PERFORMANCE CHARACTERISTICS

ACCURACY
One hundred twenty-six (126) patient samples, with EPO values ranging from 5.2 to 291 mIU/ml were assayed by the EPO ELISA procedure and an ICMA (ImmunoChemiluminescent metric assay) EPO kit. Linear regression analysis gives the following statistics:
ELISA = 0.88 ICMA Kit - 1.63 mIU/ml r = 0.96 N = 126

SENSITIVITY
The sensitivity, or minimum detection limit, of this assay is defined as the smallest single value, which can be distinguished from zero at the 95% confidence limit. The EPO ELISA has a calculated sensitivity of 1.5-2.0 mIU/ml. Sample with results below 2.0 mIU/ml should be reported as <than 2.0 mIU/ml

2. PRECISION
Intra-assay precision:

Two serum samples (mean EPO concentrations 11.9 and 121 mIU/ml) were run in 22 replicates. The samples showed good intra-assay precision with %CV of 7.6 and 3.6, respectively.

Inter-assay precision:
Two serum samples (15.5 and 134 mIU/ml) were run in duplicate in 15 independent assays. The samples showed good inter-assay precision and CV% of 10.2 and 4.1, respectively.

3. RECOVERY
A known amount of EPO (84.1 and 168.3 mIU/ml) was added to 4 samples (with original EPO concentrations of 34.8, 9.1, 14.6, and 59.6 mIU/ml) and the total EPO concentrations measured. The assay showed excellent mean recoveries of 94-116%).

4. LINEARITY
Three different samples (with original EPO concentrations of 351.1, 262.5, 113.1 mIU/ml) were diluted (1:2, 1:4, and 1:8) with the zero standard and their final EPO values determined. The samples showed mean recoveries of 88-97%).

5. SPECIFICITY AND CROSSREACTVITY

Cross-reactivity in the EPO was studied by the addition of various substances to the Zero Calibrator (Calibrator A). No significant reactivity was observed.

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 á 1-Acid Glycoprotein, 800 µg/mL; Human á 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

6. HIGH DOSE HOOK EFFECT

EPO concentration of 200,000 MIU/ml did not cause any hook effect.

7. SPECIES REACTIVITY

Human EPO kit has not been tested in other species (mouse, rat, human etc).

Instruction Manual No. M-0070

Human Erythropoietin (EPO)

ELISA KIT Cat. No. 0070

For Quantitative Determination of EPO In serum, culture medium or recombinant samples

For In Vitro Research Use Only

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DRAFT MANUAL: PLEASE CONSULT THE MANUAL SUPPLIED WITH THE KIT FOR ANY LOT SPECIFIC CHNAGES.

Human Erythropoietin (EPO) ELISA KIT # 0070

Kit Contents: (reagents for 96 tests)

Components	Cat#
Streptavidin coated microwell strip plate (96 wells). Ready-to-use	7 1 - P
EPO **Standard A**, 0 mIU/ml in human serum base; Powder, reconstitute in 4 ml water	7 2 A
EPO **Std. B**, powder, reconstitute in 2 ml water	7 2 B
EPO **Std. C**, powder, reconstitute in 2 ml water	7 2 C
EPO **Std. D**, powder, reconstitute in 2 ml water	7 2 D
EPO **Std. E**, powder, reconstitute in 2 ml water	7 2 E
EPO **Std. F**, powder, reconstitute in 2 ml water	7 2 F
EPO **Control 1**, powder, reconst. in 2 ml water	7 2 C L 1
EPO **Control 2**, powder, reconst. in 2 ml water	7 2 C L 2
**Lot specific values of stds A-F and controls are provided on the vials (range 10, 25, 150, 150, 450; EPO Stds are calibrated against recombinant WHO IS 87/684	
Anti-human EPO IgG-**biotin Conj**. 3.5 ml	7 3 - A B
Anti-hEPO-**HRP Conjugate**, 3.5 ml	7 4 - A C
Wash Buffer (20X), 30 ml	W B - 2 0
TMB substrate Soln, 20 ml	7 0 S A
Stop solution (diluted sulfuric acid), 20 ml	T - 7 0
Complete Instruction Manual	M 0 0 7 0

INTRODUCTION

Erythropoietin or EPO is a 165 amino acid glycoprotein hormone (protein accession #NP_00790, chromosome 7) that is a cytokine for erythrocyte (red blood cell) precursors in the bone marrow. Also called hematopoietin or hemopoietin, it is produced by the kidney, and is the hormone that regulates red blood cell production. EPO is produced mainly by peritubular fibroblasts of the renal cortex. EPO stimulates the production of red cells in bone marrow and it is used for the treatment for anemia in humans. It is used in treating anemia resulting from chronic kidney disease, from the treatment of cancer (chemotherapy & radiation), and from other critical illnesses (heart failure). There are two types of erythropoietin (and three brands) for people with anemia due to chronic kidney disease (not on dialysis), these are:

Epoetin (Procrit®(also known as Eprex®), NeoRecormon®), darbepoetin (Aranesp®), PDpoetin®(an erythropoietin produced in Iran by Pooyesh Darou Pharmaceuticals), Brands available in the USA include: epoetin (Procrit® and Epogen®). EPO has a history of usage as a blood doping agent in endurance sports.

