protein. Put a suitable volume of this solution into a tube for concentration (for molecular mass cut-off 10,000 or equivalent), and centrifuge until the amount of the remaining liquid becomes about 100 $\mu$L. Add 2 mL of the mobile phase A to the remaining liquid, and centrifuge until the amount of the remaining liquid becomes about 100 $\mu$L. Invert the tube for concentration, attach a receiving tube whose tare weight has been measured, centrifuge, and recover the remaining liquid. Determine the mass of the recovered remaining liquid from the mass of the receiving tube, add the mobile phase A to make a solution so that each mL contains 0.2 mg of protein, and use this solution as the sample solution. Separately, to a suitable amount of Peginterferon Alfa-2b RS for Identification add the mobile phase A to make a solution so that each mL contains 0.2 mg of protein according to the labeled amount, proceed in the same manner as the sample solution, and use this solution as the standard solution. Perform the test with exactly 100 $\mu$L each of the sample solution and standard solution as directed under Liquid Chromatography *<2.01>* according to the following conditions. Divide each peak detected in the chromatogram obtained from the sample solution to 6 groups, and regard the peak not observed in the chromatogram obtained from the standard solution as the other peak. Determine each peak area by the automatic integration method, and calculate their amounts by the area percentage method: the amount of the group 1 is 4.9 to 12.9%, the amount of the group 2 is 42.9 to 54.4%, the amount of the group 3 is 14.3 to 21.1%, the amount of the group 4 is 5.0 to 8.7%, the amount of the group 5 is 12.3 to 19.5%, the amount of the group 6 is not more than 2.1%, the amount of the other peak is not more than 0.5%. Furthermore, the total amount of the other peaks is not more than 1.0%.

Group 1: 3 to 5 peaks eluted immediately before the principal peak of the group 2
Group 2: the principal peak having the retention time between about 8 minutes and about 13 minutes
Group 3 : 3 to 4 peaks eluted immediately after the principal peak of the group 2
Group 4 : 3 peaks eluted immediately after the principal peak of the group 3
Group 5 : 1 peak eluted immediately after the principal peak of the group 4
Group 6: 1 peak eluted at about 13 to 17 minutes after the peak of the group 5
*Operating conditions*—

Detector: An ultraviolet absorption photometer (wavelength: 214 nm).

Column: A stainless steel column 7.5 mm in inside diameter and 7.5 cm in length, packed with cation-exchange sulfopropylated silica gel (10 μm in particle diameter).

Column temperature: A constant temperature of about 28℃.

Mobile phase A: To 250 mL of the mobile phase B add water to make 2000 mL, and adjust to pH 5.90 – 5.95 with a saturated solution of sodium hydroxide or phosphoric acid.

Mobile phase B: To 22.1 g of sodium dihydrogen phosphate add 1800 mL of water, adjust to pH 5.90 – 5.95 with a saturated solution of sodium hydroxide, and add water to make 2000 mL.

Flowing of mobile phase: Control the gradient by mixing the mobile phases A and B as directed in the following table.

| Time after injection of sample (min) | Mobile phase A (vol%) | Mobile phase B (vol%) |
|---|---|---|
| 0 – 50 | 95 → 0 | 5 → 100 |
| 50 – 55 | 0 | 100 |
| 55 – 57 | 0 → 95 | 100 → 5 |
| 57 – 70 | 95 | 5 |

Flow rate: Adjust so that the retention time of the principal peak is 10 – 15 minutes.

Time span of measurement: 70 minutes.

*System suitability*—

Test for required detectability: When the procedure is run with 100 μL of the standard solution under the above conditions: the SN ratio of the peak having the longest retention time among 3 peaks of the group 4 is not less than 10.

System performance: When the procedure is run with 100 μL of the standard solution under the above operating conditions, the chromatogram shows the same pattern with the chromatogram of Peginterferon Alfa-2b RS for Identification.

