Human EPO ELISA Kit

Enzyme Immunoassay for the quantitative determination of Erythropoietin (EPO) in human serum

Catalog number: ARG80825

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INTRODUCTION

Erythropoietin (EPO) is a heavily glycosylated protein with a molecular weight of about 30,000- 34,000 Daltons. Human EPO is a polypeptide consisting of 165 amino acids, containing one O-linked and three N-linked carbohydrate chains. The recombinant EPO is a good substitute for the native protein for use in an immunoassay. Serum EPO levels are dependent on the rate of production and the rate of clearance of the protein. Ninety percent of EPO is produced in the peritubular cells of the adult kidney in response to a decrease in tissue oxygenation. There is evidence indicating that the protein on these cells which detects oxygen saturation of the blood is a heme-containing moiety. As the pO2 of the plasma, a function of the hematocrit decreases, EPO concentration will increase. There are also observations suggesting that normally there is an inverse correlation between serum EPO levels and red blood cell mass.

Quantitation of serum erythropoietin concentration serves as a diagnostic adjunct in determining the cause of anemia or erythrocytosis. Aplastic anemia, hemolytic anemia and anemia due to iron deficiency all result in serum EPO elevation. Whereas, EPO levels in patients with secondary anemia due to renal failure and other disorders such as acquired immune deficiency syndrome (AIDS) are generally inappropriately low for the degree of anemia. This is mostly likely caused by an impaired ability of the diseased kidney to produce adequate quantities of EPO. Low concentrations of EPO may give an early warning of kidney transplant rejection. EPO also can be used to monitor AIDS patients undergoing Zidovudine (AZT) therapy. An increased concentration of EPO verifies that anemia associated with AZT therapy is due to red cell

hypoplasia or apliasia.

Polycythemia rubra vera, or primary erythrocytosis (an increase of red blood cell mass) results from unstimulated over production of erythrocytes. Hence, the increase in the hemoglobin causes decreased production of EPO, which results in subnormal levels of serum EPO. Secondary polycythemias, which are also characterized by an increase in the total red blood cell mass, occur as a physiological response to elevated levels of circulatory EPO caused by tissue hypoxia. The hypoxia may be due to such factors as pulmonary fibrosis, cardiovascular disease, and prolonged exposure to high altitude, abnormal forms of hemoglobin or drug treatment. Some tumors produce EPO and, in these cases, EPO may be used as a tumor marker to monitor the effectiveness of treatment.

PRINCIPLE OF THE ASSAY

This assay employs the two-site enzyme immunoassay technique. Streptavidin has been pre-coated onto a microtiter plate. Calibrators, controls, or patient samples are simultaneously incubated with the HRP labeled antibody and a biotin coupled antibody in a streptavidin-coated microplate well. After washing away any unbound antibody-enzyme reagent, a substrate solution (TMB) is added to the wells and color develops in proportion to the amount of EPO bound in the initial step. The color development is stopped by the addition of acid and the intensity of the color is measured at a wavelength of 450 nm ±2 nm. The concentration of EPO in the sample is then determined by comparing the O.D of samples to the standard curve.

MATERIALS PROVIDED & STORAGE INFORMATION

Store the unopened kit at 2-8 °C. Use the kit before expiration date.

Component	Quantity	Storage information
Streptavidin-coated microplate	1 plate	4°C.
Standard A (0 mIU/ml)	1 vial	4°C, lyophilized
Standards B-F (10.5, 25.1, 51.0, 154.0, 452.0 mIU/ml)	5 vials	4°C, lyophilized
Control 1	1 vial	4°C, lyophilized
Control 2	1 vial	4°C, lyophilized
Biotin-antibody conjugate	3.5 ml	4°C
HRP-antibody conjugate	3.5 ml	4°C
20X Wash buffer	30 ml	4°C
TMB substrate	20 ml	4°C (Protect from light)
STOP solution	20 ml	4°C

