

INTRODUCTION

Human IL-6 Ultrasensitive Immunoassay is a 3.5 - 4.5 hour solid phase ELISA designed to measure IL-6 in cell culture supernates, serum, and plasma. It contains recombinant IL-6 and antibodies raised against this protein. It has been shown to accurately quantitate recombinant IL-6. Results obtained with naturally occurring IL-6 samples showed linear curves that were parallel to the standard curves obtained using the kit standards. These results indicate that the immunoassay kit can be used to determine relative mass values for natural IL-6.

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, a biotinylated polyclonal antibody specific for IL-6 is added to the wells. Following a wash to remove any unbound antibody-biotin reagent, HRP link streptavidin is added to the wells. After washing away any unbound enzyme, 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.

_ Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.

_ 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 immunoassay, the possibility of interference cannot be excluded.

MATERIALS PROVIDED

Description	Code	Quantity
Microplate - 96 well polystyrene microplate (12 strips of 8 wells) coated with a mouse monoclonal antibody against IL-6.	110-01-01	1 plate
IL-6 Standard – 200 pg/vial of recombinant IL-6 in a buffered protein base with preservatives; lyophilized.	110-01-02	1 vial
Detection Antibody Concentrate – 120 µL / vial, 100-fold concentrated of Biotinylated polyclonal antibody against IL-6 with preservatives; lyophilized.	110-01-03	1 vial
Positive Control - one of recombinant IL-6, lyophilized	110-01-04	1 vial
Streptavidin-HRP Conjugate -120 µl/vial, 100-fold concentrated solution of Streptavidin conjugate to HRP with preservatives	SAHRP	1 vial
Dilution Buffer - 30ml/vial of buffered protein based solution with preservatives	DB01	1 vial
Wash Buffer -50 ml/vial, 10-fold concentrated buffered surfactant, with preservative.	WB01	1 vial
TMB Substrate Solution -13 ml / vial of TMB substrate solution	TMB01	1 vial
Stop Solution (2N HCl) , 13 ml /vial of 2N HCl	S-STOP	1 vial
Plate Covers – Plate sealer.	EAPS	1

STORAGE

Unopened Kit: Store at 2 - 8° C. Do not use past kit expiration date.

Opened / Reconstituted Reagents: May be stored for up to 1 month at 2 - 8°C.

Standard should be stored for up to 1 month at -70° C.

Microplate Wells: 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.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Microplate shaker (250-300rpm).
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 100 ml and 500 ml graduated cylinders.

SAMPLE COLLECTION AND STORAGE

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at $\leq -20^{\circ}$ 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 $\leq -20^{\circ}$ 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 $\leq -20^{\circ}$ C. Avoid repeated freeze-thaw cycles.

REAGENT PREPARATION

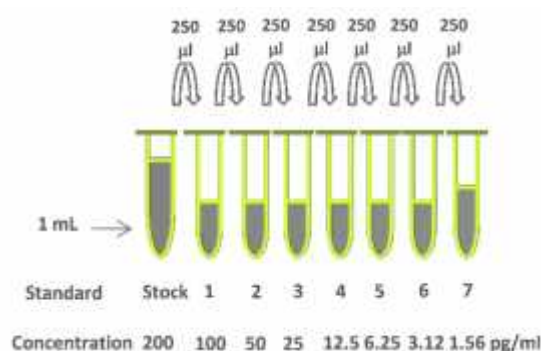
Bring all reagents to room temperature before use.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 50 ml of Wash Buffer Concentrate into deionized or distilled water (450 ml) to prepare 500 ml of Wash Buffer.

IL-6 Standard - Refer to vial label for reconstitution volume. Reconstitute the IL-6 Standard with 1 ml of Dilution Buffer. This reconstitution produces a stock solution of 200 pg/ml. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions. Pipette 250 μ L of the appropriate Dilution Buffer into the tube #1 to #7. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 200 pg/ml standard serves as the high

standard. The appropriate Dilution Buffer serves as the zero standard (0 pg/ml).

