

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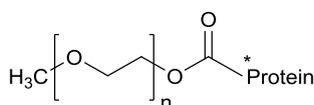
3
4
5
6 CDLPQTHSLG SRRTLMLLAG MRRISLFSCL KDRHDFGFPQ EEFGNQFQKA
7 ETIPVLHEMI QQIFNLFSTK DSSAAWDETL LDKFYTELYQ QLNDLEACVI
8 QGVGVETETPL MKEDSILAVR KYFQRITLYL KKKKYSPCAW EVVRAEIMRS
9 FSLSTNLQES LRSKE

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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 * α -amino group of N-terminal cysteine residue, ϵ -amino group of
 15 lysine residue, imidazole group of histidine residue, hydroxyl groups
 16 of tyrosine and serine residues

17 C₈₅₈H₁₃₄₉N₂₂₉O₂₅₂S₉: 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 4.8×10^7 units and not more than 8.7×10^7
 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 μ 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 μ 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 μL 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 μL . 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 μL . 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 μL 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 μm 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 μL 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 μL 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 μg
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 μg 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 μg 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 μg 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 μg 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 μL each of the sample solution, peginterferon standard
276 solution, interferon standard solution and solution for
277 confirming dyeing sensitivity and 10 μL 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 μL 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 μL 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 (μg) of monomethoxy polyethylene gly-
385 col-12000 calculated using the calibration curve

386 W: Volume (μL) 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 μm in particle di-
393 ameter and 300 Å 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 μ 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 μ g of protein. When the procedure is run with 25 μ 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 μ 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 μ 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 μ 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 μL 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 μL each of the cell culture medium for peginterferon alfa-2b
 502 in all wells. Mix thoroughly the solution in each
 503 well of the 1st column, pipet 50 μL 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 μL each of a cell suspension, containing 3.15×10^5 –
 509 3.85×10^5 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 CO₂ incubator. After the incubation,
 513 dispense 50 μL each of EMC virus solution in each well,
 514 put in a CO₂ incubator, and incubate for 16 – 21 hours.
 515 Separately, for two culture plates dispense 50 μL 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 μL 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 μL 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 μL 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 CO₂ in-
 535 cubator, and incubate for more than 3 hours. After the
 536 incubation, dispense 100 μL 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 CO₂ 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°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°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], β -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 α -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°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