

## 1 Peginterferon Alfa-2b (Genetical Recombination)

2 ペグインターフェロン アルファ-2b (遺伝子組換え)

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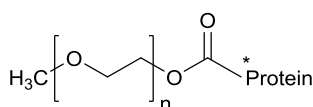
3
4
5
6 CDLPQTHSLG SRRTLMLLAG MRRISLFSCL KDRHDFGFPQ EEFGNQFQKA
7 ETIPVLHEMI QQIFNLFSTK DSSAAWDETL LDKFYTELYQ QLNDLEACVI
8 QGVGVETETPL MKEDSILAVR KYFQRITLYL KKKKYSPCAW EVVRAEIMRS
9 FSLSTNLQES LRSKE

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10 C1, H7, K31, H34, K49, K83, K112, K121, Y129, K131, K133,

11 K134, S163 and K164: PEGylation site

12 Binding mode of polyethylene glycol



13

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

17 C<sub>858</sub>H<sub>1349</sub>N<sub>229</sub>O<sub>252</sub>S<sub>9</sub>: 19192.86 (Protein moiety)

18 [215647-85-1]

19

20 The desired product of Peginterferon Alfa-2b  
 21 (Genetical Recombination) is a pegylated (polyeth-  
 22 ylene glycolated) recombinant interferon alfa-2b  
 23 (molecular mass: 38,000 to 44,000) in which one  
 24 methoxy polyethylene glycol molecule (average  
 25 molecular mass: about 12,000) is covalently bound  
 26 to one of amino residues (Cys-1, His-7, Lys-31,  
 27 His-34, Lys-49, Lys-83, Lys-112, Lys-121, Tyr-129,  
 28 Lys-131, Lys-133, Lys-134, Ser-163 and Lys-164)  
 29 through the carbonyl group. Interferon Alfa-2b (Ge-  
 30 netical Recombination) is one of the variants of hu-  
 31 man interferon alfa, and is a protein consisting of  
 32 165 amino acids where 23rd and 34th amino acid  
 33 residues are substituted with arginine and histidine  
 34 residues, respectively. It is an aqueous solution.

35 It contains not less than 3.68 mg and not more  
 36 than 5.82 mg of protein per mL, and not less than  
 37  $4.8 \times 10^7$  units and not more than  $8.7 \times 10^7$   
 38 units per mg of protein.

39 **Manufacture** Regard interferon alfa-2b (Genetical Re-  
 40 combination) as an important intermediate and prescribe  
 41 test methods and pass/fail acceptance criteria. Tests for  
 42 interferon alfa-2b (genetical recombination) contain pep-  
 43 tide mapping as the identification test and tests for  
 44 desamido substance, N-terminal methionylated substance,  
 45 host cell proteins, tetracycline and isoform as the purity  
 46 tests. Interferon alfa-2b (Genetical Recombination) is

47 purified by the method which is verified to be able to re-  
 48 duce the remained amount of DNA in a drug substance to  
 49 not more than the standard value. Methoxy polyethylene  
 50 glycol succinimidyl carbonate is used as a reagent in the  
 51 PEGylation process and tests for substitutional rate, en-  
 52 dotoxin, residual solvent and triethylamine are prescribed.  
 53 In the purification process after PEGylation, select eluted  
 54 fractions so that the content of dipeginterferon is not more  
 55 than the standard value.

56 **Description** Peginterferon Alfa-2b (Genetical Recom-  
 57 bination) occurs as a colorless to pale yellow, clear or  
 58 opalescent liquid.

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

62 (2) Compare the chromatogram of the sample solu-  
 63 tion obtained in the Assay (1) with that of the standard  
 64 solution: the form and the retention time of the principal  
 65 peaks obtained from the sample solution and the standard  
 66 solution are the same.

67 **Molecular mass** Proceed as directed in the Purity (1).  
 68 Prepare a calibration curve from the migration distances  
 69 of the molecular mass markers (proteins having molecular  
 70 mass of 14,400, 20,100, 30,000, 43,000 and 67,000) ob-  
 71 tained from the molecular mass standard solution by plot-  
 72 ting the logarithm of the molecular mass on the vertical  
 73 axis and the migration distance on the horizontal axis.  
 74 Determine the migration distance of the main band ob-  
 75 tained from the sample solution, and calculate the molec-  
 76 ular mass using the calibration curve: the molecular mass  
 77 is between 38,000 and 44,000.