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. 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.

ADI's EPO ELISA kit is a very sensitive assay for the measurement of EPO in human serum, culture medium or in recombinant proteins. Other fluids have not been tested.

QUALITY CONTROL

Control samples or serum pools should be analyzed with each run of calibrators and patient samples. Results generated from the analysis of the control samples should be evaluated for acceptability using appropriate statistical methods. When the laboratory first introduces this EPO assay, the release of patient sample results should be based on whether the kit Control results fall within the suggested acceptable ranges. If one or more of the quality control sample values lie outside the acceptable limits, the assay should be repeated. Once the laboratory has generated data of its own, the quality control parameters should be based on the statistical data by the laboratory, using either kit Control and/or serum pools made by the laboratory. Levy-Jenning plots on control results should be used. If the results for all the control samples are within mean + 2 standard deviations, with no definitive trend or bias of the quality control data, the assay should be deemed acceptable. The Westgard rule should be followed to be compliant with CLIA 88 regulations. If the control results do not fall within the stated parameters as described, assay results are invalid.

LIMITATIONS OF THE PROCEDURE

Like any analyte used as a diagnostic adjunct, EPO results must be interpreted carefully with the overall clinical presentations and other supportive diagnostic tests. Purified IgG proteins of the same species as the ones for which the capture and the label antibodies, were derived, in addition to one commercial heterophile antibody blocker, have been incorporated in the reagents to minimize the heterophile antibodies.14 Nonetheless, there can be no assurance that the heterophile interference has been completely eliminated. Therefore, it is recommended that at least three dilutions of any elevated and/or suspect positive results be assayed to detect non-parallelism compared to reference standards.

Because results obtained with one commercial EPO assay may differ significantly from those obtained with any other, it is recommended that any serial testing performed on the same patient over time should be performed
with the same commercial EPO test.

This test may not be sufficiently sensitive to consistently discriminate abnormally low EPO values from normal levels of EPO. Lower EPO levels than expected have been seen with anemias associated with the following conditions: rheumatoid arthritis, acquired immunodeficiency syndrome, cancer, and ulcerative colitis17, sickle cell disease, and in premature neonates.

After allogeneic bone marrow transplant, impaired erythropoietin response may delay erythropoietin recovery. Patients with hypergammaglobulinemia associated with multiple myeloma or Waldenstrom's disease have impaired production of erythropoietin in relation to hemoglobin concentration. This has been linked to increased plasma viscosity.

No drugs have been investigated for assay interference. EPO levels of persons living at high altitudes with erythrocytosis may rapidly fall to normal after returning to low altitudes.

EXPECTED VALUES

Determine, and Utilize Reference Intervals in the Clinical Laboratory. (NCCLS Document C28-A, Vol. 15 No. 4) **the reference ranges (2.5 . 97.5 percentile) were 4.3 . 32.9 mIU/ml** for EPO in serum. Each lab should establish their own range of normal values.

Store all reagents at 2-8 oC except the Wash Concentrate, which should be kept at room temperature (22oC to 28oC) until dilution to avoid precipitation.

2. For each of the non-zero calibrators (Calibrator B through F) and kit controls 1 and 2, reconstitute each vial with 2 mL of distilled or deionized water and mix. Allow the vial to stand for 10 minutes and then mix thoroughly by gentle inversion to insure complete reconstitution. **Use the calibrators and controls as soon as possible upon reconstitution.** ***Freeze (-15oC) the remaining calibrators and controls as soon as possible after use.*** Standards and controls are stable at -15 oC for 6 weeks after reconstitution with up to 3 freeze thaw cycles when handled as recommended in .Procedural Notes. section.

3. ***Wash buffer (20X)***: : Mix contents of wash concentrate thoroughly. If precipitate is present in the Wash Concentrate due to storage at lower temperature such as 4°C, dissolve by placing the vial in a 37°C water bath or oven with swirling or stirring. Add wash concentrate (30 mL) to **570 mL of distilled or deionized water** and mix. The diluted working wash solution is stable for 90 days when stored at room temperature.

