KAMIYA BIOMEDICAL COMPANY

Human Interleukin-6 ELISA

Measures natural and recombinant forms of the cytokine Interleukin-6 (IL-6)

Cat. No. KT-198

For Research Use Only. Not for Use in Diagnostic Procedures.

PRODUCT INFORMATION

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PRODUCT
The **K-*ASSAY*®** Human Interleukin-6 ELISA is a competitive enzyme immunoassay, which measures natural and recombinant forms of the cytokine Interleukin-6 (IL-6). For research use only, not for use in diagnostic procedures.

DESCRIPTION
Interleukin-6 is a glycoprotein of between 21 and 28 kDa produced mainly by activated monocytes and macrophages although many other cell types produce this cytokine following stimulation. IL-6 is a pleiotropic cytokine influencing antigen-specific immune responses and inflammatory reactions. It is one of the major physiologic mediators of acute phase reaction. IL-6 induces B cell differentiation and immunoglobulin secretion, T cell growth, cytotoxic T cell differentiation, and induces the production of hepatic acute phase proteins during the inflammatory response. IL-6 seems to act on very early hematopoietic progenitor cells, possibly even stem cells.

The **K-*ASSAY*®** Human Interleukin-6 ELISA is designed to measure the "total" (bound and free) amount of IL-6 in serum, plasma, and serum-free biological fluids. The assay system is not hindered by autoantibodies, soluble receptors, or binding proteins that can interfere with most commercial sandwich assays. The result is that nanogram quantities of cytokine can often be detected using this kit. The **K-*ASSAY*®** Human Interleukin-6 ELISA will also accurately measure cytokine concentration in cell culture supernatants providing the concentration is within the dynamic range of the calibration curve. There are enough reagents included in this kit for one 96-well immunoassay plate. We recommend running *duplicate* wells for the calibrators and unknowns.

PRINCIPLE
With the **K-*ASSAY*®** Human Interleukin-6 ELISA system, goat anti-rabbit antibodies are used to capture a specific IL-6 complex in each sample consisting of IL-6 antibody, biotinylated IL-6, and sample/calibrator. The biotinylated IL-6 conjugate (competitive ligand) and sample or calibrator competes for IL-6 specific antibody binding sites. Therefore, as the concentration of IL-6 in the sample increases, the amount of biotinylated IL-6 captured by the antibody decreases. The assay is visualized using a streptavidin alkaline phosphatase conjugate and an ensuing chromagenic substrate reaction. The amount of IL-6 detected in each sample is compared to an IL-6 calibration curve which demonstrates an inverse relationship between Optical Density (O.D.) and cytokine concentration: i.e. the higher the O.D. the lower the cytokine concentration in the sample.

This assay uses a two-step color generating system. In this system, alkaline phosphatase dephosphorylates NADPH (Substrate) to NADH. The NADH then serves as the cofactor, which activates a cycling redox reaction driven by alcohol dehydrogenase and diaphorase. The latter reaction forms a deep red colored product (formazan), which absorbs light at 490 nm.

COMPONENTS
- Microtiter Plate: One goat anti-rabbit pre-coated 96-well plate. Sealed in a foil pouch.
- IL-6 Calibrator: one vial (lyophilized)
- IL-6 Antibody: one vial (lyophilized)
- IL-6 Conjugate: one vial (lyophilized)
- Wash Buffer, 20X: one 50 mL bottle of 20X concentrate
- Diluent #1: one 30 mL bottle
- Diluent #2: one 30 mL bottle
- Streptavidin-Alkaline Phosphatase: one vial (lyophilized)
- Color Reagent A: one 12 mL bottle
- Color Reagent B: one 12 mL bottle
- Stop Solution: one bottle containing 10 mL of (Ready to Use) 0.5 M Sulfuric Acid. **(Caution: Caustic material. Eye, hand, face, and clothing protection should be worn when handling this material.)**
- Acetate Plate Sealers: two seals