78 **Isoelectric point** To a suitable amount of Peginterferon  
 79 Alfa-2b (Genetical Recombination) add the diluting solu-  
 80 tion to make a solution so that each mL contains 0.2 mg of  
 81 protein, and use this solution as the sample solution. Sep-  
 82 arately, to a suitable amount of Peginterferon Alfa-2b RS  
 83 for Identification add the diluting solution to make a solu-  
 84 tion so that each mL contains 0.2 mg of protein, and use  
 85 this solution as the standard solution. Separately, to a  
 86 suitable amount of Interferon Alfa-2b RS for Identifica-  
 87 tion add the diluting solution to make a solution so that  
 88 each mL contains 40  $\mu$ g of protein, and use this solution  
 89 as the control solution. Separately, to a suitable amount of  
 90 the isoelectric point marker for peginterferon alfa-2b add  
 91 the diluting solution to make a solution so that each mL  
 92 contains 2 mg of protein, and use this solution as the isoe-  
 93 lectric point standard solution. Perform the test with 25  
 94  $\mu$ L each of the sample solution, standard solution, control  
 95 solution and isoelectric point standard solution by the  
 96 isoelectric focusing method according to the following

97 conditions, and determine the migration distance of each  
98 band from the cathode. Plot the isoelectric points of the  
99 isoelectric point markers on the logarithmic axis and their  
100 migration distances on the other axis on a semi logarithmic  
101 graph, and connect each point by a straight line. Cal-  
102 culate the isoelectric point from the migration distances of  
103 the stained bands obtained from the sample solution, the  
104 standard solution and the control solution: the isoelectric  
105 point of the main band obtained from the sample solution  
106 is 5.7 to 6.1.

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

109 *Operating conditions* —

110 Equipment: Vertical type isoelectric focusing electro-  
111 phoresis device.

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

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

122 Cathode solution: 0.004 mol/L lysine solution (pH  
123 10.1).

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

126 Fixing and staining: Remove the slab gel from the  
127 equipment, and immerse in a suitable volume of a mixture  
128 of a solution of trichloroacetic acid (23 in 100) and a solu-  
129 tion of 5-sulfosalicylic acid dihydrate (69 in 1000) (1:1)  
130 for more than 1 hour. Remove the fixing solution, im-  
131 merse in colloidal coomassie blue TS overnight, and de-  
132 colorize the gel with water until a band appears against a  
133 transparent background.

134 *System suitability* —

135 (1) The bands of isoelectric point markers can be con-  
136 firmed at 3 to 7 of isoelectric point, and are distributed in  
137 the whole lanes of the gel.

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

140 (3) The isoelectric point of the main band from the con-  
141 trol solution is between 6.0 and 6.3.

142 **Protein profile** Perform the test with 25  $\mu\text{L}$  of the sam-  
143 ple solution obtained in the Assay (1) as directed under  
144 Liquid Chromatography <2.01> according to the following  
145 conditions. Determine each peak area by the automatic  
146 integration method, and calculate their amounts by the  
147 area percentage method: the amount of monopeginterfer-  
148 on alfa-2b is not less than 90.6%, the amount of dipegin-

149 terferon alfa-2b is not more than 7.5%, the amount of free  
150 interferon alfa-2b is not more than 2.4%, and the amount  
151 of the other protein is not more than 0.5%. Furthermore,  
152 the total amount of the other proteins is not more than  
153 1.0%.

154 *Operating conditions* —

155 Detector, column, column temperature, mobile phases  
156 and flow rate: Proceed as directed in the operating condi-  
157 tions in the Assay (1).

158 Time span of measurement: For 27 minutes.

159 *System suitability* —

160 Proceed as directed in the system suitability in the As-  
161 say (1).

162 **pH** <2.54> 6.8 – 7.1.

