# INTERLEUKIN-6 (IL-6) ELISA

## INTENDED USE
Reactiva IL-6 ELISA if for Quantitative Determination of IL-6 Concentration in Human Serum or Plasma by a Microplate Enzyme Immunoassay, Colorimetric.

## SUMMARY AND EXPLANATION
Interleukin-6 (IL-6) is a cytokine involved with both proinflammatory and anti-inflammatory biological processes.1 IL-6 is synthesized throughout the body, usually as a response to local tissue damage and in the initial stage of inflammation. IL-6 production induces further synthesis of other acute phase proteins including C-reactive protein (CRP), serum amyloid A (SAA), fibrinogen, haptoglobin, and α 1-antichymotrypsin. Conversely, IL-6 decreases production of fibronectin, albumin, and transferrin.2 Screening for circulating IL-6 is primarily used to evaluate the severity of systemic inflammation and infections. Elevated IL-6 can cause severe chronic inflammatory conditions through the generation of amyloid A amyloidosis. 3 Since IL-6 also regulates iron and zinc transporters, chronic inflammation characterized by elevated IL-6 causes hypoferremia, anemia, and hypozincemia. IL-6 is also essential for linking innate to acquired immune response by promoting specific differentiation of naïve CD4+T cells and T-follicular helper-cell differentiation. 2 However, overproduction of IL-6 is systematically linked to many autoimmune diseases through induction of B cells into antibodyproducing plasma cells and therefore hypergammaglobulinemia and autoantibody production. Recently, testing for serum IL-6 has become extremely important for determining the severity of COVID-19 in hospitalized patients. A study (n=89) in 2020 determined that IL-6 levels greater than 35 pg/ml correctly predicted the risk of respiratory failure in COVID-19 patients (84% sensitivity, 63% specificity). Additionally, specificity of this prediction was improved by increasing the cutoff to 80 ng/ml IL-6 (74% sensitivity, 83% specificity). 4 Therefore, serum IL-6 is a valuable marker that can be used to guide escalation of treatment in patients with COVID-19 related hyperinflammatory syndrome.

## PRINCIPLE OF THE TEST
**Sandwich Equilibrium Method (TYPE 2):**
The essential reagents required for an immunoenzymometric assay include high affinity and specificity antibodies (enzyme and immobilized), with different and distinct epitope recognition, in excess, and native antigen. In this procedure, the immobilization takes place during the assay at the surface of a microplate well through the interaction of x-IL-6 antibody coated on the well. Upon mixing the enzyme-labeled x-IL-6 antibody (separate epitope) and serum containing the native antigen, a reaction results between the native antigen and the antibodies without competition or steric hindrance to form a sandwich complex. The interaction is illustrated by the following equation:

$$^{Enz}Ab + Ag_{IL\text{-}6} + Ab_{(well)} \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} {}^{Enz}Ab - Ag_{IL\text{-}6} - Ab_{(well)}$$

Ab(well) = Antibody coated on well (Excess Quantity)
AgIL-6 = Native Antigen (Variable Quantity)
Enz Ab = Enzyme labeled Antibody (Excess Quantity)
Enz Ab - AgIL-6 - Ab(well) = Antigen-Antibodies Sandwich Complex
ka = Rate Constant of Association
k-a = Rate Constant of Dissociation

After sufficient time results, the antibody-bound fraction is separated from unbound antigen by decantation or aspiration. The enzyme activity in the antibody-bound fraction is directly proportional to the native antigen concentration. By utilizing several different serum references of known antigen values, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

## MATERIALS AND COMPONENTS
**• IL-6 Calibrators – 1.0 ml/vial**
Six (6) vials of references for IL-6 at levels of 0(A), 10(B), 50(C), 150(D), 400(E) and 1000(F) pg/ml. Store at 2-8 °C. A preservative has been added.
Note: The calibrators, human serum based, were calibrated using a reference preparation and are traceable against NIBSC code 89/548.

**• IL-6 Controls – 1.0 ml/vial**
Two (2) vials of reference controls for IL-6. Store at 2-8°C. A preservative has been added.

