Erythropoietin ELISA

For the quantitative determination of erythropoietin in human serum.

For "In Vitro Diagnostic" use within the United States of America.
This product is for "Research Use Only" outside of the United States of America.

Catalog Number: 21-EPOHU-E01
Size: 96 Wells
Version: May 2022-ALPCO 2.0

1. Intended Use

The EPO ELISA is intended for the quantitative determination of Erythropoietin (EPO) in human serum. This assay is intended for *in vitro* diagnostic use, to detect elevated or decreased EPO levels, as an aid in the diagnosis of anemias and polycythemias.

2. Introduction

Erythropoietin (EPO) is a heavily glycosylated protein with a molecular weight of about 30 - 34 kilodaltons. Human EPO is a polypeptide consisting of 165 amino acids, containing one O-linked and three N-linked carbohydrate chains[1]. Recombinant EPO is a good substitute for the native protein for use in an immunoassay[2]. Serum EPO levels are dependent on the rate of production and clearance of the protein. Ninety percent of EPO is produced in peritubular cells of the adult kidney in response to a decrease in tissue oxygenation[3,4]. Evidence suggests that the protein on these cells which detects oxygen saturation of the blood is a heme-containing moiety[5]. As the pO2 of the plasma decreases, as a function of the hematocrit, EPO concentration will increase[6]. Observations also suggest that normally there is an inverse correlation between serum EPO levels and red blood cell mass[7].

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[8]. Low concentrations of EPO may give an early warning of kidney transplant rejection[10]. 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[10].

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[9]. 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, prolonged exposure to high altitude, abnormal forms of hemoglobin or drug treatment[10]. Some tumors produce EPO and, in these cases, EPO may be used as a tumor marker to monitor the effectiveness of treatment.

3. Principle of the Test

The EPO Immunoassay is a two-site ELISA [enzyme-linked immunosorbent assay] for the measurement of the biologically active 165 amino acid chain of EPO. It utilizes two different mouse monoclonal antibodies to human EPO specific for well-defined regions on the EPO molecule. One mouse monoclonal antibody to human EPO is biotinylated and the other mouse monoclonal antibody to human EPO is labeled with horseradish peroxidase [HRP] for detection.

Streptavidin Well - Biotinylated Anti-EPO (mouse monoclonal) - EPO - HRP conjugated Anti-EPO (mouse monoclonal)

In this assay, calibrators, controls, or patient samples are simultaneously incubated with the enzyme labeled antibody and a biotin coupled antibody in a streptavidin-coated microplate well. At the end of the assay incubation, the microwell is washed to remove unbound components and the enzyme bound to the solid phase is incubated with the substrate, tetramethylbenzidine (TMB). An acidic stop solution is then added to stop the reaction and converts the color to yellow. The intensity of the

yellow color is directly proportional to the concentration of EPO in the sample. A dose response curve of absorbance units versus concentration is generated using results obtained from the calibrators. Concentrations of EPO present in the controls and patient samples are determined directly from this curve. The standards have been calibrated against the World Health Organization (WHO) erythropoietin international standard that consists of recombinant DNA derived EPO. The WHO reference standard used was the erythropoietin 1st international standard (87/684).