163 **Positional isomer** To a suitable amount of Peginterfer-  
164 on Alfa-2b (Genetical Recombination) add the mobile  
165 phase A to make a solution so that each mL contains 0.2  
166 mg of protein. Put a suitable volume of this solution into a  
167 tube for concentration (for molecular mass cut-off 10,000  
168 or equivalent), and centrifuge until the amount of the re-  
169 maining liquid becomes about 100  $\mu\text{L}$ . Add 2 mL of the  
170 mobile phase A to the remaining liquid, and centrifuge  
171 until the amount of the remaining liquid becomes about  
172 100  $\mu\text{L}$ . Invert the tube for concentration, attach a receiv-  
173 ing tube whose tare weight has been measured, centrifuge,  
174 and recover the remaining liquid. Determine the mass of  
175 the recovered remaining liquid from the mass of the re-  
176 ceiving tube, add the mobile phase A to make a solution  
177 so that each mL contains 0.2 mg of protein, and use this  
178 solution as the sample solution. Separately, to a suitable  
179 amount of Peginterferon Alfa-2b RS for Identification add  
180 the mobile phase A to make a solution so that each mL  
181 contains 0.2 mg of protein according to the labeled  
182 amount, proceed in the same manner as the sample solu-  
183 tion, and use this solution as the standard solution. Per-  
184 form the test with exactly 100  $\mu\text{L}$  each of the sample solu-  
185 tion and standard solution as directed under Liquid Chro-  
186 matography <2.01> according to the following conditions.  
187 Divide each peak detected in the chromatogram obtained  
188 from the sample solution to 6 groups, and regard the peak  
189 not observed in the chromatogram obtained from the  
190 standard solution as the other peak. Determine each peak  
191 area by the automatic integration method, and calculate  
192 their amounts by the area percentage method: the amount  
193 of the group 1 is 4.9 to 12.9%, the amount of the group 2  
194 is 42.9 to 54.4%, the amount of the group 3 is 14.3 to  
195 21.1%, the amount of the group 4 is 5.0 to 8.7%, the  
196 amount of the group 5 is 12.3 to 19.5%, the amount of the  
197 group 6 is not more than 2.1%, the amount of the other  
198 peak is not more than 0.5%. Furthermore, the total amount  
199 of the other peaks is not more than 1.0%.

200 Group 1: 3 to 5 peaks eluted immediately before the prin-  
201 cipal peak of the group 2

202 Group 2: the principal peak having the retention time be-  
203 tween about 8 minutes and about 13 minutes

204 Group 3 : 3 to 4 peaks eluted immediately after the prin-  
205 cipal peak of the group 2

206 Group 4 : 3 peaks eluted immediately after the principal  
207 peak of the group 3

208 Group 5 : 1 peak eluted immediately after the principal  
209 peak of the group 4

210 Group 6: 1 peak eluted at about 13 to 17 minutes after the  
211 peak of the group 5

212 *Operating conditions* –

213 Detector: An ultraviolet absorption photometer (wave-  
214 length: 214 nm).

215 Column: A stainless steel column 7.5 mm in inside di-  
216 ameter and 7.5 cm in length, packed with cation-exchange  
217 sulfopropylated silica gel (10  $\mu\text{m}$  in particle diameter).

218 Column temperature: A constant temperature of about  
219 28°C.

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

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

228 Flowing of mobile phase: Control the gradient by mix-  
229 ing the mobile phases A and B as directed in the following  
230 table.  
231

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

232 Flow rate: Adjust so that the retention time of the prin-  
233 cipal peak is 10 – 15 minutes.

234 Time span of measurement: 70 minutes.

235 *System suitability* –

236 Test for required detectability: When the procedure is  
237 run with 100  $\mu\text{L}$  of the standard solution under the above  
238 conditions: the SN ratio of the peak having the longest  
239 retention time among 3 peaks of the group 4 is not less  
240 than 10.

241 System performance: When the procedure is run with  
242 100  $\mu\text{L}$  of the standard solution under the above operating  
243 conditions, the chromatogram shows the same pattern  
244

245 with the chromatogram of Peginterferon Alfa-2b RS for  
246 Identification.