**Purity** **(1)** Impurities 1—To a suitable amount of Peginterferon Alfa-2b (Genetical Recombination) add water to make a solution so that each mL contains 50 μg of protein, dilute to 2 times its volume with the diluting solution, and use this solution as the sample solution. Separately, to a suitable amount of Peginterferon Alfa-2b RS for Identification add water to make a solution so that each mL contains 50 μg of protein according to the labeled amount, dilute to 2 times its volume with the diluting solution, and use this solution as the peginterferon standard solution. Separately, to a suitable amount of Interferon Alfa-2b RS for Identification add water to make a solution so that each mL contains 28 μg of protein according to the labeled amount, dilute to 2 times its volume with the diluting solution, and use this solution as the interferon standard solution. To a suitable amount of the interferon standard solution add the diluted diluting solution (1 in 2) to make a solution so that each mL contains 0.5 μg of protein, and use this solution as the solution for confirming dyeing sensitivity. Separately, dissolve molecular mass marker for peginterferon alfa-2b in the diluted diluting solution (1 in 2) to make a solution so that each mL contains about 3 μg of protein, and use this solution as the molecular mass standard solution. Heat the sample solution, the peginterferon standard solution, the interferon standard solution, the solution for confirming dyeing sensitivity and the molecular mass standard solution in water bath for 2 minutes. Perform the test with 20 μL each of the sample solution, peginterferon standard solution, interferon standard solution and solution for confirming dyeing sensitivity and 10 μL of the molecular mass standard solution by SDS-polyacrylamide gel electrophoresis according to the following conditions. Determine the integrated optical density (IOD) of each stained band obtained from the sample solution, and calculate their amounts by the percentage method: the total amount of impurities other than free interferon alfa-2b, monopeginterferon alfa-2b and dipeginterferon alfa-2b is not more than 2.0%.

Diluting solution: To 50 mL of 0.126 mol/L tris buffer solution (pH 6.8) add 20 mL of glycerin, 10 g of sodium lauryl sulfate, 0.1 g of bromophenol blue and 5 mL of 2-mercaptoethanol to dissolve, and add 0.126 mol/L tris buffer solution (pH 6.8) to make 100 mL.

*Operating conditions*—

Equipment: An electrophoresis apparatus consisting of a vertical type slab gel electrophoretic vessel and a constant voltage power unit.

Slab gel: 14% polyacrylamide slab gel (for tris-glycine) (size: 8 cm × 8 cm, thick: 1 mm).

Buffer solution for electrophoresis: Dissolve 2.9 g of 2-amino-2-hydroxymethyl-1,3-propanediol, 14.4 g of glycine and 1g of sodium laurylsulfate in water to make 1000 mL.

Electrophoresis: Energize at a constant voltage of 125 V. Stop the electrophoresis when the front of the migration of bromophenol blue reaches the lower end of the slab gel.

Staining: Immerse the slab gel in a mixture of methanol, water and acetic acid (5:4:1) for more than 2 hours to decolorize, rinse with water, immerse in a solution of glutaraldehyde (1 in 10) for 30 minutes, and wash with water for 20 minutes three times. Then, immerse the gel in the silver staining solution for peginterferon alfa-2b for 5 minutes, wash quickly with water, and immerse in a solution, prepared by dissolving 5 mg of citric acid monohydrate in water and adding 50 $\mu$L of formaldehyde and water to make 100 mL, until the band corresponding to free interferon alfa-2b is obtained from the solution for confirming dyeing sensitivity. Rinse the gel with water more than three times.

Analysis equipment: Equipment which measure integrated optical density (IOD) using an integrating densitometer or image analysis.

*System suitability* —

(1) The staining intensities of the main band of the sample solution and the peginterferon standard solution are the same, and the bands corresponding to monopeginterferon alfa-2b and free interferon alfa-2b are observed, respectively.

(2) The stained band of the protein contained in each standard solution is obtained.

(3) The stained bands of all molecular mass markers are distributed in the range of about 80% of the lane of the gel.