4. Kit Components

Kit Components	Description	Quantity
RGT 1 = Reagent 1	Biotinylated EPO Antibody [mouse monoclonal anti-human EPO] containing ProClin 300 as preservative	1 x 3.5 mL
RGT 2 = Reagent 2	Peroxidase (Enzyme) labeled EPO Antibody [mouse monoclonal anti-human EPO]	1 x 3.5 mL
RGT A = Reagent A	ELISA Wash Concentrate [saline with surfactant with the preservative ciprofloxacin hydrochloride]	1 x 30 mL
RGT B = Reagent B	TMB Substrate [tetramethylbenzidine]	1 x 20 mL
SOLN = Stop Solution	ELISA Stop Solution [1 N sulfuric acid]	1 x 20 mL
PLA = Microplate	One holder with Streptavidin Coated Strips	12 x 8 well strips
CAL = Calibrators A: 0 mIU/mL B: C: Refer to vial D: labels for exact E: concentrations F:	Lyophilized synthetic h-EPO. Lyophilized Zero calibrator is a buffered protein solution and all other calibrators consist of synthetic h-EPO (1-165) in buffered protein solution. These standards have been calibrated against the World Health Organization erythropoietin 1st international standard [recombinant DNA derived EPO] (87/684). Each calibrator contains the preservative ciprofloxacin hydrochloride.	1 x 4 mL for the zero calibrator 1 x 2 mL for all other calibrators
CTRL = Controls 1 & 2 Refer to vial label for exact ranges	Lyophilized. 2 Levels. Synthetic h-EPO (1-165) in a buffered protein solution. Each control contains the preservative ciprofloxacin hydrochloride.	1 x 2 mL per level

MATERIAL AND EQUIPMENT REQUIRED BUT NOT PROVIDED

- Microplate reader capable of reading at 450 nm and 405 nm
- Microplate washer [if washer is unavailable, manual washing is acceptable]
- Precision Pipettors to deliver 25, 200, 100 and 150 µL
- *(Optional):* A multi-channel dispenser or a repeating dispenser for 25, 100 and 150 µL
- Timer capable of ± 2 minute accuracy
- Distilled or Deionized water
- Plate rotator or shaker
- Microplate shakers: For the shaker diameters indicated below, it has been found that the Streptavidin kits will maintain optimal performance response at the following speed settings:

Microplate Shaker	Shaking diameter	Speed Setting
Orbital	3 mm (0.118 in)	600 ± 10 rpm
	19 mm (0.75 in)	170 ± 10 rpm
Linear	25 mm (0.98 in)	170 ± 10 rpm

5. Warning and Precautions

For *in vitro* diagnostic use.

Although the reagents provided in this kit have been specifically designed to contain no human blood components, the human patient samples, which might be positive for HBsAg, HBcAg, or

HIV antibodies, must be treated as potentially infectious hazards. Common precautions in handling should be exercised, as applied to any untested patient sample.

This kit contains material of animal origin and should be handled as a potential carrier and transmitter of disease.

The Stop Solution consists of 1 N Sulfuric Acid. This is a strong acid. Although diluted, it still must be handled with care. It can cause burns and should be handled with gloves, eye protection, and appropriate protective clothing. Any spill should be wiped up immediately with copious quantities of water. Do not breathe vapor and avoid inhalation.

ELISA Reagent 1, Biotinylated EPO Antibody contains ProClin 300 as a preservative. Avoid contact and wear gloves while handling this reagent. Promptly wash skin with mild soap and water if accidental skin contact should occur. Flush eyes with water for 15 minutes if reagent should be in contact with eye(s). If ingested, avoid vomiting and give large amount of water. Contact a physician immediately.

ELISA Reagent A, ***Wash Concentrate***, and ***EPO Calibrators and Controls*** all contain ciprofloxacin hydrochloride as a preservative. Keep from personnel who have demonstrated a sensitivity to Quinoline-based drug products. Females who are or may be pregnant should avoid any contact with ciprofloxacin.

If turbidity is observed in any reagent, do not perform the assay, and please contact ALPCO.

Various types of shakers with different specifications are commercially available. If the microplate shaker does not fall within the specified range above, each laboratory is encouraged to set their own optimal range.

6. Sample Collection and Storage

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.[11,12] Collect whole blood without anticoagulant and allow blood to clot at 2°C to 8°C, if possible. It has been reported that serum samples clotted at room temperature (22°C to 28°C) caused a decrease in EPO values, 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 -15°C 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 (22°C to 28°C) and mix by gentle inversion or swirling. Avoid grossly hemolyzed or grossly lipemic samples.

7. Reagent Preparation and Storage

Store all kit components at 2-8°C.