247 **Purity (1)** Impurities 1 – To a suitable amount of  
248 Peginterferon Alfa-2b (Genetical Recombination) add  
249 water to make a solution so that each mL contains 50  $\mu\text{g}$   
250 of protein, dilute to 2 times its volume with the diluting  
251 solution, and use this solution as the sample solution.  
252 Separately, to a suitable amount of Peginterferon Alfa-2b  
253 RS for Identification add water to make a solution so that  
254 each mL contains 50  $\mu\text{g}$  of protein according to the la-  
255 beled amount, dilute to 2 times its volume with the dilut-  
256 ing solution, and use this solution as the peginterferon  
257 standard solution. Separately, to a suitable amount of In-  
258 terferon Alfa-2b RS for Identification add water to make a  
259 solution so that each mL contains 28  $\mu\text{g}$  of protein ac-  
260 cording to the labeled amount, dilute to 2 times its volume  
261 with the diluting solution, and use this solution as the in-  
262 terferon standard solution. To a suitable amount of the  
263 interferon standard solution add the diluted diluting solu-  
264 tion (1 in 2) to make a solution so that each mL contains  
265 0.5  $\mu\text{g}$  of protein, and use this solution as the solution for  
266 confirming dyeing sensitivity. Separately, dissolve mole-  
267 cular mass marker for peginterferon alfa-2b in the di-  
268 luted diluting solution (1 in 2) to make a solution so that  
269 each mL contains about 3  $\mu\text{g}$  of protein, and use this solu-  
270 tion as the molecular mass standard solution. Heat the  
271 sample solution, the peginterferon standard solution, the  
272 interferon standard solution, the solution for confirming  
273 dyeing sensitivity and the molecular mass standard solu-  
274 tion in water bath for 2 minutes. Perform the test with 20  
275  $\mu\text{L}$  each of the sample solution, peginterferon standard  
276 solution, interferon standard solution and solution for  
277 confirming dyeing sensitivity and 10  $\mu\text{L}$  of the molecular  
278 mass standard solution by SDS-polyacrylamide gel elec-  
279 trophoresis according to the following conditions. Deter-  
280 mine the integrated optical density (IOD) of each stained  
281 band obtained from the sample solution, and calculate  
282 their amounts by the percentage method: the total amount  
283 of impurities other than free interferon alfa-2b, mono-  
284 opeginterferon alfa-2b and dipeginterferon alfa-2b is not  
285 more than 2.0%.

286 Diluting solution: To 50 mL of 0.126 mol/L tris buffer  
287 solution (pH 6.8) add 20 mL of glycerin, 10 g of sodium  
288 lauryl sulfate, 0.1 g of bromophenol blue and 5 mL of  
289 2-mercaptoethanol to dissolve, and add 0.126 mol/L tris  
290 buffer solution (pH 6.8) to make 100 mL.

291 *Operating conditions* –

292 Equipment: An electrophoresis apparatus consisting of  
293 a vertical type slab gel electrophoretic vessel and a con-  
294 stant voltage power unit.

295 Slab gel: 14% polyacrylamide slab gel (for tris-glycine)  
296 (size: 8 cm  $\times$  8 cm, thick: 1 mm).

297 Buffer solution for electrophoresis: Dissolve 2.9 g of  
298 2-amino-2-hydroxymethyl-1,3-propanediol, 14.4 g of  
299 glycine and 1g of sodium laurylsulfate in water to make  
300 1000 mL.

301 Electrophoresis: Energize at a constant voltage of 125  
302 V. Stop the electrophoresis when the front of the migra-  
303 tion of bromophenol blue reaches the lower end of the  
304 slab gel.

305 Staining: Immerse the slab gel in a mixture of methanol,  
306 water and acetic acid (5:4:1) for more than 2 hours to de-  
307 colorize, rinse with water, immerse in a solution of glutar-  
308 aldehyde (1 in 10) for 30 minutes, and wash with water  
309 for 20 minutes three times. Then, immerse the gel in the  
310 silver staining solution for peginterferon alfa-2b for 5  
311 minutes, wash quickly with water, and immerse in a solu-  
312 tion, prepared by dissolving 5 mg of citric acid monohy-  
313 drate in water and adding 50  $\mu\text{L}$  of formaldehyde and  
314 water to make 100 mL, until the band corresponding to  
315 free interferon alfa-2b is obtained from the solution for  
316 confirming dyeing sensitivity. Rinse the gel with water  
317 more than three times.

318 Analysis equipment: Equipment which measure inte-  
319 grated optical density (IOD) using an integrating densi-  
320 tometer or image analysis.

321 *System suitability* —

322 (1) The staining intensities of the main band of the  
323 sample solution and the peginterferon standard solution  
324 are the same, and the bands corresponding to monopegin-  
325 terferon alfa-2b and free interferon alfa-2b are observed,  
326 respectively.

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

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

332 (4) The band corresponding to free interferon alfa-2b is  
333 obtained from the solution for confirming dyeing sensitiv-  
334 ity.