Materials or equipment required but not provided

- Multi-channel or repeating pipettes
- Pipettors & tips capable of accurately measuring 10 to 1,000 µL
- Graduated serological pipets, 25 mL and/or 10 mL
- 96-well microtiter plate reader with 490 nm filter
- 96-well plate washer with gravity feed (optional)
- Graph paper for manual plotting of data
- 12 x 75 and 13 x 100 test tubes
- Mechanical vortex
- One 1-liter container
- 15 mL and 25 mL centrifuge tubes
- Deionized water for dilution of the Wash Buffer

SAMPLE COLLECTION & STORAGE

Serum Samples

a) If using serum separator tube (SST) - allow samples to clot for 30 minutes at room temperature. Centrifuge for 10 minutes at 5,000 rpm. Remove serum and assay promptly or aliquot and store the samples at -20°C. Avoid multiple freeze-thaw cycles.

b) If not using serum separator tube (SST) - allow samples to clot overnight at 4°C. Centrifuge for 10 minutes at 5,000 rpm. Remove serum and assay promptly or aliquot and store the samples at -20°C. Avoid multiple freeze-thaw cycles.

Plasma Samples

Collect plasma using EDTA or heparin (pyrogen free) as an anticoagulant. Within 30 minutes of collection, centrifuge at 5,000 rpm. Assay promptly or aliquot and store the samples at -20°C. Avoid multiple freeze-thaw cycles.

Cell Culture Supernatant Samples

Centrifuge to remove particulate matter and assay immediately or aliquot and store samples at -20°C. Avoid multiple freeze-thaw cycles.

Serum Free Biological Samples

(CSF, synovial fluid, saliva, etc.) Upon sample collection, assay promptly or aliquot and store the samples at -20°C. Avoid multiple freeze-thaw cycles.

PREPARATION OF REAGENTS

Rabbit Anti-Human Interleukin-6 Antibody

a) **Serum/Plasma Samples**: Reconstitute the lyophilized IL-6 antibody with 3.5 mL of Diluent #1 and vortex.

b) **Tissue Culture or Biological Samples other than Serum/Plasma**: Reconstitute the lyophilized IL-6 antibody with 3.5 mL of Diluent #2 and vortex.

Recombinant Interleukin-6 Calibrator

Use of Different Sample Types: Use one of the 3 methods below to make up the kit calibrators in the serial dilution.

Method #1: For serum/plasma use Diluent #1 provided to reconstitute the IL-6 Calibrator vial and to dilute the calibrators.

Method #2: For tissue culture samples, use your tissue culture media to reconstitute the IL-6 Calibrator vial and to dilute the calibrators.

Method #3: For serum-free samples (e.g. CSF, urine samples, synovial fluid, etc.) use Diluent #2 provided to reconstitute the IL-6 Calibrator vial and to dilute the calibrators.

a) Label 6 12 x 75 test tubes # 2-6 and “0 dose”. Add 750 µL of the appropriate diluent to the 6 Calibrator tubes.

b) Reconstitute the lyophilized Interleukin-6 Calibrator with 1,000 µL of the appropriate diluent and vortex. This solution is Calibrator #1, which has a concentration of 200 ng/mL.

c) Calibrators # 2-6 are then prepared by performing a 1:4 dilution of the preceding calibrator. For example, to make Calibrator #2, add 250 µL of Calibrator #1 to tube #2 and vortex and so on. Do not add any Interleukin-6 Calibrator to the "0 Dose" Calibrator tube.

