

Quantikine[®] ELISA

Human IL-6 Immunoassay

Catalog Number D6050

S6050

PD6050

For the quantitative determination of human interleukin 6 (IL-6) concentrations in cell culture supernates, serum, and plasma.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

Interleukin 6 (IL-6) is a pleiotropic α -helical 22-28 kDa phosphorylated and variably glycosylated (1-5) cytokine that plays important roles in acute phase reactions, inflammation, hematopoiesis, bone metabolism, and cancer progression. Mature human IL-6 is 183 amino acids (aa) in length and shares 41% aa sequence identity with mouse and rat IL-6 (6). Alternate splicing generates several isoforms with internal deletions, some of which exhibit antagonistic properties (7-10). Cells known to express IL-6 include CD8⁺ T cells, fibroblasts, synoviocytes, adipocytes, osteoblasts, megakaryocytes, endothelial cells (under the influence of endothelins), sympathetic neurons, cerebral cortex neurons, adrenal medulla chromaffin cells, retinal pigment cells, mast cells, keratinocytes, Langerhans cells, fetal and adult astrocytes, neutrophils, monocytes, eosinophils, colonic epithelial cells, B1 B cells, and pancreatic islet beta cells (2, 7, 10-33). IL-6 production is generally correlated with cell activation and is normally kept in control by glucocorticoids, catecholamines and secondary sex steroids (2). Normal human circulating IL-6 is in the 1 pg/mL range, with slight elevations during the menstrual cycle, modest elevations in certain cancers, and large elevations after surgery (34-38).

IL-6 induces signaling through a cell surface heterodimeric receptor complex composed of a ligand binding subunit (IL-6 R) and a signal transducing subunit (gp130). IL-6 binds to IL-6 R, triggering IL-6 R association with gp130 and gp130 dimerization (39). gp130 is also a component of the receptors for CLC, CNTF, CT-1, IL-11, IL-27, LIF, and OSM (40). Soluble forms of IL-6 R are generated by both alternate splicing and proteolytic cleavage (3). In a mechanism known as trans-signaling, complexes of soluble IL-6 and IL-6 R elicit responses from gp130-expressing cells that lack cell surface IL-6 R (1, 3). Trans-signaling enables a wider range of cell types to respond to IL-6, as the expression of gp130 is ubiquitous, while that of IL-6 R is predominantly restricted to hepatocytes, monocytes, and resting lymphocytes (1-3). Soluble splice forms of gp130 block trans-signaling from IL-6/IL-6 R but not from other cytokines that use gp130 as a co-receptor (3, 41).

IL-6, along with TNF- α and IL-1, drives the acute inflammatory response, is almost solely responsible for fever and the acute phase response in the liver, and is important in the transition from acute inflammation to either acquired immunity or chronic inflammatory disease (1-4). It contributes to chronic inflammation in conditions such as obesity, insulin resistance, inflammatory bowel disease, inflammatory arthritis, and sepsis when dysregulated, often involving IL-6 trans-signaling (1, 2). It plays an important role in differentiation of naïve T cells to Th17 inflammatory cells in the presence of TGF- β . IL-6 modulates bone resorption and is a major effector in inflammatory joint destruction in rheumatoid arthritis through promoting Th17 T cell activity (1). It contributes to atherosclerotic plaque development and destabilization (2). IL-6 can also have anti-inflammatory effects, however, such as in skeletal muscle where it is secreted in response to exercise (2). It promotes hematopoiesis by being a growth factor for hematopoietic stem cells, induces B cell maturation to plasma cells, and perpetuates multiple myeloma (1, 42). IL-6 also promotes, but probably does not initiate, other types of inflammation-associated carcinogenesis, such as colitis-associated cancer (1).

The Quantikine Human IL-6 Immunoassay is a 4.5 hour solid phase ELISA designed to measure IL-6 in cell culture supernates, serum, and plasma. It contains recombinant human IL-6 and antibodies raised against recombinant human IL-6 and has been shown to accurately quantitate the recombinant factor. Results obtained using natural IL-6 showed linear curves that were parallel to the standard curves obtained using the *E. coli*-expressed Quantikine kit standards. These results indicate that this kit can be used to determine relative mass values for natural human IL-6.