335 (5) Prepare a calibration curve from the migration dis-  
336 tances of the bands of the molecular mass markers (pro-  
337 teins having molecular masses 14,400, 20,100, 30,000,  
338 43,000 and 67,000) obtained from the molecular mass  
339 standard solutions by plotting the logarithm of the molec-  
340 ular mass on the vertical axis and the migration distance  
341 on the horizontal axis. Determine the migration distance  
342 of the main band obtained from the peginterferon standard  
343 solution, and calculate the molecular mass using the cali-  
344 bration curve: the molecular mass is between 38,000 and  
345 44,000.

346 (2) Impurities 2—Proceed as directed in (1) according  
347 to the following conditions. Determine the integrated op-  
348 tical density (IOD) of each stained band obtained from the

349 sample solution, and calculate their amounts by the per-  
350 centage method: the total amount of impurities other than  
351 free interferon alfa-2b, monopeginterferon alfa-2b and  
352 dipeginterferon alfa-2b is not more than 3.5%.

353 Diluting solution: To 50 mL of 0.126 mol/L tris buffer  
354 solution (pH 6.8) add 20 mL of glycerin, 10 g of sodium  
355 lauryl sulfate and 0.1 g of bromophenol blue to dissolve,  
356 and add 0.126 mol/L tris buffer solution (pH 6.8) to make  
357 100 mL.

358 *Operating conditions* —

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

360 *System suitability* —

361 Proceed as directed in the system suitability in (1) ex-  
362 cept for the system suitability (5).

363 (3) Monomethoxy polyethylene glycol-12000—Use  
364 Peginterferon Alfa-2b (Genetical Recombination) as the  
365 sample solution. Separately, weigh accurately 0.15 g of  
366 monomethoxy polyethylene glycol-12000, add water to  
367 make exactly 100 mL, if necessary heat to about 40°C to  
368 dissolve, and use this solution as the standard solution.  
369 Perform the test with a volume of the sample solution,  
370 equivalent to about 0.5 mg of protein, and 10, 15, 20, 25  
371 and 30  $\mu\text{L}$  of the standard solution as directed under Liq-  
372 uid Chromatography <2.01> according to the following  
373 conditions, and determine each peak area. Prepare a cali-  
374 bration curve from the injection volumes and the peak  
375 areas of monomethoxy polyethylene glycol-12000 ob-  
376 tained from the standard solution, and calculate the  
377 amount of monomethoxy polyethylene glycol-12000 in  
378 Peginterferon Alfa-2b from the peak area of monomethoxy  
379 polyethylene glycol-12000 from the sample solution: not  
380 more than 5.9%.

381 Amount (%) of monomethoxy polyethylene gly-  
382 col-12000

$$383 = X/W \times 100$$

384 X: Amount ( $\mu\text{g}$ ) of monomethoxy polyethylene gly-  
385 col-12000 calculated using the calibration curve

386 W: Volume ( $\mu\text{L}$ ) of the sample solution taken

387 *Operating conditions* —

388 Detector: An evaporative light scattering detector.

389 Detector temperature: 50°C

390 Column: A stainless steel column 4.6 mm in inside di-  
391 ameter and 15 cm in length, packed with butylsilanized  
392 silica gel for liquid chromatography (5  $\mu\text{m}$  in particle di-  
393 ameter and 300  $\text{\AA}$  in pore diameter).

394 Column temperature: A constant temperature of about  
395 35°C.

396 Mobile phase A: Diluted trifluoroacetic acid (1 in  
397 1000).

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

400 Flowing of mobile phase: Control the gradient by mix-  
401 ing the mobile phases A and B as directed in the following  
402 table.  
403

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

404  
405 Flow rate: 1.0 mL per minute.

406 *System suitability* –

407 (1) When the procedure is run with a volume of Pegin-  
408 terferon Alfa-2b RS for Identification, equivalent to about  
409 0.3 mg of protein, under the above conditions,  
410 monomethoxy polyethylene glycol-12000 and peginter-  
411 feron alfa-2b are eluted in this order with the resolution  
412 between these peaks being not less than 1.5.

413 (2) The coefficient of determination ( $r^2$ ) of the calibra-  
414 tion curve obtained from the peak areas of the standard  
415 solutions is not less than 0.98.