(4) The band corresponding to free interferon alfa-2b is obtained from the solution for confirming dyeing sensitivity.

(5) Prepare a calibration curve from the migration distances of the bands of the molecular mass markers (proteins having molecular masses 14,400, 20,100, 30,000, 43,000 and 67,000) obtained from the molecular mass standard solutions by plotting the logarithm of the molecular mass on the vertical axis and the migration distance on the horizontal axis. Determine the migration distance of the main band obtained from the peginterferon standard solution, and calculate the molecular mass using the calibration curve: the molecular mass is between 38,000 and 44,000.

**(2)** Impurities 2—Proceed as directed in (1) according to the following conditions. Determine the integrated optical density (IOD) of each stained band obtained from the sample solution, and calculate their amounts by the percentage method: the total amount of impurities other than free interferon alfa-2b, monopeginterferon alfa-2b and dipeginterferon alfa-2b is not more than 3.5%.

Diluting solution: To 50 mL of 0.126 mol/L tris buffer solution (pH 6.8) add 20 mL of glycerin, 10 g of sodium lauryl sulfate and 0.1 g of bromophenol blue to dissolve, and add 0.126 mol/L tris buffer solution (pH 6.8) to make 100 mL.

*Operating conditions* —

Proceed as directed in the operating conditions in (1).

*System suitability* —

Proceed as directed in the system suitability in (1) except for the system suitability (5).

**(3)** Monomethoxy polyethylene glycol-12000—Use Peginterferon Alfa-2b (Genetical Recombination) as the sample solution. Separately, weigh accurately 0.15 g of monomethoxy polyethylene glycol-12000, add water to make exactly 100 mL, if necessary heat to about 40℃ to dissolve, and use this solution as the standard solution. Perform the test with a volume of the sample solution, equivalent to about 0.5 mg of protein, and 10, 15, 20, 25 and 30 $\mu$L of the standard solution as directed under Liquid Chromatography *<2.01>* according to the following conditions, and determine each peak area. Prepare a calibration curve from the injection volumes and the peak areas of monomethoxy polyethylene glycol-12000 obtained from the standard solution, and calculate the amount of monomethoxy polyethylene glycol-12000 in Pegiterferon Alfa-2b from the peak area of monomethoxy polyethylene glycol-12000 from the sample solution: not more than 5.9%.

Amount (%) of monomethoxy polyethylene glycol-12000
$= X / W \times 100$

*X*: Amount ($\mu$g) of monomethoxy polyethylene glycol-12000 calculated using the calibration curve
*W*: Volume ($\mu$L) of the sample solution taken

*Operating conditions* —

Detector: An evaporative light scattering detector.

Detector temperature: 50℃

Column: A stainless steel column 4.6 mm in inside diameter and 15 cm in length, packed with butylsilanized silica gel for liquid chromatography (5 $\mu$m in particle diameter and 300 Å in pore diameter).

Column temperature: A constant temperature of about 35℃.

Mobile phase A: Diluted trifluoroacetic acid (1 in 1000).

Mobile phase B: A mixture of acetonitrile and diluted trifluoroacetic acid (1 in 1000) (9:1).

Flowing of mobile phase: Control the gradient by mixing the mobile phases A and B as directed in the following table.

| Time after injection of sample (min) | Mobile phase A (vol%) | Mobile phase B (vol%) |
|---|---|---|
| 0 – 13 | 95 → 5 | 5 → 95 |
| 13 – 20 | 5 | 95 |
| 20 – 30 | 5 → 95 | 95 → 5 |

Flow rate: 1.0 mL per minute.

*System suitability* ―

(1) When the procedure is run with a volume of Peginterferon Alfa-2b RS for Identification, equivalent to about 0.3 mg of protein, under the above conditions, monomethoxy polyethylene glycol-12000 and peginterferon alfa-2b are eluted in this order with the resolution between these peaks being not less than 1.5.