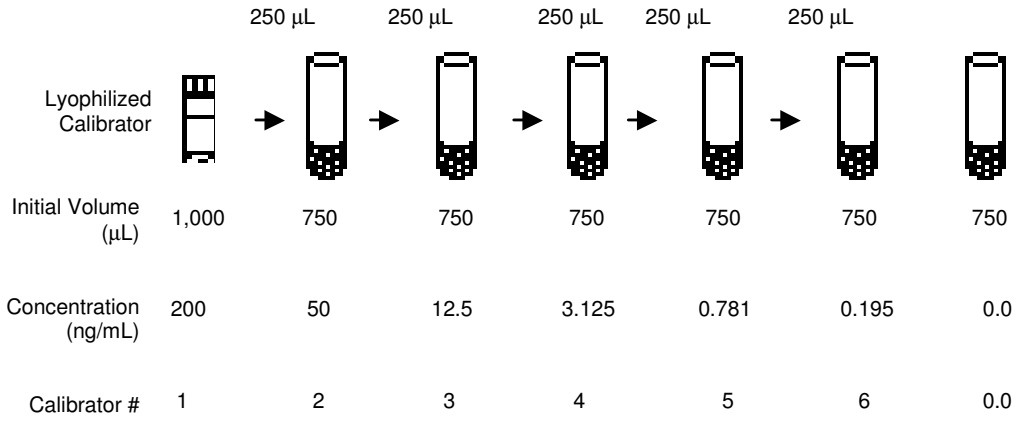

Figure 2: Serial Dilution of Interleukin-6 Calibrator

Human Interleukin-6 Conjugate

a) **Serum/Plasma Samples**: Reconstitute the lyophilized IL-6 Conjugate with 3.5 mL of Diluent #1 and vortex.

b) **Tissue Culture or Biological Samples other than Serum/Plasma**: Reconstitute the lyophilized IL-6 conjugate with 3.5 mL of Diluent #2 and vortex.

Diluting Wash Buffer

Dilute entire contents of concentrated Wash Buffer to 1.0 Liter with deionized water. Stir to homogenize.

Streptavidin-Alkaline Phosphatase

Reconstitute the lyophilized Streptavidin-Alkaline Phosphatase with 6.0 mL of Diluent #2 and vortex.

Color Reagents

a) Allow Color Reagent A and Color Reagent B to come to room temperature. **Do not mix reagents in advance.**

b) Approximately 20 mL of the Color Reagent Solution is needed for an entire plate. If only a portion of the immunoplate is being used at one sitting, use the table below for the appropriate volumes of each to use.

c) Mix appropriate volumes of each reagent in a clean 15 mL or 25 mL screw-cap centrifuge tube and vortex just prior to use.

Table 1:

	Volume of Color Reagent A Required	Volume of Color Reagent B Required
100% of Plate Used	12 mL	12 mL
75% of Plate Used	9 mL	9 mL
50% of Plate Used	6 mL	6 mL
25% of Plate Used	3 mL	3 mL

Mixing & Storage of Color Reagents

- The cycling reaction is temperature sensitive, therefore these reagents must be at room temperature prior to use.
- **Do not mix reagents in advance**. Allow Color Reagent A and Color Reagent B to come to room temperature and mix appropriate volumes (refer to Table 1) of room temperature Color Reagents in a clean screw-top tube just prior to use and vortex.
- Discard any remaining mixed Color Reagent that was not used.
- Store unmixed/unused Color Reagent A and Color Reagent B at 4°C for a maximum of 6 months.

PROCEDURE

1. In duplicate, dispense 100 µL of the calibrators # 0-6 into their designated wells.

   NOTE: A CALIBRATION CURVE MUST BE OBTAINED FOR EACH RUN.
2. Unknown Samples
   - Serum/Plasma Samples*: For each individual sample, add 100 µL of sample + 200 µL of Diluent #1 + 100 µL of Diluent #2 to a 12x75 test tube and vortex. In duplicate, add 100 µL of each serum/plasma sample preparation to their designated wells.
   - Tissue Culture Samples: In duplicate, add 100 µL of each undiluted tissue culture sample to designated wells.
   - Biological Fluid Samples Other Than Serum/Plasma** (e.g. CSF, urine, synovial fluid, etc.): In duplicate, add 50 µL of each undiluted sample + 50 µL of Diluent #2 to designated wells.