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

420 **Assay** (1) Protein content—Weigh accurately a suita-  
421 ble volume of Peginterferon Alfa-2b (Genetical Recom-  
422 bination), add the diluting solution to make a solution so  
423 that each mL contains about 1 mg of protein, and use this  
424 solution as the sample solution. Separately, dissolve one  
425 Peginterferon Alfa-2b RS in a suitable volume of water.  
426 Pipet a suitable volume of this solution, add exactly the  
427 diluting solution to make a solution so that each mL con-  
428 tains 0.2 mg of protein, and use this solution as the stand-  
429 ard solution. Perform the test with 25  $\mu$ L each of the sam-  
430 ple solution and standard solution as directed under Liq-  
431 uid Chromatography <2.01> according to the following  
432 conditions, and determine the peak areas,  $A_{TM}$ ,  $A_{TD}$  and  
433  $A_{TY}$ , of monopeginterferon alfa-2b, dipeginterferon al-  
434 fa-2b and free interferon alfa-2b obtained from the sample  
435 solution, and the peak areas,  $A_{SM}$ ,  $A_{SD}$  and  $A_{SY}$ , of mon-  
436 opeginterferon alfa-2b, dipeginterferon alfa-2b and free  
437 interferon alfa-2b from the standard solution.

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

442 Protein concentration (mg/mL)

$$443 = M_S \times (A_{TM} + A_{TD} + A_{TY}) / (A_{SM} + A_{SD} +$$

$$444 A_{SY}) \times D$$

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

447  $D$ : Dilution factor for the sample solution

448 *Operating conditions* –

449 Detector: An ultraviolet absorption photometer (wave-  
450 length: 214 nm).

451 Column: A stainless steel column 8.0 mm in inside di-  
452 ameter and 50 cm in length, packed with hydroxypropy-  
453 lysilized silica gel for liquid chromatography (molec-  
454 ular mass cut-off: 4000 to hundreds of thousands).

455 Column temperature: A constant temperature of about  
456 25°C.

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

459 Flow rate: 1.0 mL per minute.

460 *System suitability* –

461 Test for required detectability: To a suitable amount of  
462 Interferon Alfa-2b RS for Identification add the diluting  
463 solution to make a solution so that each mL contains 0.25  
464  $\mu$ g of protein. When the procedure is run with 25  $\mu$ L of  
465 this solution under the above operating conditions, the  
466 peak of free interferon alfa-2b is detected.

467 System performance: To a suitable amount of Peginter-  
468 feron Alfa-2b RS for Identification add the diluting solu-  
469 tion to make a solution so that each mL contains 1 mg of  
470 protein. When the procedure is run with 25  $\mu$ L of this  
471 solution under the above operating conditions, dipeginter-  
472 feron alfa-2b, monopeginterferon alfa-2b and free interferon  
473 alfa-2b are eluted in this order with the resolution between  
474 dipeginterferon alfa-2b and monopeginterferon alfa-2b  
475 being not less than 1.0.

476 System repeatability: When the test is repeated 4 times  
477 with 25  $\mu$ L of the standard solution under the above oper-  
478 ating conditions, the relative standard deviation of the  
479 retention time of monopeginterferon alfa-2b is not more  
480 than 2.0%, and the relative standard deviation of the total  
481 of  $A_{SM}$ ,  $A_{SD}$  and  $A_{SY}$  is not more than 3.0%.

482 (2) Specific activity – Pipet a suitable volume of  
483 Peginterferon Alfa-2b (Genetical Recombination), add the  
484 cell culture medium for peginterferon alfa-2b to make a  
485 solution so that each mL contains about 600 units, and use  
486 this solution as the sample solution. Separately, dissolve  
487 one Peginterferon Alfa-2b RS in 0.7 mL of water. Pipet a  
488 suitable volume of this solution, add the cell culture me-  
489 dium for peginterferon alfa-2b to make a solution so that  
490 each mL contains 600 units, and use this solution as the  
491 standard solution. Perform the test with the sample solu-  
492 tion and standard solution according to the following  
493 method, and determine the potency.