(2) The coefficient of determination ($r^2$) of the calibration curve obtained from the peak areas of the standard solutions is not less than 0.98.

(3) The difference between the elution times of monomethoxy polyethylene glycol-12000 in the sample solution and the standard solution is not more than 0.3 minutes.

**Assay (1)** Protein content―Weigh accurately a suitable volume of Peginterferon Alfa-2b (Genetical Recombination), add the diluting solution to make a solution so that each mL contains about 1 mg of protein, and use this solution as the sample solution. Separately, dissolve one Peginterferon Alfa-2b RS in a suitable volume of water. Pipet a suitable volume of this solution, add exactly the diluting solution to make a solution so that each mL contains 0.2 mg of protein, and use this solution as the standard solution. Perform the test with 25 μL each of the sample solution and standard solution as directed under Liquid Chromatography *<2.01>* according to the following conditions, and determine the peak areas, $A_{TM}$, $A_{TD}$ and $A_{TY}$, of monopeginterferon alfa-2b, dipeginterferon alfa-2b and free interferon alfa-2b obtained from the sample solution, and the peak areas, $A_{SM}$, $A_{SD}$ and $A_{SY}$, of monopeginterferon alfa-2b, dipeginterferon alfa-2b and free interferon alfa-2b from the standard solution.

Diluting solution: To 1 mL of polysorbate 80 add water to make 100 mL. To 1 mL of this solution add 0.05 mol/L sodium phosphate buffer solution (pH 7.0) to make 100 mL.

Protein concentration (mg/mL)

$$= M_S \times (A_{TM} + A_{TD} + A_{TY}) / (A_{SM} + A_{SD} + A_{SY}) \times D$$

$M_S$: Protein concentration of the standard solution (mg/mL)

$D$: Dilution factor for the sample solution

*Operating conditions*―

Detector: An ultraviolet absorption photometer (wavelength: 214 nm).

Column: A stainless steel column 8.0 mm in inside diameter and 50 cm in length, packed with hydroxypropylsilanized silica gel for liquid chromatography (molecular mass cut-off: 4000 to hundreds of thousands).

Column temperature: A constant temperature of about 25°C.

Mobile phase: A mixture of phosphate buffer solution (pH 6.8) and acetonitrile (4:1).

Flow rate: 1.0 mL per minute.

*System suitability* ―

Test for required detectability: To a suitable amount of Interferon Alfa-2b RS for Identification add the diluting solution to make a solution so that each mL contains 0.25 μg of protein. When the procedure is run with 25 μL of this solution under the above operating conditions, the peak of free interferon alfa-2b is detected.

System performance: To a suitable amount of Peginterferon Alfa-2b RS for Identification add the diluting solution to make a solution so that each mL contains 1 mg of protein. When the procedure is run with 25 μL of this solution under the above operating conditions, dipeginterferon alfa-2b, monopeginterferon alfa-2b and free interferon alfa-2b are eluted in this order with the resolution between dipeginterferon alfa-2b and monopeginterferon alfa-2b being not less than 1.0.

System repeatability: When the test is repeated 4 times with 25 μL of the standard solution under the above operating conditions, the relative standard deviation of the retention time of monopeginterferon alfa-2b is not more than 2.0%, and the relative standard deviation of the total of $A_{SM}$, $A_{SD}$ and $A_{SY}$ is not more than 3.0%.

(2) Specific activity ―Pipet a suitable volume of Peginterferon Alfa-2b (Genetical Recombination), add the cell culture medium for peginterferon alfa-2b to make a solution so that each mL contains about 600 units, and use this solution as the sample solution. Separately, dissolve one Peginterferon Alfa-2b RS in 0.7 mL of water. Pipet a suitable volume of this solution, add the cell culture medium for peginterferon alfa-2b to make a solution so that each mL contains 600 units, and use this solution as the standard solution. Perform the test with the sample solution and standard solution according to the following method, and determine the potency.