**• IL-6 Enzyme Reagent – 6 ml/vial**
One (1) vial contains anti-IL-6 conjugate reagent. Store at 2-8°C.

**• IL-6 Antibody Coated Plate – 96 wells**
One 96-well microplate coated with x-IL-6 antibody. Store at 2-8 °C.

**• Wash Solution Concentrate – 20 ml/vial**
One (1) vial contains a surfactant in buffered saline. A preservative has been added. Store at 2-8 °C. See Reagent Preparation section.

**• Substrate Reagent – 12 ml/vial**
One (1) vial contains tetramethylbenzidine (TMB) and hydrogen peroxide (H2O2) in buffer. Store at 2-8 °C.
**• Stop Solution – 8 ml/vial**
One (1) vial contains a strong acid (0.5 M H2SO4). Store at 2-8°C.
**Note 1:** Do not use reagents beyond the kit expiration date.
**Note 2:** Do not expose reagents to heat, sun, or strong light.
**Note 3:** The above components are for one 96-well microplate

## MATERIALS REQUIRED BUT NOT PROVIDED
• Pipette capable of delivering 0.050ml (50µl) and 0.100ml (100µl) volumes with a precision of better than 1.5%.
• Dispenser(s) for repetitive deliveries of 0.100ml (100µl) and 0.350ml (350µl) volumes with a precision of better than 1.5%.
• Microplate washers or a squeeze bottle (optional).
• Microplate reader with 450nm and 620nm wavelength absorbance capability.
• Absorbent paper for blotting the microplate wells.
• Plastic wrap or microplate cover for incubation steps.
• Vacuum aspirator (optional) for wash steps.
• Timer.
• Quality control materials.

## SPECIMEN COLLECTION
The specimens shall be blood serum or plasma in type and the usual precautions in the collection of venipuncture samples should be observed. For accurate comparison to established normal values, a fasting morning serum sample should be obtained. The blood should be collected in a plain redtop venipuncture tube without additives or anti-coagulants for serum or EDTA/heparin containing tubes for plasma. Allow the blood to clot for serum samples. Centrifuge the specimen to separate the serum or plasma from the cells. If the specimen(s) cannot be assayed immediately after blood withdrawal, the sample(s) may be stored at temperatures of 2-8 °C for up to seven (7) days or -20 °C for up to 30 days. Avoid use of contaminated devices. Avoid repetitive freezing and thawing (a maximum of two freeze/thaw cycles prior to use).
When assayed in duplicate, 0.100 ml (100 µl) of the specimen is required.

## PRECAUTIONS
**For In Vitro Diagnostic Use Not for Internal or External Use in Humans or Animals.** All products that contain human serum have been found to be non-reactive for Hepatitis B Surface Antigen, HIV 1&2 and HCV Antibodies by FDA licensed reagents. Since no known test can offer complete assurance that infectious agents are absent, all human serum products should be handled as potentially hazardous and capable of transmitting disease. Good laboratory procedures for handling blood products can be found in the Center for Disease Control / National Institute of Health, "Biosafety in Microbiological and Biomedical Laboratories," 2nd Edition, 1988, HHS Publication No. (CDC) 88-8395. Safe Disposal of kit components must be according to local regulatory and statutory requirement.

## REAGENT PREPARATION
**Wash Buffer**
Dilute contents of wash solution to 1000ml with distilled or deionized water in a suitable storage container. Diluted buffer can be stored at 2-30°C for up to 60 days.
**Note: Do not use reagents that are contaminated or have bacterial growth.**

## TEST PROCEDURE
Before proceeding with the assay, bring all reagents, serum reference calibrators and controls to room temperature (20-27°C).
****Test Procedure should be performed by a skilled individual or trained professional****
1. Format the microplates' wells for each serum reference, control and patient specimen to be assayed in duplicate**. Replace any unused microwell strips back into the aluminum bag, seal and store at 2-8 °C.**
2. Pipette 0.050 ml (50 µl) of the appropriate serum reference calibrator, control or specimen into the assigned well.
3. Add 0.050 ml (50 µl) of the IL-6 Enzyme Reagent to each well.
**It is very important to dispense all reagents close to the bottom of the coated well.**
4. Swirl the microplate gently for 20-30 seconds, cover and incubate for 60 minutes at room temperature.
5. Discard the contents of the microplate by decantation or aspiration. If decanting, tap and blot the plate dry with absorbent paper.
6. Add 0.350 ml (350 µl) of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat four (4) additional times for a total of five (5) washes.
**An automatic or manual plate washer can be used. Follow the manufacturer's instruction for proper usage. If a squeeze bottle is employed, fill each well by depressing the container (avoiding air bubbles) to dispense the wash. Decant the wash and repeat four (4) additional times.**
7. Add 0.100 ml (100 µl) of Substrate Reagent to all wells.
**Always add reagents in the same order to minimize reaction time differences between wells.**
**DO NOT SHAKE PLATE AFTER SUBSTRATE ADDITION**
8. Incubate at room temperature for twenty (20) minutes.
9. Add 0.050 ml (50 µl) of stop solution to each well and mix gently for 15-20 seconds. Always add reagents in the same order to minimize reaction time differences between wells.
10. Read the absorbance in each well at 450nm (using a reference wavelength of 630nm to minimize well imperfections) in a microplate reader. **The results should be read within fifteen (15) minutes of adding the stop solution.**
**Note:** For re-assaying specimens with concentrations greater than 1000 pg/ml, dilution should be performed in human serum or plasma with low IL-6 values and multiplied accordingly.