494 *Procedure*

495 Use a 96-well culture plate in a horizontally long (8  
496 lines  $\times$  12 columns), dispense 50  $\mu$ L each of the sample

497 solution in the wells of the 1st and the 4th or the 5th and  
 498 the 8th lines of the 1st column. Dispense 50  $\mu\text{L}$  each of  
 499 the standard solution into the wells of the 2nd and the 3rd  
 500 or the 6th and the 7th lines of the 1st column. Dispense 50  
 501  $\mu\text{L}$  each of the cell culture medium for peginterferon alfa-  
 502 fa-2b in all wells. Mix thoroughly the solution in each  
 503 well of the 1st column, pipet 50  $\mu\text{L}$  each, and add in each  
 504 well of the 2nd column of the same lines. Repeat this  
 505 procedure to the 12th column, dilute the sample solution  
 506 and the standard solution in serially two-fold steps on the  
 507 culture plate, and use this plate as the test plate. Dispense  
 508 100  $\mu\text{L}$  each of a cell suspension, containing  $3.15 \times 10^5$  –  
 509  $3.85 \times 10^5$  cells per mL prepared using the culture medi-  
 510 um for peginterferon alfa-2b, in each well, and incubate  
 511 under atmosphere of 4 – 6% carbon dioxide at 36 – 37°C  
 512 for about 4 hours in a CO<sub>2</sub> incubator. After the incubation,  
 513 dispense 50  $\mu\text{L}$  each of EMC virus solution in each well,  
 514 put in a CO<sub>2</sub> incubator, and incubate for 16 – 21 hours.  
 515 Separately, for two culture plates dispense 50  $\mu\text{L}$  each of  
 516 the cell culture medium for peginterferon alfa-2b in the  
 517 wells of the 1st to the 4th lines of the 1st column, dispense  
 518 50  $\mu\text{L}$  each of the standard solution in the wells of the 5th  
 519 to the 8th lines of the 1st column, proceed in the same  
 520 manner as the test plate, and use these plates as the refer-  
 521 ence plates. In the middle of the incubation for 16 – 21  
 522 hours, take the reference plates, remove the solution in  
 523 each well, dispense 50 – 100  $\mu\text{L}$  each of crystal violet  
 524 staining solution in each well, stand for more than 10  
 525 minutes, remove the solution in each well, and wash each  
 526 well. When 0 – 10% of the cells in the 1st to 4th lines are  
 527 observed to be stained and when the well in which nearly  
 528 50% of the cells in the 5th to 8th lines are stained is ob-  
 529 served in the 4th to 9th columns, take the test plate. Re-  
 530 move the solution in each well, dispense 50  $\mu\text{L}$  each of a  
 531 solution, prepared by dissolving 0.5 g of  
 532 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium  
 533 bromide in the culture medium for peginterferon alfa-2b  
 534 to make 100 mL, in each well, put the plate in a CO<sub>2</sub> in-  
 535 cubator, and incubate for more than 3 hours. After the  
 536 incubation, dispense 100  $\mu\text{L}$  each of a solution of sodium  
 537 laurylsulfate in 0.01 mol/L hydrochloric acid TS (1 in 10)  
 538 in each well, and incubate in a CO<sub>2</sub> incubator overnight.  
 539 After the incubation, perform the test with the solution in  
 540 each well as directed under Ultraviolet-visible Spectro-  
 541 photometry <2.24>, and determine the absorbance at 570  
 542 nm from the baseline drawn at 690 nm. Calculate cor-  
 543 rected absorbances by subtracting the average of the ab-  
 544 sorbances of blank areas from the absorbance of each well  
 545 on the culture plate, plot the corrected absorbances on the  
 546 vertical axis and the column numbers on the horizontal  
 547 axis for the sample solution and the standard solution, and  
 548 connect each point. Determine the column numbers

549 showing 50% of the maximum corrected absorbance on  
 550 the graph, and use the column numbers as  $x$  and  $y$ , respec-  
 551 tively. Estimate the potency (Unit/mL) by the following  
 552 equation, and calculate the ratio of biological activity to  
 553 protein content.

$$554 \quad \text{Potency (Unit/mL)} \\ 555 \quad = \text{Potency of the standard solution (Unit/mL)} \times 2^{x-y} \times \\ 556 \quad D$$

557 2: Dilution factor of the dilution series  
 558  $D$ : Dilution factor for the sample solution

559 *System suitability*–

560 The column number showing 50% of the maximum  
 561 corrected absorbance obtained from the standard solution  
 562 on the test plate is in the range of 4 to 9.

563 **Containers and storage** Containers – Tight containers.  
 564 Storage – At  $-80^\circ\text{C}$ .