MATERIALS REQUIRED BUT NOT PROVIDED

- Microplate reader capable of measuring absorbance at 450nm or 405nm.
- Pipettes and pipette tips
- Deionized or distilled water
- Orbital microplate shaker: 3 mm (0.1118 in) 600 ± 10 rpm or 19 mm (0.75 in) 170 ± 10 rpm. (Alternatively: linear microplate shaker 2.5 mm (0.098 in) 170 ± 10 rpm.
- Automated microplate washer (optional)

TECHNICAL HINTS AND PRECAUTIONS

- Wear protective gloves, clothing, eye, and face protection especially while handling blood or body fluid samples.
- Store the kit at 4°C at all times.
- If crystals are observed in the 20X Wash buffer, warm to RT until the crystals are completely dissolved. 20X Wash buffer could be kept at room temperature (22- 28 °C) until dilution to avoid precipitation.
- Ensure complete reconstitution and dilution of reagents prior to use.
- It is highly recommended that the standards, samples and controls be assayed in duplicates.
- Change pipette tips between the addition of different reagent or samples.

SAMPLE COLLECTION & STORAGE INFORMATION

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

Serum – It is recommended to collect at least 400 µl of samples to assay EPO in duplicate. Since Dr. Wide and Dr. Cahan reported the diurnal variation of erythropoietin, it is highly recommended that the specimen be collected between 7:30 a.m. to 12:00 noon [L. Wide, et al., Br J Haematol 1989; 72: 85-90; C. Cahan, et al., J Appl Physiol 1992; 72: 2112-7]. Serum samples clotted at room temperature (22 °C- 28 °C) might cause a decrease in EPO value as assessed by radioimmunoassay of about 30% over clotting on ice, so it is suggested collect the samples on ice or at 2-8 °C. Use a serum separator tube (SST) and allow samples to clot on ice or 2-8 °C before centrifugation for 15

minutes at 1000 x g at 2-8 °C. Remove serum and assay immediately or aliquot and store samples at 2-8 °C up to 24 hours or ≤ -20 °C up to 12 months. Do not store samples in self-defrosting freezers. Avoid repeated freeze-thaw cycles. Prior to use, allow all specimens to come to room temperature (22 °C- 28 °C) and mix by gentle inversion or swirling. Avoid grossly hemolyzed or grossly lipemic samples.

REAGENT PREPARATION

- 1X Wash buffer: Dilute 20X Wash buffer into distilled water to yield 1X Wash buffer.
- Standards: Standard Zero (A) reconstituted in 4 ml distilled water. Non-zero standards (B-F) reconstituted in 2 ml distilled water. Allow the vials to stand for 10 minutes and then mix thoroughly by gentle inversion to insure complete reconstitution.
- Controls: Reconstituted in 2 ml (each) distilled water. Allow the vials to stand for 10 minutes and then mix thoroughly by gentle inversion to insure complete reconstitution.

  Note: Use the standards and controls as soon as possible upon reconstitution. Freeze (-20°C) the remaining calibrators and controls as soon as possible after use. Standards and controls are stable at -20 °C for 6 weeks after reconstitution with up to 3 freeze thaw cycles.

ASSAY PROCEDURE

Standards, samples and controls should be assayed in duplicates.

1. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal it.
2. Add 200 µl of Standards, Controls and samples into respective wells.
   Note: Freeze (-20°C) the remaining calibrators and controls as soon as possible after use. (Keep another 2 empty wells for blank)
3. Add 25 µl of Biotin-antibody conjugate into each well.
4. Add 25 µl of HRP-antibody conjugate into each well. Tapping the sides of the microplate for few times to achieve thorough mixing of the sample with Reagents. Be careful to avoid spillage.
   Note: The Biotin-antibody conjugate and HRP-antibody conjugate could be mixed with equal volume prior use, and add 50 µl of the mixed solution in the well.
5. Cover wells and incubate for 120 minutes at RT on a microplate shaker (~600 rpm; please refer MATERIALS REQUIRED BUT NOT PROVIDED for other setting)
6. Aspirate each well and wash, repeating the process 4 times for a total 5 washes. Wash by filling each well with 1× Wash Buffer (350 µl) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating, decanting or blotting against clean paper towels.
7. Add 150 µl of TMB Reagent to each well. Incubate for 30 minutes at room

temperature on a microplate shaker (~600 rpm) in dark.