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## INTERPRETATION OF RESULTS

A dose response curve is used to ascertain the concentration of IL-6 in unknown specimens.

1. Plot the absorbance for each duplicate serum reference versus the corresponding IL-6 concentration in pg/ml on linear graph paper (do not average the duplicates of the serum references before plotting).
2. Draw the best-fit curve through the plotted points.
3. To determine the concentration of IL-6 for an unknown, locate the average absorbance of the duplicates for each unknown on the vertical axis of the graph, find the intersecting point on the curve, and read the concentration (in pg/ml) from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated). In the following example, the average absorbance (0.801) intersects the dose response curve at 244 pg/ml IL-6 concentration (See Figure 1).

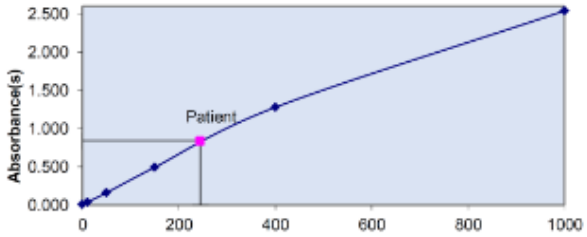
**Note:** Computer data reduction software designed for ELISA assays may also be used for the data reduction. **If such software is utilized, the validation of the software should be ascertained.**

**EXAMPLE 1**

| Sample I.D. | Well Number | Abs (A) | Mean Abs (B) | Value (pg/ml) |
|---|---|---|---|---|
| Cal A | A1<br>B1 | 0.008<br>0.010 | 0.009 | 0 |
| Cal B | C1<br>D1 | 0.038<br>0.036 | 0.037 | 10 |
| Cal C | E1<br>F1 | 0.153<br>0.168 | 0.161 | 50 |
| Cal D | G1<br>H1 | 0.480<br>0.505 | 0.493 | 150 |
| Cal E | A2<br>B2 | 1.302<br>1.255 | 1.278 | 400 |
| Cal F | C2<br>D2 | 2.562<br>2.517 | 2.540 | 1000 |
| Patient | E2<br>F2 | 0.798<br>0.803 | 0.801 | 244 |

*The data presented in Example 1 and Figure 1 is for illustration only and should not be used in lieu of a dose response curve prepared with each assay.

**FIGURE 1**




**IL-6 Values in pg/ml**

*If the absorbance readout is off-scale or higher than the average absorbance of the highest calibrator, sample should be repeated with dilution.

## Q.C. PARAMETERS

In order for the assay results to be considered valid the following criteria should be met:

1. Maximum Absorbance (Calibrator 'F') ≥ 1.3
2. Absorbance of Calibrator B ≥ 0.02
3. Four out of six quality control pools should be within the established ranges.