It has been observed in our laboratories that the measurement of IL-6 is insensitive to the addition of the recombinant form of the IL-6 soluble receptor. Therefore it is probable that experimental sample measurements reflect the total amount of IL-6 present, i.e., the total amount of free IL-6 plus the amount of IL-6 initially bound to soluble receptors, if any are present in the samples. High levels of high-affinity autoantibodies to IL-6 in the serum of some blood donors have been reported (36, 37). Such autoantibodies have the potential to interfere with the measurement of IL-6 by ELISA immunoassays.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for IL-6 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any IL-6 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for IL-6 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of IL-6 bound in the initial step. The color development is stopped and the intensity of the color is measured.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- It is important that the Calibrator Diluent selected for the standard curve be consistent with the samples being assayed.
- If samples generate values higher than the highest standard, dilute the samples with the appropriate Calibrator Diluent and repeat the assay. If cell culture supernate samples require larger dilutions, perform an intermediate dilution with culture media and the final dilution with the appropriate Calibrator Diluent.
- Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Variations in sample collection, processing, and storage may cause sample value differences.
- This assay is designed to eliminate interference by soluble receptors, binding proteins, and other factors present in biological samples. Until all factors have been tested in the Quantikine Immunoassay, the possibility of interference cannot be excluded.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- When using an automated plate washer, adding a 30 second soak period following the addition of Wash Buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

| PART | PART # | CATALOG # D6050 | CATALOG # S6050 | DESCRIPTION | STORAGE OF OPENED/ RECONSTITUTED MATERIAL |
|----------------------------|--------|--------------------|--------------------|---|--|
| IL-6 Microplate | 890045 | 1 plate | 6 plates | 96 well polystyrene microplate (12 strips of 8 wells) coated with a mouse monoclonal antibody against IL-6. | Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of zip-seal. May be stored for up to 1 month at 2-8 °C.* |
| IL-6 Standard | 890047 | 1 vial | 6 vials | 1.5 ng/vial of recombinant human IL-6 in a buffered protein base with preservatives; lyophilized. | Aliquot and store for up to 1 month at ≤ -20 °C in a manual defrost freezer.* Avoid repeated freeze-thaw cycles. |
| IL-6 Conjugate | 890046 | 1 vial | 6 vials | 21 mL/vial of polyclonal antibody against IL-6 conjugated to horseradish peroxidase with preservatives. | May be stored for up to 1 month at 2-8 °C.* |
| Assay Diluent RD1W | 895117 | 1 vial | 6 vials | 11 mL/vial of a buffered protein base with preservatives. | |
| Calibrator Diluent RD5T | 895175 | 1 vial | 6 vials | 21 mL/vial of a buffered protein base with preservatives. <i>For cell culture supernate samples.</i> | |
| Calibrator Diluent RD6F | 895018 | 1 vial | 6 vials | 21 mL/vial of animal serum with preservatives. <i>For serum/plasma samples.</i> | |
| Wash Buffer Concentrate | 895003 | 1 vial | 6 vials | 21 mL/vial of a 25-fold concentrated solution of buffered surfactant with preservatives. | |
| Color Reagent A | 895000 | 1 vial | 6 vials | 12 mL/vial of stabilized hydrogen peroxide. | |
| Color Reagent B | 895001 | 1 vial | 6 vials | 12 mL/vial of stabilized chromogen (tetramethylbenzidine). | |
| Stop Solution | 895032 | 1 vial | 6 vials | 6 mL/vial of 2 N sulfuric acid. | |
| Plate Sealers | N/A | 4 strips | 24 strips | Adhesive strips. | |

* Provided this is within the expiration date of the kit.

D6050 contains sufficient materials to run an ELISA on one 96 well plate.

S6050 (SixPak) contains sufficient materials to run ELISAs on six 96 well plates.

This kit is also available in a PharmPak (R&D Systems, Catalog # PD6050). PharmPaks contain sufficient materials to run ELISAs on 50 microplates. Specific vial counts of each component may vary. Please refer to the literature accompanying your order for specific vial counts.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- 500 mL graduated cylinder.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- Test tubes for dilution of standards.
- Human IL-6 Controls (optional; available from R&D Systems).

PRECAUTIONS

Calibrator Diluent RD6F contains sodium azide which may react with lead and copper plumbing to form explosive metallic azides. Flush with large volumes of water during disposal.

The Stop Solution provided with this kit is an acid solution. Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling.

SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA, heparin, or citrate as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

REAGENT PREPARATION

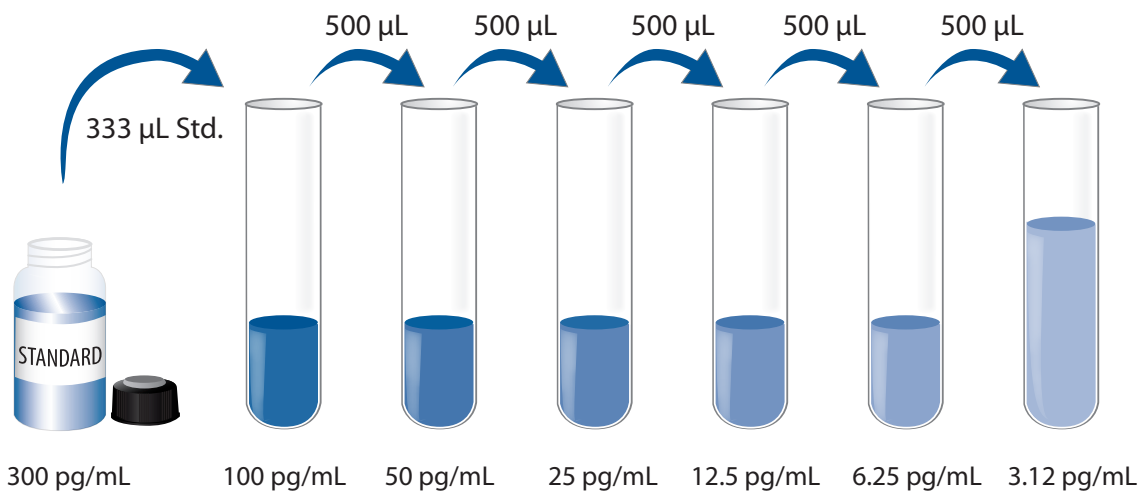
Bring all reagents to room temperature before use.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 μL of the resultant mixture is required per well.

IL-6 Standard - Reconstitute the IL-6 Standard with 5.0 mL of Calibrator Diluent RD5T (*for cell culture supernate samples*) or Calibrator Diluent RD6F (*for serum/plasma samples*). This reconstitution produces a stock solution of 300 pg/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 667 μL of the appropriate Calibrator Diluent into the 100 pg/mL tube and 500 μL of diluent into each remaining tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted standard serves as the high standard (300 pg/mL). The appropriate Calibrator Diluent serves as the zero standard (0 pg/mL).



ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all standards, samples, and controls be assayed in duplicate.

1. Prepare all reagents and working standards as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 100 μL of Assay Diluent RD1W to each well.
4. Add 100 μL of Standard, sample, or control per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature. A plate layout is provided to record standards and samples assayed.
5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 μL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
6. Add 200 μL of IL-6 Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.
7. Repeat the aspiration/wash as in step 5.
8. Add 200 μL of Substrate Solution to each well. Incubate for 20 minutes at room temperature. **Protect from light.**
9. Add 50 μL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

CALCULATION OF RESULTS

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density.

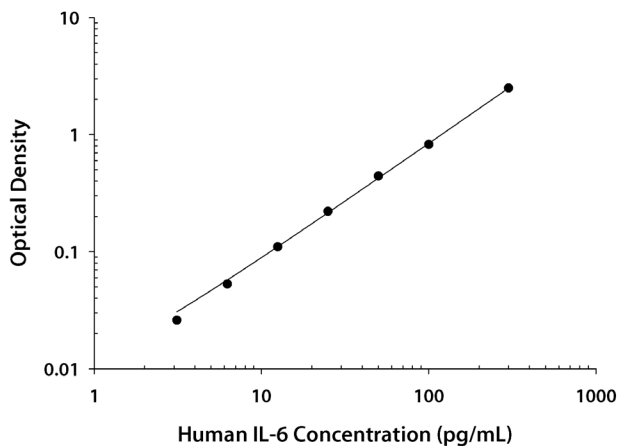
Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the IL-6 concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