8. Add 100 µl of Stop Solution to each well. Tapping the sides of the microplate for few times to achieve thorough mixing of the sample with Reagents. Be careful to avoid spillage.
9. Add 250 µl of distilled water in 2 empty wells as blank.
10. Read the OD with a microplate reader at 450 nm immediately. (In case of overflow, read the absorbance of the solution in the wells using a microplate reader set to 405 nm within 10 minutes).

CALCULATION OF RESULTS

1. Calculate the average absorbance values for each set of standards, controls and patient samples.

2. Using linear graph paper, construct a standard curve by plotting the mean absorbance obtained from each standard against its concentration with absorbance value on the vertical (Y) axis and concentration on the horizontal (X) axis.

3. Using the mean absorbance value for each sample determine the corresponding concentration from the standard curve.

4. In general, samples and controls should be read using the 450 nm for EPO concentrations up to 154.0 mIU/ml (Using the O.D. value of Standard A-E reading at 450 nm for calculation). EPO concentrations above 154.0 mIU/ml should be interpolated using the 405 nm reading (Using the OD value of Standard A, D, E and F reading at 405 nm for calculation).

5. Automated method: The results in the IFU have been calculated automatically using a 4 PL (4 Parameter Logistics) or point-to-point curve fit. 4

Parameter Logistics is the preferred method. Other data reduction functions may give slightly different results.

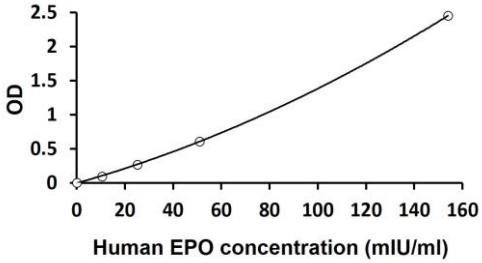
6. Samples that have values below the limit of detection (1.1 mIU/mL) should be reported as " <1.1 mIU/mL".

7. Samples that have values higher the highest standard (452 mIU/mL) should be reported as " > 452 mIU/mL".

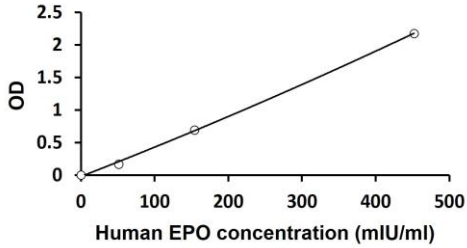
EXAMPLE OF TYPICAL STANDARD CURVE

The following data is for demonstration only and cannot be used in place of data generations at the time of assay.

O.D. 450 nm:



O.D. 405 nm



QUALITY ASSURANCE

Sensitivity

The minimum detectable dose (MDD) of EPO was 1.1 mIU/ml.

Intra-assay and Inter-assay precision

The CV value of intra-assay precision was 6.6% and inter-assay precision was 6.95%.

Specificity

Cross-reactivity in the EPO was studied by the addition of various substances to the Zero Calibrator (Calibrator A).

Cross reactant	Amount of Cross reactant Added
Human Transferrin	400 µg/ml
Human Bilirubin (unconjugated)	200 µg/ml
Human Hemoglobin	5 mg/ml
Human Alpha –Globulin	60 mg/ml
Human Alpha2-Macroglobulin	500 µg/ml
Human α 1-Acid Glycoprotein	800 µg/ml
Human α 1-Antitrypsin	500 µg/ml
Triglycerides	30 mg/ml
Human Albumin	60 mg/ml
Human Gamma Globulin	60 mg/ml
ACTH (intact molecule: amino acid sequence1-39)	5,000 pg/ml
TSH	100 µIU/ml

None of the cross reactants interferes with this EPO ELISA in the concentrations studied. The very small changes in EPO seen for some cross reactants were well within the statistical limits of intra-assay variation.

Recovery

92.5-108.9%