TEST PROCEDURE *(ALLOW ALL REAGENTS TO REACH ROOM TEMPERATURE 22-28 oC BEFORE USE).*

Arrange required # of streptavidin coated strips and arrange them on the plate. Store unused strips in the bag. Label or mark the microtiter well strips to be used on the plate.

1. Pipet **200 ul** of standards, controls and serum samples into appropriate wells in *duplicate*.
2. Immediately, dispense **25 ul** of **biotinylated anti-EPO** conj into each well. Dispense **25 ul** of **anti-EPO-HRP** conjugate into each well. **Note**: It is recommended to mix the biotin and HRP conjugate in 1:1 ratio and dispense 50 ul as a single step.
3. Tap the microplate firmly against a rigid object, such as a pen, to achieve thorough mixing of the sample with Reagents. For complete assurance of mixing, repeat the tapping for a minimum of 5 times for each of the remaining three of the four sides of the plate.
4. Be careful to avoid spillage. Cover the microplate(s) with aluminum foil or a tray to avoid exposure to light, and **incubate for 2 hrs at room temperature (22 o- 28oC) on an orbital shaker (**in the absence of shaker it is possible to manually mix the plate manually every 30 min or so and increasing the time to 3 hrs**).**
5. First Aspirate the well contents completely and **wash the wells 5 times with 1x wash buffer (350 ul/well)** We recommend using an automated ELISA plate washer for better consistency. Failure to wash the wells properly will lead to high blank or zero values. If washing manually, plate must be tapped over paper towel between washings to ensure proper washing.
6. Dispense **150 ul of TMB substrate** into the wells. Mix gently for 5-10 seconds, cover the plate and incubate at room temp on an orbital shaker. for **30± 5 min at room temp (22-28oC)**. Positive wells will develop blue color.
7. Add **100 ul stop solution** into each well (blue color turns into yellow). **Read absorbance at 450 nm** within 15 minutes.

Note: The second reading is designed to extend the analytical validity of the calibration curve to the value represented by the highest calibrator, which is approximately 350 mIU/ml (the exact concentration is printed on the vial label and will change slightly from one lot to another). Hence, patient samples with EPO > the penultimate [2nd to the highest] calibrator, i.e. Calibrator E. can be quantified against a calibration curve consisting of the readings all the way up to the concentration equivalent to the highest calibrator using the 405 nm reading, away from the wavelength of maximum absorbance. Patient and control samples should be read using the 450 nm for EPO concentrations up to the concentration of Calibrator E. EPO concentrations reading above that of Calibrator E should be interpolated using the 405 nm reading.

8. By using the final absorbance values obtained in the previous step, construct two calibration curves using 405 nm reading and 450 nm reading via cubic spline, 4 parameter logistics, or point-to-point interpolation to quantify the concentration of EPO.

CALCULATION OF RESULTS

Manual Method

1. For the 450 nm readings, construct a dose response curve (calibration curve) using the first five calibrators provided, i.e. Calibrators A, B, C, D and E. For the 405 nm readings, construct a second dose response curve using Calibrators A, D, E and F.

2. Assign the concentration for each calibrator stated on the vial in mIU/ml. Plot the data from the calibration curve on linear graph paper with the concentration on the X-axis and the corresponding A.U. on the Y-axis.

3. Draw a straight line between 2 adjacent points. This mathematical algorithm is commonly known as the "point-to-point" calculation. Obtain the concentration of the sample by locating the absorbance unit on the Y-axis and finding the corresponding concentration value on the X-axis. Patient and control samples should be read using the 450 nm for EPO concentrations up to the penultimate [2nd to the highest] calibrator, i.e. Calibrator E. EPO concentrations above the concentration of the penultimate calibrator (in the example shown below as 113 mIU/ml) should be interpolated using the 405 nm reading.

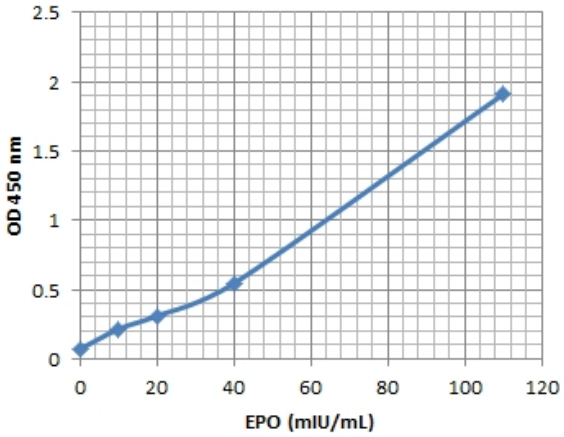
Automated Method:

4. Computer programs using cubic spline or 4 PL [4 Parameter Logistics] or Point-to-Point can generally give a good fit. For the 450 nm readings, construct a dose response curve (calibration curve) using the first five calibrators provided, i.e. Calibrators A, B, C, D and E. For the 405 nm readings, construct a second dose response curve using Calibrators A, D, E and F.