   ** See note in Analysis of Results section on pages 5 and 6 for data analysis.*
3. Dispense 25 µL of reconstituted Rabbit Anti-Human Interleukin-6 Antibody into each well. Cover plate with Acetate Plate Sealer to prevent evaporation and incubate for 3 hours at room temperature.

   Note: At this point you should allow Color Reagent A and Color Reagent B solutions to come to room temperature.
4. Gently remove the Plate Sealer, dispense 25 µL of reconstituted Interleukin-6 Conjugate into each well; reseal plate, and incubate for 30 minutes at room temperature.
5. IMPORTANT WASH STEP:

   Gently remove the Plate Sealer and wash the plate 5 times. A thorough washing of the plate is extremely important to reduce background. We recommend using a multi-channel pipette to fill each well with 250 µL of diluted Wash Buffer. Fluid removal from the wells is best accomplished by inverting the plate over a sink and flicking the fluid out of the wells and then blotting the plate on clean paper towels. Using the multichannel pipet add 250 µL of Wash Buffer to each well; flick and blot the plate. Repeat this procedure for a total of 4 times. Dispense 250 µL of diluted Wash Buffer a fifth time and let plate soak for 10 minutes. <u>After the 10 minute soak, blot and aspirate each well to remove any excess fluid.</u>

   For users of automatic plate washers: It is important to ensure that the wash apparatus is properly maintained and operating correctly. Tubing and tips can easily become clogged, leading to incomplete washing and inadequate aspiration of wells. The result may be poor precision and an unsuitable calibration curve. For best results, we recommend 2 cycles of 5 washes each, dispensing 250 µL of 1X wash buffer per well with a 10 minute soak interval between the 2 cycles.
6. Allow Color Reagent A and Color Reagent B to come to room temperature, refer to page 4 for reagent preparation. Dispense 50 µL of the diluted Streptavidin-Alkaline Phosphatase into each well. Reseal the plate and incubate at room temperature for 30 minutes.
7. Gently remove the plate sealer. Wash the plate 5 times using the wash method described above being sure to soak plate with wash buffer for 10 minutes on wash cycle #5 before final fluid removal and aspiration of each well.
8. Dispense 200 µL of the prepared Color Reagent Solution into each well. Incubate for 15 minutes at room temperature. WHEN PREPARING TO READ THE PLATE, DO NOT BLANK THE PLATE READER ON THE "0 DOSE" CALIBRATOR WELLS.
9. It is very important that the color generation is monitored very closely, as the time for development may vary according to laboratory conditions. Therefore, we recommend that you read the plate at 490 nm during the 15 minute incubation to monitor the speed at which color is generated.
   If the plate reader being used has a shake mode, set the shaker for 3.0 seconds. If no shaker is available, hold the plate firmly and carefully tap the edges to insure good mixing of the color reagent fluid in the wells.

   When the O.D. for "O Dose" has reached 1.6, take a reading and save it. Dispense 50 µL of Stop Solution at this time into each well IN THE SAME ORDER that the Color Reagent Solution was added. The addition of the Stop Solution is an optional step, which you may choose to omit.
10. Read the plate a final time at 490 nm.

ANALYSIS OF RESULTS

Manual Plotting: Plot the calibration curve on semi-log graph paper. Known concentrations of Interleukin-6 are plotted on the log scale (X-axis) and the corresponding OD on the linear scale (Y-axis). The calibration curve should have a sigmoidal shape that shows an inverse relationship between Interleukin-6 concentrations and the corresponding OD's (absorbances). In other words, the greater the concentration of Interleukin-6 in the sample, the lower the OD, or less red color. The concentration of Interleukin-6 in unknown samples may be determined by plotting the sample OD on the Y-axis, then drawing a horizontal line to intersect with the calibration curve. A vertical line dropped from this point intersects the X-axis at the concentration of Interleukin-6 in the unknown sample.

***Note**: If analyzing serum/plasma samples, which were diluted 1:4 in the Assay Procedure, the resulting potency estimates will need to be multiplied by a dilution factor of 4.

