⟨92⟩ GROWTH FACTORS AND CYTOKINES USED IN CELL THERAPY MANUFACTURING

INTRODUCTION

Qualification of reagents, source materials, and control of the manufacturing process are key elements that ensure the quality and safety of cell therapies. Growth factors and cytokines are important for the maintenance, growth, selection, and purification of cultures of cell therapy products. This chapter describes the accepted tests, procedures, and acceptance criteria for growth factors and cytokines that may be involved in the manufacturing of cell therapy products.

RECOMBINANT HUMAN INTERLEUKIN 4 (rhIL-4)

MHKCDITLQE	IIKTLNSLTE	QKTLCTELTV	TDIFAASKNT
TEKETFCRAA	TVLRQFYSHH	EKDTRCLGAT	AQQFHRHKQL
IRFLKRLDRN	LWGLAGLNSC	PVKEANQSTL	ENFLERLKTI
MREKYSKCSS			

$C_{658}H_{1071}N_{193}O_{197}S_8$ 15,096 Da

rhIL-4 is a single-chain polypeptide of 130 amino acid residues expressed in *Escherichia coli*. It is produced as a lyophilized powder and contains NLT 0.5×10^7 USP Units of IL-4/mg of total protein. Process specific host-cell DNA impurities in IL-4 with limits of less than 1 ng/mg are determined as described in *Nucleic Acid-Based Techniques—Approaches for Detecting Trace Nucleic Acids (Residual DNA Testing)* ⟨1130⟩. Neither manufacturing license nor market approval is required for IL-4 intended for use as an ancillary material during manufacturing. Following are typical IL-4 quality attributes.

IDENTIFICATION

- **A.** Amino-terminal sequence analysis of at least eight amino acids is performed with an automated sequencer. Stepwise-released phenylthiohydantoin amino acids are identified with on-line reversed-phase high-performance liquid chromatography, on the basis of their elution times.
- **B.** Use the electrophoresis method followed by western blotting analysis to visualize the IL-4 protein. The method is sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), described in the test for *Purity*.

  Phosphate buffered saline; Laemmli sample buffer, reducing; and **Laemmli sample buffer, nonreducing:** Proceed as directed in the test for *Purity* in the *Assay*.

  Standard stock solution: 50 µg/mL of reconstituted USP rHuman Interleukin 4 RS in *Phosphate buffered saline*. [NOTE—Do not agitate while mixing; swirl gently.]

  Standard solution: 20 µg/mL of IL-4, from *Standard stock solution*, in *Phosphate buffered saline*

  Standard solution, reducing: Combine 20 µL of *Standard solution* and 5 µL of *Laemmli sample buffer, reducing*.

  Standard solution, nonreducing: Combine 20 µL of *Standard solution* and 5 µL of *Laemmli sample buffer, nonreducing*.

  Sample stock solution: 50 µg/mL of reconstituted IL-4 in *Phosphate buffered saline*. [NOTE—Do not agitate while mixing; swirl gently.]

  Sample solution: 20 µg/mL of IL-4, from *Sample stock solution*, in *Phosphate buffered saline*

  Sample solution, reducing: Combine 20 µL of *Sample solution* and 5 µL of *Laemmli sample buffer, reducing*.

  Sample solution, nonreducing: Combine 20 µL of *Sample solution* and 5 µL of *Laemmli sample buffer, nonreducing*.

  Analysis

  Samples: *Standard solution, reducing; Standard solution, nonreducing; Sample solution, reducing;* and *Sample solution, nonreducing*

  Western blotting: After electrophoresis, the proteins are transferred onto a polyvinylidene fluoride (PVDF) membrane using standard procedures. Incubate the membrane for 1 h at room temperature with *Phosphate buffered saline* containing 0.1% Tween 20 and 5% skim milk powder. The membrane is then incubated with an anti-IL-4 antibody[1] (diluted appropriately in *Phosphate buffered saline*), followed by incubation with a secondary antibody at room temperature under gentle agitation for 1 h for each of the antibodies. The IL-4 protein band is identified by developing the membrane using a suitable detection system.[2]

  Acceptance criteria: The developed Western blot should give a positive signal equivalent to the USP rHuman Interleukin 4 RS.