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

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

568 **Peginterferon alfa-2b RS**

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

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

572 **Cell culture medium for peginterferon alfa-2b** To  
 573 Eagle's minimal essential medium (EMEM) containing  
 574 Earle's salts add inactivated fetal bovine serum (final  
 575 concentration: 10%), sodium hydrogen carbonate (final  
 576 concentration: 2.7 mg/mL), HEPES-isotonic sodium chlo-  
 577 ride buffer solution (final concentration: 0.02 mol/L),  
 578 L-glutamine (final concentration: 0.004 mol/L) and peni-  
 579 cillin-streptomycin (final concentration: penicillin 125  
 580 U/mL, streptomycin 125  $\mu\text{g/mL}$ )

581 **Cell for peginterferon alfa-2b** Incubate diploid cell  
 582 derived from human foreskin (HFB4 cell) using the cul-  
 583 ture medium for peginterferon alfa-2b, and store. Incubate  
 584 the stored cell at  $36.5^\circ\text{C} \pm 0.5^\circ\text{C}$  using a flask and a roller  
 585 bottle. Subculture every 5 to 10 days by taking cells with  
 586 trypsin and diluting to 2-fold.

587 **Colloidal coomassie blue TS** Prepare for electro-  
 588 phoresis.

589 **Crystal violet staining solution** To 15 mg of crystal  
 590 violet and 5 mg of sodium chloride add 17.05 mL of a  
 591 mixture of water, ethanol (95) and formaldehyde  
 592 (20:10:1).

593 **EMC virus** Encephalomyocarditis (EMC) virus (for  
594 examples, ATCC VR-129 or VR-129B). Incubate the  
595 virus using Vero cells. Collect the culture supernatant,  
596 determine the virus titer, aliquot into small volume, and  
597 store at  $-70^{\circ}\text{C}$  or lower.

598 **Glutaraldehyde**  $\text{C}_5\text{H}_8\text{O}_2$  Oily liquid.

599 **Isoelectric point marker for peginterferon alfa-2b**  
600 A mixture of proteins for isoelectric point electrophoresis  
601 containing eight proteins having known isoelectric points  
602 in the range of pH 3 to 10 (amyloglucosidase [pI: 3.50],  
603 soybean trypsin inhibitor [pI: 4.55],  $\beta$ -lactoglobulin [pI:  
604 5.20], bovine carbonic anhydrase b [pI: 5.85], human  
605 carbonic anhydrase b [pI: 6.55], horse myoglobin [pI of  
606 the band in the acidic side: 6.85, pI of the band in the  
607 basic side: 7.35], lentil lectin [pI of the band in the acidic  
608 side: 8.15, pI of the band in the middle: 8.45, pI of the  
609 band in the basic side: 8.65] and trypsinogen [pI: 9.30])

610 **Molecular mass marker for peginterferon alfa-2b**  
611 A mixture of proteins for electrophoresis containing  
612  $\alpha$ -lactalbumin (molecular mass: 14,400), soybean trypsin  
613 inhibitor (molecular mass: 20,100), bovine carbonic an-  
614 hydrase (molecular mass: 30,000), ovalbumin (molecular  
615 mass: 43,000) and bovine serum albumin (molecular  
616 mass: 67,000).

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

619 *Molecular mass:* 11,000 – 13,000

620 *Polydispersity:* not more than 1.1.

621 *Moisture content:* not more than 0.50%.

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

623 *Storage:* At  $-30^{\circ}\text{C}$  or lower

624 **Phosphate buffer solution (pH 6.8)** Dissolve 27.6 g  
625 of sodium dihydrogen phosphate monohydrate and 28.4 g  
626 of anhydrous sodium sulfate in 1800 mL of water, adjust  
627 to pH 6.8 with sodium hydroxide, and add water to make  
628 2000 mL.

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

633 **Sodium dihydrogen phosphate monohydrate**  
634  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$

635 **0.05 mol/mL Sodium phosphate buffer solution (pH**  
636 **7.0)** Dissolve 3.55 g of anhydrous disodium hydrogen  
637 phosphate in 450 mL of water, adjust to pH 7.0 with  
638 phosphoric acid, and add water to make 500 mL.

639 **0.126 mol/L Tris buffer solution (pH 6.8)** Dissolve  
640 15.3 g of 2-amino-2-hydroxymethyl-1,3-propanediol in

641 about 800 mL of water, adjust to pH 6.8 with hydrochloric  
642 acid, and add water to make 1000 mL.

643 **Add the following to 9.42 Solid Sup-**  
644 **ports/Column Packings for Chromatog-**  
645 **raphy:**

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

648

649