1. All reagents except the calibrators, kit controls and the Wash Concentrate are ready-to-use. Store all reagents at 2-8°C.
2. For Zero Calibrator (Calibrator A) reconstitute vial with 4 mL of distilled or deionized water and mix. 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 vials to stand for 10 minutes and then mix thoroughly by gentle inversion to ensure complete reconstitution. **Use the calibrators and controls as soon as possible upon reconstitution. *Freeze (-***

15°C) the remaining calibrators and controls as soon as possible after use. Standards and controls are stable at -15°C for 6 weeks after reconstitution with up to 3 freeze-thaw cycles when handled as recommended in "Procedural Notes" section.

3. ***ELISA Reagent A***: Wash Concentrate: 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.

8. Assay Procedure

1. Place sufficient **Streptavidin Coated Strips** in a holder to run all six (6) calibrators, A – F of the EPO CALIBRATORS [exact concentration stated on the vial label], Controls, and patient samples. At a minimum, designate two wells to serve as "blanks". Refer to Step 9 for final plate reading.
2. Pipet **200 µL** of calibrators, controls, and samples into the designated or mapped well. ***Freeze (-15°C) the remaining calibrators and controls as soon as possible after use.***
3. Add or dispense **25 µL** of **Reagent 1 (Biotinylated Antibody**) into each of the wells, which already contain the calibrators, controls, and samples, leaving the blank wells empty.
4. Add or dispense **25 µL** of **Reagent 2 (Enzyme Labeled Antibody**) into each of the same wells, leaving the blank wells empty. 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. Be careful to avoid spillage. Cover the microplate(s) with aluminum foil or a tray to avoid exposure to light and place it on a **shaker set at recommended settings (see Section 4)** for **2 hours ± 15 minutes** at room temperature (22°- 28°C).
5. First aspirate the fluid completely and then wash/aspirate each well **five (5) times** with the 1X **Working Wash Solution (prepared from Reagent A**), using an automatic microplate washer. The wash solution volume should be set to dispense 0.35 mL into each well.
6. Add or dispense **150 µL** of the ***ELISA Reagent B*** (TMB Substrate) into each of the wells. Tap the microplate as described in Step # 4.
7. With appropriate cover to avoid light exposure, place the microplate(s) on a **shaker at recommended settings (see Section 4)** for **30 ± 5 minutes** at room temperature (22°- 28°C).
8. Add or dispense **100 µL** of the **Stop Solution** into each of the wells, except the blank wells. Tap the microplate as described in Step # 4. Be careful to avoid spillage.
9. Prior to reading, ensure both "blank wells" as mentioned in Step # 1 are filled with 250 µL of distilled or deionized water. Blank the plate reader according to the manufacturer's instructions by using the blank wells.* Read the absorbance of the solution in the wells within 10 minutes, using a microplate reader set to **450 nm**. **Read** the plate **again** with the reader set to **405 nm** against distilled or deionized water**.**

   **If due to technical reasons the ELISA plate reader cannot be adjusted to zero using "blank", subtract the "blank" absorbance value from all other absorbance values to obtain results.*

   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 450 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 greater than the 2nd 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.

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

9. Procedural Notes

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

- It is recommended that all calibrators, controls, and patient samples are assayed in duplicate.
- The samples should be pipetted into the well with minimum amounts of air-bubbles present.
- Patient samples with values greater than the highest calibrator (Calibrator F), which is approximately 450 mIU/mL (see exact concentration on vial label, because it can vary from one lot to another), must be diluted with Calibrator A (Zero Calibrator) and re-assayed. Multiply the result by the dilution factor. Alternatively, the result may be reported as greater than the highest calibrator concentration (Calibrator F). For example, if Calibrator F has an assigned EPO value of 494 mIU/mL, the reported value should be ">494 mIU/mL".
- Reagents from different lot numbers must not be interchanged.
- If preferred, mix in equal volumes, in sufficient quantities for the assay, Reagent 1 (Biotinylated Antibody) and Reagent 2 (Enzyme Labeled Antibody) in a clean amber bottle. The combined reagent is stable for seven (7) days when stored at 4°C. Then use 50 µL of the mixed antibody in each well. This alternative method should replace Steps # 3 and 4, to be followed with the incubation.
- When mixing, avoid splashing of reagents from wells. This will affect assay precision and accuracy.