ASSAY

- **PURITY:** [NOTE—Purity is determined on the bulk material.] SDS-PAGE is performed as described under *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* ⟨1056⟩ under reducing and nonreducing conditions.

  Molecular weight marker: Use a suitable molecular weight marker containing protein bands between 10 and 200 kDa.

  Phosphate buffered saline: 2.67 mM of potassium chloride, 1.47 mM of potassium phosphate (KH_2PO_4), 137.93 mM of sodium chloride, and 8.06 mM of dibasic sodium phosphate in water. Adjust to a pH of 7.0–7.3.

  Laemmli sample buffer, nonreducing: 100 mM TRIS-HCl, pH 6.8, 50% glycerol, 0.25% bromophenol blue indicator, and 10% sodium lauryl sulfate in water

  Laemmli sample buffer, reducing: Add 2.5 µL mercaptoethanol to 50 µL of *Laemmli sample buffer, nonreducing*.

  Sample stock solution: 400 µg/mL of bulk IL-4 in *Phosphate buffered saline*

[1] A suitable anti-IL-4 antibody can be obtained from commercial sources (e.g., Dianova Inc.).
[2] A suitable detection system can be obtained from commercial sources (e.g., Pierce/Perbio Science).

Sample solution 1: Combine 20 µL of *Sample stock solution* and 5 µL of *Laemmli sample buffer, nonreducing*.
Sample solution 2: Combine 20 µL of *Sample stock solution* and 5 µL of *Laemmli sample buffer, reducing*.
Control A stock solution: 4 µg/mL of IL-4, from *Sample stock solution*, in *Phosphate buffered saline*. [NOTE—*Control A solutions* are run in triplicates in both reducing and nonreducing conditions.]
Control A solution 1: Combine 20 µL of *Control A stock solution* and 5 µL of *Laemmli sample buffer, nonreducing*.
Control A solution 2: Combine 20 µL of *Control A stock solution* and 5 µL of *Laemmli sample buffer, reducing*.
Control B stock solution: 12 µg/mL of IL-4, from *Sample stock solution*, in *Phosphate buffered saline*. [NOTE—*Control B solutions* are run in duplicates in both reducing and nonreducing conditions.]
Control B solution 1: Combine 20 µL of *Control B stock solution* and 5 µL of *Laemmli sample buffer, nonreducing*.
Control B solution 2: Combine 20 µL of *Control B stock solution* and 5 µL of *Laemmli sample buffer, reducing*.
Electrophoretic conditions
(See *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* ⟨1056⟩.)
Mode: Discontinuous PAGE gel
Stacking gel: 4% acrylamide
Resolving gel: 12% acrylamide
Run conditions: 10 min at 100 V; then 30 min at 200 V
Protein detection: Silver stain
Analysis
Samples: *Sample solution 1, Sample solution 2, Control A solution 1, Control A solution 2, Control B solution 1*, and *Control B solution 2*
Incubate 25 µL of each *Sample solution* and *Control solution* under nonreducing conditions for 5 min at 60°, and load onto the gel. Incubate 20 µL of each *Sample solution* and *Control solution* under reducing conditions for 5 min at 60°, and load onto the gel. After silver staining and scanning the whole gel, determine the intensity of all detectable protein bands by densitometry, and calculate the percentage of each detectable protein band, in the *Sample solution*, twice by comparing the pixel intensity of each contaminating band with the mean value of *Control solutions A* and *B*, respectively, by the formulas:

$$\text{Result} = (A_{100}) \times 1/(A_1) \text{ and}$$

$$\text{Result} = (A_{100}) \times 3/(A_3)$$

A_{100} = intensity of one contaminating band of the *Sample solution*
A_1 = mean intensity of all detectable bands of *Control A solution*
A_3 = mean intensity of all detectable bands of *Control B solution*