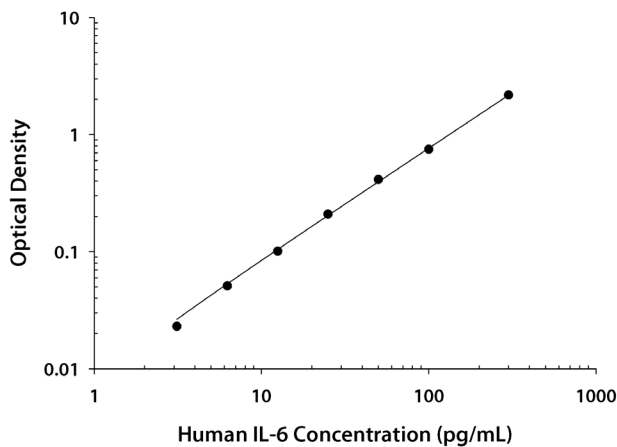
These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.

CALIBRATOR DILUENT RD5T



| (pg/mL) | O.D. | Average | Corrected |
|---------|----------------|---------|-----------|
| 0 | 0.022 0.028 | 0.025 | — |
| 3.12 | 0.050 0.052 | 0.051 | 0.026 |
| 6.25 | 0.078 0.078 | 0.078 | 0.053 |
| 12.5 | 0.134 0.136 | 0.135 | 0.110 |
| 25 | 0.247 0.245 | 0.246 | 0.221 |
| 50 | 0.472 0.465 | 0.468 | 0.443 |
| 100 | 0.865 0.836 | 0.850 | 0.825 |
| 300 | 2.524 2.515 | 2.520 | 2.495 |

CALIBRATOR DILUENT RD6F



| (pg/mL) | O.D. | Average | Corrected |
|---------|----------------|---------|-----------|
| 0 | 0.025 0.029 | 0.027 | — |
| 3.12 | 0.049 0.051 | 0.050 | 0.023 |
| 6.25 | 0.078 0.077 | 0.078 | 0.051 |
| 12.5 | 0.127 0.129 | 0.128 | 0.101 |
| 25 | 0.236 0.236 | 0.236 | 0.209 |
| 50 | 0.438 0.442 | 0.440 | 0.413 |
| 100 | 0.780 0.773 | 0.776 | 0.749 |
| 300 | 2.176 2.221 | 2.198 | 2.171 |

PRECISION

Intra-assay Precision (Precision within an assay)

Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays)

Three samples of known concentration were tested in twenty separate assays to assess inter-assay precision.

CELL CULTURE SUPERNATE ASSAY

| Sample | Intra-Assay Precision | | | Inter-Assay Precision | | |
|--------------------|-----------------------|------|-----|-----------------------|------|-----|
| | 1 | 2 | 3 | 1 | 2 | 3 |
| n | 20 | 20 | 20 | 20 | 20 | 20 |
| Mean (pg/mL) | 15.8 | 95.6 | 179 | 16.4 | 98.8 | 188 |
| Standard deviation | 0.7 | 3.0 | 3.1 | 0.6 | 2.5 | 3.7 |
| CV (%) | 4.4 | 3.1 | 1.7 | 3.7 | 2.5 | 2.0 |

SERUM/PLASMA ASSAY

| Sample | Intra-Assay Precision | | | Inter-Assay Precision | | |
|--------------------|-----------------------|------|-----|-----------------------|-----|-----|
| | 1 | 2 | 3 | 1 | 2 | 3 |
| n | 20 | 20 | 20 | 20 | 20 | 20 |
| Mean (pg/mL) | 16.8 | 97.7 | 186 | 17.2 | 101 | 191 |
| Standard deviation | 0.7 | 1.6 | 3.8 | 1.1 | 3.3 | 7.2 |
| CV (%) | 4.2 | 1.6 | 2.0 | 6.4 | 3.3 | 3.8 |

RECOVERY

The recovery of IL-6 spiked to three different levels in samples throughout the range of the assay in various matrices was evaluated.

| Sample Type | Average % Recovery | Range |
|--------------------------|--------------------|---------|
| Cell culture media (n=5) | 98 | 94-103% |
| Serum (n=5) | 93 | 86- 99% |
| EDTA plasma (n=5) | 95 | 84-101% |
| Heparin plasma (n=5) | 90 | 88-98% |
| Citrate plasma (n=5) | 91 | 82-95% |

SENSITIVITY

The minimum detectable dose (MDD) of IL-6 is typically less than 0.70 pg/mL.