****Note:** If analyzing biological samples other than serum/plasma, which were diluted 1:2 in the Assay Procedure, the resulting potency estimates will need to be multiplied by a dilution factor of 2.

Plate Reader/PC Interface: An alternative approach is to enter the data into a computer program capable of performing many functions of data plotting and curve fitting. The data fit a logit-log, a spline fit, or 4-parameter logistic. Currently existing spreadsheet software can also perform some simple plotting.

PERFORMANCE CHARACTERISTICS

Analytical Sensitivity and Detection Limits

Sensitivity:	0.195 ng/mL
Range of Detection:	0.195 ng/mL to 200 ng/mL
Cross-reactivity:	<0.5% against cytokine calibrators
Intra-Assay Variation:	±7.0 %
Inter-Assay Variation:	±11.3 %

STORAGE

Unused reconstituted kit reagents should be stored at 4°C for a maximum of 14 days.
Unused plate strips should be placed back in the foil pouch with the desiccant and stored at 4°C for a maximum of 14 days.

WARNINGS AND PRECAUTIONS

- Wash Buffer, Diluent #1, Diluent #2, Color Reagent A and Color Reagent B contain sodium azide. Sodium azide may react with copper and lead plumbing to form highly explosive metal azides. Upon disposal, flush with large amounts of water to prevent azide build-up. Avoid contact with skin.
- *Caustic Material:* Stop Solution (0.5 M Sulfuric Acid). ***Caution: Eye, hand, face, and clothing protection should be worn when handling this material.***
- Human Source Material: The human blood material supplied has been tested with a method currently acceptable to FDA and found non-reactive for HIV-1/2 antibody, HCV Antibody, a Serologic Test for Syphilis and Hepatitis B surface antigen. However, all materials should be handled carefully and in accordance with good laboratory practices.
- The instructions provided have been designed to optimize the kit's performance. Deviation from the instructions may result in the sub-optimal performance of the kit and the failure to produce accurate data.

TECHNICAL HINTS

- Store Kit and kit reagents at 4°C. Do not freeze.
- Manual Plate Washing: Vigorous washing and complete removal of all liquid by aspiration at the end of each step is very important to obtain low background values.
- Recommended Method for Manual Plate Washing:
  1. Remove existing fluid from each well by flicking the plate over a sink. Subsequently, blot the plate on clean paper towels.
  2. Forcefully pipet 250 µL of diluted Wash Buffer into each well with a multi-channel pipette.
  3. Remove fluid from each well by flicking the plate over a sink. Subsequently blot the plate on clean paper towels.
  4. Repeat washing and flicking 4 times.
  5. Dispense 250 µL of diluted Wash Buffer a fifth time and let soak for 10 minutes. After soaking the plate for 10 minutes, blot and aspirate each well to remove any excess fluid.
- All samples and calibrators should be run in duplicate.
- All samples and kit reagents should be at room temperature (20-25°C) prior to use.
- Thaw samples at room temperature. Do not use a water bath to thaw samples.
- If using a multi-channel pipettor, always use clean disposable reagent reservoirs for the addition of the IL-6 Antibody. IL-6 Conjugate, Streptavidin Alkaline Phosphatase solution, the Color Reagent solution and Stop Solution.
- Use clean pipet tips for each transfer to avoid cross contamination.
- Do not mix reagents from different kit lots.

- Avoid microbial contamination of kit reagents.
- Avoid exposure of kit reagents to excessive heat or light during storage and incubation.
- Do not freeze kit reagents.
- If using samples that are clotted, hemolyzed, or microbially contaminated, or if there is any question about the integrity of a sample, make a notation regarding the sample and its location on the plate and interpret the results accordingly.
- Individual components of the assay contain preservatives. Gloves should be worn and good laboratory practices should be followed while performing the assay to avoid skin contact.
- Do not mix the Color Reagents in advance. Care must be taken not to contaminate the Color Reagent solution. If the solution turns red immediately after mixing, do not use.

FOR RESEARCH USE ONLY

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