10. Calculation of Data

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 Absorbance Units (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 2nd highest calibrator, i.e. Calibrator E. EPO concentrations above the concentration of Calibrator E (in the example shown below as 156 mIU/mL) should be interpolated using the 405 nm reading.

Automated Method:

4. Computer programs using 4-PL [4-Parameter Logistics], cubic spline, 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.

Sample Data *at 450 nm* [raw A.U. readout against distilled or deionized water]

Microplate Well	1st Reading Absorbance Unit	2nd Reading Absorbance Unit	Average Absorbance Unit	EPO mIU/mL
Calibrator A	0.006	0.006	0.006	0
Calibrator B	0.094	0.092	0.093	10.3
Calibrator C	0.232	0.219	0.226	24.8
Calibrator D	0.509	0.474	0.492	48
Calibrator E	1.918	1.799	1.859	156
Control 1	0.171	0.170	0.171	18.2
Control 2	2.27	2.20	2.24	184
Patient Sample 1	0.012	-------	0.012	1.1
Patient Sample 2	0.031	-------	0.031	3.2
Patient Sample 3	0.089	-------	0.089	9.6
Patient Sample 4	0.508	-------	0.508	50.1
Patient Sample 5	3.283	-------	3.283	>156*

* Because the concentration of these samples is greater than the concentration of Calibrator E, e.g., 156 mIU/mL, it is recommended to analyze this raw data using the 405 nm calibration curve as shown in **Sample Data** *at 405 nm* in the table below.

Sample Data *at 405 nm* [raw A.U. readout against distilled or deionized water]

Microplate Well	1st Reading Absorbance Unit	2nd Reading Absorbance Unit	Average Absorbance Unit	EPO mIU/mL
Calibrator A	0	0	0	0
Calibrator D	0.14	0.13	0.135	48
Calibrator E	0.538	0.508	0.523	156
Calibrator F	2.06	2.03	2.04	523
Control 1	0.046	0.044	0.045	<156**
Control 2	0.649	0.626	0.638	184
Patient Sample 1	0.000	------	0.000	<156**
Patient Sample 2	0.007	------	0.007	<156**
Patient Sample 3	0.023	------	0.023	<156**
Patient Sample 4	0.14	------	0.14	<156**
Patient Sample 5	1.161	------	1.161	302

** For samples with concentrations less than the concentration of Calibrator E, e.g., 156 mIU/mL, it is recommended to analyze this raw data using the 450 nm calibration curve as shown in **Sample Data** *at 450 nm* in the table above. This practice should give the results with optimum sensitivity of the assay.

NOTE: The data presented are for illustration purposes only and must not be used in place of data generated at the time of the assay.

11. 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. 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 the mean ± 2 standard deviations, with no definitive trend or bias of the quality control data, the assay should be deemed acceptable. The Westgard rules 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.

12. Limitations of the Procedure

Like any analyte used as a diagnostic adjunct, EPO results must be interpreted carefully along with the overall clinical presentation 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 interference from 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.[15]

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.[16] 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, ulcerative colitis[17], sickle cell disease, and in premature neonates.[18]

After allogeneic bone marrow transplant, impaired erythropoietin response may delay erythropoietin recovery.[17] 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.[17] 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.[19]

Supplements containing high biotin levels such as those marketed for hair, skin, and nail benefits, may contain interfering biotin amounts. Biotin levels higher than the recommended daily allowance may cause interference with the assay. Therefore, it is important to communicate with health care providers and patients about biotin intake when collecting samples to prevent incorrect test results. Results show that the highest concentration at which no significant interference was observed is 1 ng/mL of D-Biotin.

The use of full or semi-automated equipment for dispensing of reagents and/or plate washing must be validated for equivalency to manual results by the laboratory.