WORKSHEET OF TYPICAL ASSAY

Wells	Stds/samples (mIU/ml)	Net Abs.	Calculated Conc (mIU/l)
A1, A2	**Std. A**	0.070	
B1, B2	**Std. B**	0.215	
C1, C2	**Std. C**	0.310	
D1, D2	**Std. D**)	0.545	
E1, E2	**Std. E**	1.920	
F1, F2	Control 1	0.281	17.8
G1, G2	**Sample 1**	0.459	32.3

NOTE: These data are for demonstration purpose only. A complete standard curve must be run in every assay to determine sample values. Each laboratory should determine their own normal reference values. The above values are derived from values at 450nM. Samples contains concn >113 mIU/ml then data should be used from readings at 405nM.



Kit-spec-XL

A typical std assay curve (do not use this for calculating sample values)

In patients with erythrocytosis due to uncompensated hypoxia, serum immunoreactive EPO is elevated; in those with compensated hypoxia, the serum immunoreactive EPO level is usually within the range of normal, and in patients with polycythemia vera, serum immunoreactive EPO is either normal or low. Thus, while an elevated serum EPO level suggests that erythrocytosis is a secondary phenomenon and a low EPO level supports the possibility of autonomous erythropoiesis, a normal serum EPO level excludes neither hypoxia nor autonomous EPO production as the cause of erythrocytosis.

PRINCIPLE OF THE TEST

EPO ELISA kit is based on sequential binding of human EPO from samples to two antibodies, one immobilized on microtiter well plates (biotinylated anti-EPO is captured on streptavidin coated plates), and other conjugated to the enzyme horseradish peroxidase. After a washing step, chromogenic substrate is added and colors developed. The enzymatic reaction (color) is directly proportional to the amount of EPO present in the sample. Adding stopping solution terminates the reaction. Absorbance is then measured on a microtiter well ELISA reader at 450 nm or 405 nM. The unknown sample values are then read-off the standard curve.

MATERIALS AND EQUIPMENT REQUIRED

Adjustable micropipet (25-100 µl) and multichannel pipet with disposable plastic tips. Reagent troughs, plate washer (recommended) and ELISA plate Reader.

PRECAUTIONS

The Alpha Diagnostic International EPO ELISA kit is intended for *in vitro research* use only. The Control and Standards have been prepared from human sera shown to be negative for HBsAg and HIV antibodies. Nevertheless, such tests are unable to prove the complete absence of viruses, therefore, sera should be handled with appropriate precautions.

Applicable **MSDS**, if not already on file, for the following reagents can be obtained from ADI or the web site.
TMB (substrate), H2SO4 (stop solution), and Prolcin-300 (0.1% v/v in standards, sample diluent and HRP-conjugates).
All waste material should be properly disinfected before disposal. Avoid contact with the stop solution (1N sulfuric acid).

SPECIMEN COLLECTION AND HANDLING

The determination of EPO should be performed on human serum. To assay the specimen in duplicate, 400 µL of human serum is required. It is highly recommended that the specimen be collected between 7:30 a.m. to 12:00 noon, because diurnal variation of erythropoietin has been reported in literature. Collect whole blood without anticoagulant and allow blood to clot between 2-8°C, if possible. It has been reported that serum samples clotted at room temperature (22oC to 28oC) caused a decrease in EPO value as assessed by radioimmunoassay of about 30% over clotting on ice.13 Then, the serum should be promptly separated, preferably in a refrigerated centrifuge, and stored at -15oC or lower. Serum samples may be stored up to 24 hours at 2-8°C. Serum samples frozen at -15°C are stable for up to 12 months. Do not store samples in self-defrosting freezers. Avoid repeated freezing and thawing of samples. For long term storage of samples, it is recommended that samples should be aliquoted into sample tubes or vials prior to freezing. Prior to use, allow all specimens to come to room temperature (22oC to 28oC) and mix by gentle inversion or swirling. Avoid grossly hemolyzed or grossly lipemic samples.

Reagent Preparation

Store all kit components at 2-8oC except the Wash Concentrate and the Stop Solution.

1. All reagents except the non-zero calibrators, kit controls and the Wash Concentrate are ready-to-use.