IL-4 control solutions analysis should yield one detectable band with an apparent molecular weight of approximately 15 kDa. If values calculated by means of *Control A solution* are different from those revealed by comparison with *Control B solution*, the value corresponding to the highest amount of impurity should be taken. If the intensity of one of the contaminating bands is lower than the value of *Control A solution* (corresponding to 1%), the value of this contamination is set to 1%. The purity of the sample solution is then calculated:

$$\text{Result} = 100 - \Sigma\, C_n$$

C = percentage of each contamination given in rounded whole numbers
n = number of contaminants of the IL-4 *Sample solution*

Acceptance criteria: The purity of IL-4 is NLT 97%, as determined by SDS-PAGE.

• **PROTEIN CONTENT:** [NOTE—Protein content is determined on the basis of the packaged product.]
Phosphate buffered saline: Proceed as directed in the test for *Purity*.
Sample solution: 50 µg/mL of IL-4 in *Phosphate buffered saline*. [NOTE—Do not agitate while mixing; swirl gently.]
Blank: *Phosphate buffered saline*
Spectrometric conditions
(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)
Mode: UV
Pathlength: 1 cm
Analytical wavelength: 280 nm
Analysis
Samples: *Sample solution* and *Blank*
Calculate the protein concentration:

$$C = A_{280}/0.63$$

C = IL-4 concentration of the *Sample solution* (mg/mL)
A_{280} = absorbance at 280 nm

SPECIFIC TESTS

• **BIOIDENTITY:** [NOTE—The biological activity measurement is determined on the basis of the packaged product.]
RPMI 1640 medium with L-glutamine: Prepare a mixture of the ingredients in the quantities shown in sufficient water to obtain 1 L of medium, and sterilize by filtration:

Material	Quantity
Calcium nitrate ($Ca(NO_3) \cdot 4H_2O$)	100 mg
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	100 mg
Potassium chloride	400 mg
Sodium chloride	6000 mg
Sodium phosphate, dibasic anhydrous	800 mg
Sodium bicarbonate	2000 mg
Glycine	10 mg
L-Arginine	200 mg
L-Asparagine	50 mg
L-Aspartic acid	20 mg
L-Polyvinylidene fluoride L-cystine dihydrochloride	20 mg
L-Glutamic acid	20 mg
L-Glutamine	300 mg
L-Histidine	15 mg
L-Hydroxyproline	20 mg
L-Isoleucine	50 mg
L-Leucine	50 mg
L-Lysine hydrochloride	40 mg
L-Methionine	15 mg
L-Phenylalanine	15 mg
L-Proline	20 mg
L-Serine	30 mg
L-Threonine	20 mg
L-Tryptophan	5 mg
L-Tyrosine disodium salt dihydrate	20 mg
L-Valine	20 mg
Biotin	0.2 mg
Choline chloride	3 mg
D-Calcium pantothenate	0.25 mg
Folic acid	1 mg
i-Inositol	35 mg
Niacinamide	1 mg
para-Aminobenzoic acid	1 mg
Pyridoxine hydrochloride	1 mg
Riboflavin	0.2 mg
Thiamine hydrochloride	1 mg
Vitamin B_{12}	0.005 mg
D-Glucose (dextrose)	2000 mg
Glutathione (reduced)	1 mg
Phenol red	5 mg

Growth medium: Using aseptic procedures, prepare the following tissue culture medium:

RPMI-1640 with L-glutamine	500 mL
Sodium pyruvate 100 mM	5 mL
Fetal bovine serum	50 mL

Human rGM-CSF[a]	3×10^4 International Units

[a] Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) is added extemporaneously.

Sterilize by filtration, and store at between 2° to 8°. Use within 1 month. Add GM-CSF immediately before use.