13. Expected Values

EPO levels were measured in 120 apparently normal individuals in the U.S. with the EPO ELISA. The samples consist of 61 males and 59 females, ranging from 18 to 96 years of age. There is no significant statistical difference in the reference ranges obtained from the female and male population of data. This finding, that there is no gender difference, is consistent with the

literature[21]. Further, the EPO values do not appear to have significant age dependence, except higher values were obtained in samples from early phases of adulthood, i.e. approximately 22 to 42 years of age. Using the nonparametric method for the analysis of reference values outlined in the NCCLS publication "How to Define, 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 3.22 - 31.9 mIU/mL** for EPO in serum. Each laboratory should establish their own range of expected normal 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." [20]

14. Performance Characteristics

Accuracy

Eighty-five (85) patient samples, with EPO values ranging from 3.8 to 304 mIU/mL, were assayed by the EPO ELISA procedure and another ELISA EPO kit. Linear regression analysis gives the following statistics:

EPO ELISA = 0.94 (previous ELISA Kit) – 0.41 mIU/mL
r = 0.989 N = 85

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 1.1 mIU/mL. Hence, patient sample results below 1.1 mIU/mL should be reported as "Less than 1.1 mIU/mL".

Precision and Reproducibility

The intra-assay precision of the EPO ELISA was calculated from 22 replicate determinations on each of the two samples.

Intra-Assay Variation

Sample	Mean Value (mIU/mL)	N	Coefficient of Variation %
A	14.4	22	8.4
B	189	22	4.8

The inter-assay precision of the EPO ELISA was calculated from data on two samples obtained in 22 different assays.

Inter-Assay Variation

Sample	Mean Value (mIU/mL)	N	Coefficient of Variation %
A	20.4	22	8.8
B	183	22	5.1

Specificity and Cross-Reactivity
Cross-reactivity in the EPO ELISA 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)	5000 pg/mL
TSH	100 µIU/mL

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

Recovery
Various amounts of EPO were added to four different patient sera to determine the recovery. The results are described in the following table:

Serum Sample	Endogenous EPO mIU/mL	EPO added mIU/mL	Expected Value mIU/mL	Measured Value mIU/mL	Recovery (%)
A	7.9	--	--	--	--
	7.1	50.0	57.1	52.8	92.5%
	5.5	150.0	155.5	150.0	96.5%
B	6.0	--	--	--	--
	5.4	50.0	55.4	57.2	103.2%
	4.2	150.0	154.2	168.0	108.9%
C	53.6	--	--	--	--
	48.2	50.0	98.2	105.0	106.9%
	37.5	150.0	187.5	202.0	107.7%
D	0	--	--	--	--
	0	50.0	50.0	50.2	100%
	0	150.0	150.0	145.0	96.7%

Linearity of Patient Sample Dilutions: Parallelism
Three patient serum samples were diluted with Calibrator A (Zero Calibrator). Results (in mIU/mL) were obtained as shown:

Sample	Dilution	Expected	Observed	% Observed ÷ Expected
A	Undiluted	--	247.0	--
	1:2	123.5	119.0	96%
	1:4	61.8	58.5	95%
	1:8	30.9	28.8	93%

B	Undiluted 1:2 1:4 1:8	-- 69.5 34.8 17.4	139.0 74.0 39.9 19.8	-- 106% 114% 114%
C	Undiluted 1:2 1:4 1:8	-- -- 126.5 63.3	>500.0 253.0 116.0 57.0	-- -- 92% 90%

High Dose Hook Effect

The EPO ELISA kit has exhibited no "high dose hook effect" in standard diluent spiked with 200,000 mIU/mL of EPO. Additionally, three samples with known high EPO values (1,920 mIU/mL, 1,520 mIU/mL, and 966 mIU/mL) were tested without dilution and their results read much greater than the highest standard. Samples with EPO levels greater than the highest calibrator, however, should be diluted and re-assayed for correct values.