*Procedure*

Use a 96-well culture plate in a horizontally long (8 lines × 12 columns), dispense 50 μL each of the sample

solution in the wells of the1st and the 4th or the 5th and the 8th lines of the 1st column. Dispense 50 μL each of the standard solution into the wells of the 2nd and the 3rd or the 6th and the 7th lines of the 1st column. Dispense 50 μL each of the cell culture medium for peginterferon alfa-2b in all wells. Mix thoroughly the solution in each well of the 1st column, pipet 50 μL each, and add in each well of the 2nd column of the same lines. Repeat this procedure to the 12th column, dilute the sample solution and the standard solution in serially two-fold steps on the culture plate, and use this plate as the test plate. Dispense 100 μl each of a cell suspension, containing $3.15 \times 10^5$ – $3.85 \times 10^5$ cells per mL prepared using the culture medium for peginterferon alfa-2b, in each well, and incubate under atmosphere of 4 – 6% carbon dioxide at 36 – 37℃ for about 4 hours in a $CO_2$ incubator. After the incubation, dispense 50 μl each of EMC virus solution in each well, put in a $CO_2$ incubator, and incubate for 16 – 21 hours. Separately, for two culture plates dispense 50 μL each of the cell culture medium for peginterferon alfa-2b in the wells of the 1st to the 4th lines of the 1st column, dispense 50 μL each of the standard solution in the wells of the 5th to the 8th lines of the 1st column, proceed in the same manner as the test plate, and use these plates as the reference plates. In the middle of the incubation for 16 – 21 hours, take the reference plates, remove the solution in each well, dispense 50 – 100 μL each of crystal violet staining solution in each well, stand for more than 10 minutes, remove the solution in each well, and wash each well. When 0 – 10% of the cells in the 1st to 4th lines are observed to be stained and when the well in which nearly 50% of the cells in the 5th to 8th lines are stained is observed in the 4th to 9th columns, take the test plate. Remove the solution in each well, dispense 50 μL each of a solution, prepared by dissolving 0.5 g of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide in the culture medium for peginterferon alfa-2b to make 100 mL, in each well, put the plate in a $CO_2$ incubator, and incubate for more than 3 hours. After the incubation, dispense 100 μL each of a solution of sodium laurylsulfate in 0.01 mol/L hydrochloric acid TS (1 in 10) in each well, and incubate in a $CO_2$ incubator overnight. After the incubation, perform the test with the solution in each well as directed under Ultraviolet-visible Spectrophotometry <*2.24*>, and determine the absorbance at 570 nm from the baseline drawn at 690 nm. Calculate corrected absorbances by subtracting the average of the absorbances of blank areas from the absorbance of each well on the culture plate, plot the corrected absorbances on the vertical axis and the column numbers on the horizontal axis for the sample solution and the standard solution, and connect each point. Determine the column numbers showing 50% of the maximum corrected absorbance on the graph, and use the column numbers as $x$ and $y$, respectively. Estimate the potency (Unit/mL) by the following equation, and calculate the ratio of biological activity to protein content.

Potency (Unit/mL)
$= \text{Potency of the standard solution (Unit/mL)} \times 2^{x-y} \times D$

2: Dilution factor of the dilution series
$D$: Dilution factor for the sample solution

*System suitability*–
The column number showing 50% of the maximum corrected absorbance obtained from the standard solution on the test plate is in the range of 4 to 9.