## RISK ANALYSIS

The MSDS and Risk Analysis Form for this product are available on request from Reactiva Search

### Assay Performance

1. It is important that the time of reaction in each well is held constant to achieve reproducible results.
2. Pipetting of samples should not extend beyond ten (10) minutes to avoid assay drift.
3. Highly lipemic, hemolyzed or grossly contaminated specimen(s) should not be used.
4. If more than one (1) plate is used, it is recommended to repeat the dose response curve.
5. The addition of substrate solution initiates a kinetic reaction, which is terminated by the addition of the stop solution. Therefore, the substrate and stop solution should be added in the same sequence to eliminate any time-deviation during reaction.
6. Plate readers measure vertically. Do not touch the bottom of the wells.
7. Failure to remove adhering solution adequately in the aspiration or decantation wash step(s) may result in poor replication and spurious results.
8. Use components from the same lot. No intermixing of reagents from different batches.
9. Accurate and precise pipetting, as well as following the exact time and temperature requirements prescribed are essential. Any deviation from Reactiva IFU may yield inaccurate results.
10. All applicable national standards, regulations and laws, including, but not limited to, good laboratory procedures, must be strictly followed to ensure compliance and proper device usage.
11. It is important to calibrate all the equipment e.g. pipettes, readers, washers and/or the automated instruments used with this device, and to perform routine preventative maintenance.

### Interpretation

1. Measurements and interpretation of results must be performed by a skilled individual or trained professional.
2. Laboratory results alone are only one aspect for determining patient care and should not be the sole basis for therapy, particularly if the results conflict with other determinants.
3. For valid test results, adequate controls and other parameters must be within the listed ranges and assay requirements.
4. If test kits are altered, such as by mixing parts of different kits, which could produce false test results, or if results are incorrectly interpreted, Monocent shall have no liability.
5. If computer controlled data reduction is used to interpret the results of the test, it is imperative that the predicted values for the calibrators fall within 10% of the assigned concentrations.

## EXPECTED RANGES OF VALUES

IL-6 levels were measured by the Reactiva IL-6 ELISA in apparently normal serum and plasma samples. The values obtained are given in Table 2. Positive criteria was obtained from literature (See Reference 4)

**Table 2**

| Reference Ranges for the IL-6 Test System | |
|---|---|
| Normal Patients | < 10 pg/ml |
| Positive Patients | ≥ 35 pg/ml |

It is important to keep in mind that establishment of a range of values, which can be expected to be found by a given method for a population of "normal" persons, is dependent upon a multiplicity of factors: the specificity of the method, the population tested and the precision of the method in the hands of the analyst. For these reasons, each laboratory should depend upon the range of expected values established by the manufacturer only until an in-house range can be determined by the analysts using the method with a population indigenous to the area inwhich the laboratory is located.

## PERFORMANCE CHARACTERISTICS:

### Sensitivity

The Reactiva IL-6 ELISA has an analytical sensitivity of 0.9 pg/ml.

### Accuracy

#### Linearity

The linearity of the Reactiva IL-6 ELISA was tested by diluting human serum samples containing high levels of IL-6 (200 to 1100 pg/ml) with low IL-6 (<10 pg/ml) human serum samples or the "0" Calibrator. The system demonstrates excellent linearity through the range of the test.

#### Recovery

The recovery of the Reactiva IL-6 ELISA was calculated for five serum samples spiked with 10, 50, 150, 400, and 1000 pg/ml IL-6 using WHO Standard 89/548. Recoveries were determined to be within 15% of the expected values for all samples.

## QUALITY CONTROL

Each laboratory should assay controls at levels in the low, medium and high ranges of the dose response curve for monitoring assay performance. These controls should be treated as unknowns and values determined in every test procedure performed. Quality control charts should be maintained to follow the performance of the supplied reagents. Pertinent statistical methods should be employed to ascertain trends. Significant deviation from established performance can indicate unnoticed change in experimental conditions or degradation of kit reagents. Fresh reagents should be used to determine the reason for the variations.

## REFERENCES

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3. Gillmore JD, Lovat LB, Persey MR, Pepys MB, Hawkins PN. Amyloid load and clinical outcome in AA amyloidosis in relation to circulating concentration of serum amyloid A protein. Lancet. 2001 Jul 7;358(9275):24-9. doi: 10.1016/S0140-6736(00)05252-1. PMID: 11454373.
4. Herold T, Jurinovic V, Arnreich C, Lipworth BJ, Hellmuth JC, von Bergwelt-Baildon M, Klein M, Weinberger T, Elevated levels of interleukin-6 and CRP predict the need for mechanical ventilation in COVID-19, Journal of Allergy and Clinical Immunology (2020), doi: https://doi.org/10.1016/j.jaci.2020.05.008.

**PRESENTACIÓN:**

CONT. 96 TEST CODIGO: RSET062-2