The MDD was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

LINEARITY

To assess the linearity of the assay, samples were spiked with high concentrations of IL-6 in various matrices and diluted with the appropriate Calibrator Diluent to produce samples with values within the dynamic range of the assay.

| | | Cell culture media (n=4) | Serum (n=4) | EDTA plasma (n=4) | Heparin plasma (n=4) | Citrate plasma (n=4) |
|------|-----------------------|--------------------------|-------------|-------------------|----------------------|----------------------|
| 1:2 | Average % of Expected | 99 | 97 | 101 | 103 | 101 |
| | Range (%) | 96-101 | 92-100 | 98-105 | 96-109 | 96-106 |
| 1:4 | Average % of Expected | 100 | 101 | 104 | 106 | 105 |
| | Range (%) | 93-110 | 93-107 | 97-110 | 97-113 | 101-109 |
| 1:8 | Average % of Expected | 96 | 102 | 100 | 104 | 106 |
| | Range (%) | 92-100 | 96-108 | 86-112 | 93-111 | 101-111 |
| 1:16 | Average % of Expected | 94 | 103 | 99 | 105 | 101 |
| | Range (%) | 83-108 | 93-111 | 90-110 | 99-107 | 90-114 |

CALIBRATION

This immunoassay is calibrated against highly purified *E. coli*-expressed recombinant human IL-6 produced at R&D Systems. The NIBSC/WHO International Standard for IL-6 (89/548), which was intended as a potency standard, was evaluated in this kit. The NIBSC/WHO standard is a CHO cell-derived recombinant human IL-6.

The dose response curve of the International Standard (89/548) parallels the Quantikine standard curve. To convert sample values obtained with the Quantikine Human IL-6 kit to approximate NIBSC 89/548 units, use the equation below.

NIBSC (89/548) approximate value (IU/mL) = 0.131 x Quantikine Human IL-6 value (pg/mL)

SAMPLE VALUES

Serum/Plasma - Forty serum and plasma samples from apparently healthy volunteers were evaluated for the presence of IL-6 in this assay. No medical histories were available for the donors used in this study. Thirty-three samples measured less than the lowest standard, 3.12 pg/mL. Seven samples measured between 3.12 and 12.5 pg/mL.

Cell Culture Supernates - Human peripheral blood mononuclear cells (1×10^6 cells/mL) were cultured in RPMI supplemented with 10% fetal calf serum, 50 μ M β -mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μ g/mL streptomycin sulfate and stimulated for 1, 3, and 5 days with 10 μ g/mL PHA. Aliquots of the culture supernates were removed on days 1, 3, and 5 and assayed for levels of natural IL-6. Results are listed in the following table.

| Condition | Day 1 (pg/mL) | Day 3 (pg/mL) | Day 5 (pg/mL) |
|--------------|---------------|---------------|---------------|
| Unstimulated | 575 | 311 | 660 |
| Stimulated | 17,130 | 17,520 | 16,340 |

SPECIFICITY

This assay recognizes natural and recombinant human IL-6.

The factors listed below were prepared at 50 ng/mL in Calibrator Diluent RD5T and at 100 ng/mL in Calibrator Diluent RD6F and assayed for cross-reactivity. Preparations of the following factors at 50 ng/mL in a mid-range rhIL-6 control prepared in Calibrator Diluent RD5T and 100 ng/mL in a mid-range rhIL-6 control prepared in Calibrator Diluent RD6F were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

CNTF
G-CSF
GM-CSF
sgp130
IL-1 α
IL-1 β
IL-2
IL-3
IL-4
IL-6 sR
IL-6 sR/sgp130
IL-7
IL-8
IL-11
IL-12
LIF
LIF R
OSM
TNF- α
TNF- β

Recombinant mouse:

GM-CSF
IL-2
IL-3
IL-4
IL-5
IL-6
IL-7
IL-11
IL-12

Recombinant rat:

CNTF

Natural proteins:

bovine FGF acidic
bovine FGF basic
human PDGF
porcine PDGF
human TGF- β 1
porcine TGF- β 1.2
porcine TGF- β 2

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PLATE LAYOUT

Use this plate layout to record standards and samples assayed.

| | | | | | | | | |
|----|---|---|---|---|---|---|---|---|
| | | | | | | | | |
| 12 | | | | | | | | |
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