**Containers and storage** Containers—Tight containers.
Storage—At −80℃.

***Add the following under 9.01 Reference Standards (1) to read:***

**Interferon alfa-2b RS for Identification**

**Peginterferon alfa-2b RS**

**Peginterferon alfa-2b RS for Identification**

***Add the following to 9.41 Reagents, Test Solutions:***

**Cell culture medium for peginterferon alfa-2b** To Eagle's minimal essential medium (EMEM) containing Earle's salts add inactivated fetal bovine serum (final concentration: 10%), sodium hydrogen carbonate (final concentration: 2.7 mg/mL), HEPES-isotonic sodium chloride buffer solution (final concentration: 0.02 mol/L), L-glutamine (final concentration: 0.004 mol/L) and penicillin-streptomycin (final concentration: penicillin 125 U/mL, streptomycin 125 μg/mL)

**Cell for peginterferon alfa-2b** Incubate diploid cell derived from human foreskin (HFB4 cell) using the culture medium for peginterferon alfa-2b, and store. Incubate the stored cell at 36.5℃±0.5℃ using a flask and a roller bottle. Subculture every 5 to 10 days by taking cells with trypsin and diluting to 2-fold.

**Colloidal coomassie blue TS** Prepare for electrophoresis.

**Crystal violet staining solution** To 15 mg of crystal violet and 5 mg of sodium chloride add 17.05 mL of a mixture of water, ethanol (95) and formaldehyde (20:10:1).

**EMC virus** Encephalomyocarditis (EMC) virus (for examples, ATCC VR-129 or VR-129B). Incubate the virus using Vero cells. Collect the culture supernatant, determine the virus titer, aliquot into small volume, and store at −70℃ or lower.

**Glutaraldehyde** $C_5H_8O_2$ Oily liquid.

**Isoelectric point marker for peginterferon alfa-2b** A mixture of proteins for isoelectric point electrophoresis containing eight proteins having known isoelectric points in the range of pH 3 to 10 (amyloglucosidase [pI: 3.50], soybean trypsin inhibitor [pI: 4.55], β-lactoglobulin [pI: 5.20], bovine carbonic anhydrase b [pI: 5.85], human carbonic anhydrase b [pI: 6.55], horse myoglobin [pI of the band in the acidic side: 6.85, pI of the band in the basic side: 7.35], lentil lectin [pI of the band in the acidic side: 8.15, pI of the band in the middle: 8.45, pI of the band in the basic side: 8.65] and trypsinogen [pI: 9.30])

**Molecular mass marker for peginterferon alfa-2b** A mixture of proteins for electrophoresis containing α-lactalbumin (molecular mass: 14,400), soybean trypsin inhibitor (molecular mass: 20,100), bovine carbonic anhydrase (molecular mass: 30,000), ovalbumin (molecular mass: 43,000) and bovine serum albumin (molecular mass: 67,000).

**Monomethoxy polyethylene glycol 12000** White granular powder.

*Moecular mass*: 11,000 – 13,000

*Polydispersity*: not more than 1.1.

*Moisture content*: not more than 0.50%.

*Purity* polyethylene glycoldiol: not more than 1.0%.

*Storage*: At −30℃ or lower

**Phosphate buffer solution (pH 6.8)** Dissolve 27.6 g of sodium dihydrogen phosphate monohydrate and 28.4 g of anhydrous sodium sulfate in 1800 mL of water, adjust to pH 6.8 with sodium hydroxide, and add water to make 2000 mL.

**Silver staining solution for peginterferon alfa-2b** Dissolve 76 mg of sodium hydroxide, 1.4 mL of ammonia water (28) and 0.8 g of silver nitrate in water to make 100 mL. Store protected from light.

**Sodium dihydrogen phosphate monohydrate** $NaH_2PO_4.H_2O$

**0.05 mol/mL Sodium phosphate buffer solution (pH 7.0)** Dissolve 3.55 g of anhydrous disodium hydrogen phosphate in 450 mL of water, adjust to pH 7.0 with phosphoric acid, and add water to make 500 mL.

**0.126 mol/L Tris buffer solution (pH 6.8)** Dissolve 15.3 g of 2-amino-2-hydroxymethyl-1,3-propanediol in about 800 mL of water, adjust to pH 6.8 with hydrochloric acid, and add water to make 1000 mL.

***Add the following to 9.42 Solid Supports/Column Packings for Chromatography:***

**Cation-exchange sulfopropylated silica gel for liquid chromatography** Prepare for liquid chromatography.