15. References

1. Sawyer, S.T., Krantz, S.B., Sawada, K. *Receptors for Erythropoietin in Mouse and Human Erythroid Cells and Placenta.* **Blood** 1989; 74: 103-109.
2. Imai, N., Kawamura, A., Higuchi, M., et al. *Physicochemical and Biological Comparison of Recombinant Human Erythropoietin with Human Urinary Erythropoietin.* **J Biochem** 1990; 107: 352-359.
3. Jacobson, L.O., Goldwasser, E., Fried, W., Pizak, L.F. *The Role of the Kidney in Erythropoiesis.* **Nature** 1957; 179: 633-634.
4. Koury, S.T., Bondurant, M.C., Koury, M.J. *Localization of Erythropoietin Synthesizing Cells in Murine Kidney by in-situ Hybridization.* **Blood** 1988; 71: 524-527.
5. Goldberg, M.A., Dunning, S.P., Bunn, H.F. *Regulation of the Erythropoietin Gene: Evidence that the Oxygen Sensor is a Heme Protein.* **Science** 1988; 242: 1412-1415.
6. Erslev, A.J., Caro, J., Birgegard, G., Silver, R., Miller, O. *The Biogenesis of Erythropoietin.* **Experimental Hematology** 1980; Suppl 8: 1-13.
7. Spivak, J.L. *The Mechanism of Action of Erythropoietin.* **Int J Cell Cloning** 1986; 4: 139-166.
8. Erslev, A.J. *Erythropoietin.* **New Eng J Med** 1991; 324:1339-1344.
9. Garcia, J.F., Ebbbe, S.N., Hollander, L., Cutting, H.O., Miller, M.E., Cronkits, E.P. *Radioimmunoassay of Erythropoietin: Circulating Levels in Normal and Polycythemic Human Beings.* **J Lab Clin Med** 1982; 99: 624-635.
10. Wild, D., editor. **The Immunoassay Handbook**, Stockton Press, 1994, p. 428.
11. Wide L., Bengtsson C., Birgegard G. *Circadian Rhythm of Erythropoietin in Human Serum*. **Br J Haematol** 1989; 72: 85-90.
12. Cahan C., Decker M.J., Arnold J.L., Washington L.H., Veldhuis J.D., Goldwasser E., Strohl K.P. *Diurnal Variations in Serum Erythropoietin Levels in Healthy Subjects and Sleep Apnea Patients*. **J Appl Physiol** 1992; 72: 2112-7.
13. Goldwasser E. and Sherwood J.B. *Annotation, Radioimmunoassay of Erythropoietin*. **Br J Haematol** 1981; 48: 359-63.
14. Kricka L.J. *Human Anti-Animal Antibody Interferences in Immunological Assays.* **Clin Chem** 1999; 45: 942-956.
15. Cotes P.M. and Spivak J.L. *Erythropoietin in Health and Disease*. **Erythropoietin Molecular, Cellular and Clinical Biology**, Editors: Erslev A.J., Adamson J.W., Wschbach J.W., Winearls C.G. 1991; Chapter 11:184-207.
16. Jelkmann W. *Renal Erythropoietin: Properties and Production*. **Rev Physiol Biochem Pharmacol** 1986; 104: 139-215.
17. Cotes M.P. *Anomalies in Circulating Erythropoietin Levels*. **Annals of NY Acad, Sci** 1994; 718:103-9.

18. **Wintrobe's Clinical Hematology**, ninth edition, edited by Lee G.R., Bithell T.C., Foerster J., Athens J.W., Lukens J.N. Lea & Febiger, Philadelphia 1993.
19. Fairbanks V. *Q & A*. **CAP Today** Nov 1996, pg. 88.
20. Spivak, JL**. "Erythrocytosis", Hematology: Basic Principles and Practice**; editors: Hoffman R, Benz EJ Jr., Shattil, SJ; Furie B., Cohen HJ; Silberstein LE; 1995; Chapter 37:484-491
21. Miller, ME, Chandra M, Garcia JF: "Clinical applications of measurement of serum immunreactive levels of erythropoietin", Ann. N.Y.Acad.Sci. 459: 375-381, 1985.