Assay medium: Use *Growth medium* containing no GM-CSF.

Phosphate buffered saline: Proceed as directed in the test for *Purity* in the *Assay*.

Resazurin solution: 11 mg of resazurin in 100 mL of *Phosphate buffered saline*. [NOTE—Sterile filter and store solution protected from light at 4°. *Resazurin solution* is stable for at least 6 months if treated under sterile conditions.]

[NOTE—For all *Standard* and *Sample solutions*, IL-4 concentration is determined by photometry at 280 nm using an extinction coefficient (ε) of 0.63 $mg^{-1}cm^{-1}$.]

Standard stock solution: 50 µg/mL of USP rHuman Interleukin 4 RS in *Phosphate buffered saline*. [NOTE—Do not agitate while mixing; swirl gently.]

Standard solutions: 36, 12, 4, 1.33, 0.44, 0.15, 0.05, 0.016, 0.006 ng/mL of IL-4, from *Standard stock solution* in *Assay medium*

Sample stock solution: 50 µg/mL of IL-4 in *Phosphate buffered saline*. [NOTE—Do not agitate while mixing; swirl gently.]

Sample solutions: 36, 12, 4, 1.33, 0.44, 0.15, 0.05, 0.016, 0.006 ng/mL of IL-4, from *Sample stock solution* in *Assay medium*

Control solution: Use the *Assay medium*.

Cell culture preparation: Prepare cell cultures of the human factor-dependent TF-1 cell line (ATCC No. CRL-2003), following the protocol described in the ATCC information sheet. Passage the cultures every 2–3 days, using 1:3 subcultures of the cells for up to 1 month. Seed density should be 0.5×10^6 cells/mL, and maximal density should be 3×10^6 cells/mL. Viability of the cells should be >90%. Maximal passage number is 24, and maximal cultivation time from thawing is 28 days. After 28 days, initiate a new culture. Cells are propagated using *Growth medium* at 37°, supplemented with air and 5% carbon dioxide.

Analysis

Samples: *Standard solutions, Sample solutions,* and *Control solution*

The activity of the *Sample solution* is determined in duplicate. Wash the cells three times in *Phosphate buffered saline*. Plate 2×10^4 TF-1 cells resuspended in 100 µL of *Assay medium* per well in 96-well, flat-bottom microplates. Incubate for 72 h at 37° and 5% CO_2 atmosphere in a humidified incubator in the presence or absence of various concentrations of *Standard solution, Sample solution*, or *Control solution* by adding 100 µL of the corresponding solution to each well. Add 30 µL of *Resazurin solution* to each well and incubate for another 24 h. Determine the fluorescence intensity per well by reading the plate with a microplate reader using 544 nm (excitation) and 590 nm (emission). Convert the fluorescence intensity in each well to a percentage of the maximum fluorescence intensity. For the *Sample solution* and *Standard solution*, plot the percentage of fluorescence intensity versus the concentration of the respective solution. By using the least squares method of regression analysis, compute the ED50 in ng/mL of the *Sample solution* and the *Standard solution*. The coefficient of determination for curve regression should be ≥ 0.98. Calculate the potency in USP Interleukin 4 Units/mg:

$$\text{Result} = A \times E_S/E_U$$

A = activity of USP rHuman Interleukin 4 RS (USP units/mg)
E_S = determined ED_{50} of *Standard solution* (ng/mL)
E_U = determined ED_{50} of *Sample solution* (ng/mL)

Acceptance criteria: NLT 0.5×10^7 USP IL-4 Units/mg

- **STERILITY TESTS** ⟨71⟩: Meets the requirements
- **BACTERIAL ENDOTOXINS TEST** ⟨85⟩: It contains NMT 50 USP Endotoxin Units/mg.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, and store at –80°.
- **LABELING:** Material is of recombinant DNA origin.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Endotoxin RS
USP rHuman Interleukin 4 RS