Standard	Standard	Reagent Diluent	Concentration
stock	Powder	1000 μ L	200 pg/ml
# 1	250 μ L of stock	250 μ L	100 pg/ml
# 2	250 μ L of 1	250 μ L	50 pg/ml
# 3	250 μ L of 2	250 μ L	25 pg/ml
# 4	250 μ L of 3	250 μ L	12.5 pg/ml
# 5	250 μ L of 4	250 μ L	6.25 pg/ml
# 6	250 μ L of 5	250 μ L	3.125 pg/ml
# 7	250 μ L of 6	250 μ L	1.56 pg/ml



Detection Antibody- Reconstitute the **Detection**

Antibody concentrated with 120 μ L of Dilution Buffer to produce a 100-fold concentrated stock solution. Pipette 11.88 ml of the appropriate Dilution Buffer into the 15 ml centrifuge tube and transfer 120 μ L of 100-fold concentrated stock solution to prepare working solution.

Streptavidin-HRP Conjugate - Pipette 11.88 ml of Dilution Buffer into the 15 ml centrifuge tube and transfer 120 μ L of 100-fold concentrated stock solution to prepare working solution.

ASSAY PROCEDURE

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

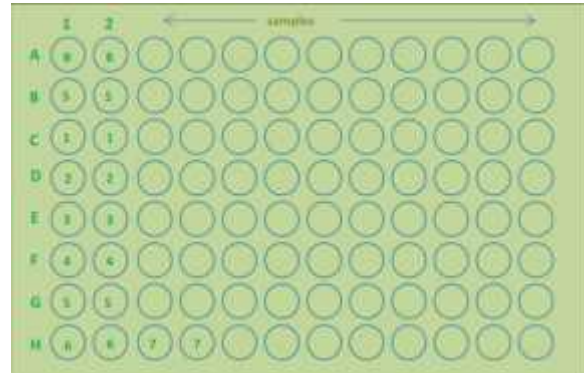
1. Prepare all reagents and working standards as directed in the previous sections.
2. Remove excess micro-plate strips from the plate frame, return them to the foil pouch containing the desiccant pack, reseal.

3. Add 100 μ L of Dilution Buffer to Blank well (A1, A2).
4. Add 100 μ L of Standard (from B1 to H2), sample, or control per well. Cover with the Sealer. Incubate for 2 hours on micro-plate shaker 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 (250 μ L) 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 100 μ L of Detection Antibody working solution to each well. Cover with sealer. Incubate for 2 hours on micro-plate shaker at room temperature.
7. Repeat the aspiration/wash as in step 5.
8. Add 100 μ L of **Streptavidin-HRP Conjugate** working solution to each well. Incubate for 1 hour on micro-plate shaker at room temperature.
9. Repeat the aspiration/wash as in step 5.
10. Add 100 μ L of Substrate Solution to each well. Incubate for 30-45 minutes at room temperature.
Protect from light.
11. Add 100 μ L 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 if the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
12. Determine the optical density of each well within 30 minutes, using a micro-plate 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 standard 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.

CALIBRATION

This immunoassay is calibrated against a highly purified E. Coli-expressed recombinant IL-6.

SENSITIVITY

Twenty-five assays were evaluated and the minimum detectable dose (MDD) of IL-6 was 1.56 pg/ml.

SPECIFICITY

This assay recognizes both natural and recombinant human IL-6. The factors listed below were prepared at 50 ng/ml in Dilution Buffer, and assayed for cross reactivity. Preparations of the following factors at 50 ng/ml in a mid-range rh IL-6 control were assayed for interference. No significant cross-reactivity or interference was observed.

Human Recombinant Proteins:
IL-4, IL-1beta, IL-10, IL-12, IL-2, IL-8

Mouse Recombinant Proteins:
IL-6

SUMMARY OF ASSAY PROCEDURE

