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EPO ELISA

Catalog Number M046013

For the quantitative determination of erythropoietin (EPO) in human serum samples.

For research use only.
This product insert must be read in its entirety before use.

INTENDED USE

The EPO ELISA is intended for the quantitative determination of Erythropoietin (EPO) in human serum. This assay is intended for research use, as an aid in the diagnosis of anemias and polycythemias. With the advent of the administration of recombinant erythropoietin as a biologic therapy to increase red blood cell mass, an erythropoietin assay may be used also to aid in the prediction and monitoring of response to recombinant erythropoietin treatment in persons with anemias.

SUMMARY AND EXPLANATION

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 (1). The 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 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 (3,4). There is evidence indicating 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, a function of the hematocrit decreases, EPO concentration will increase (6). There are also observations suggesting 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.

PRINCIPLE OF THE ASSAY

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 stopping 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 unit vs. 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 erythropoietin 1st international standard (87/684).

KIT COMPONENTS

Microplate Plate - The plate contains 12 x 8-well strips coated with streptavidin. Ready for use.

Calibrator A - 1 vial (0 mIU/mL) of a buffered protein solution. Lyophilized.

Calibrators B through F - Synthetic human EPO (1-165) produced in a buffered protein solution. Lyophilized. See vial label for concentrations.

Controls - Synthetic human EPO (1-165) produced in a buffered protein solution. Lyophilized. See vial label for concentration ranges.

Biotinylated EPO Antibody - 1 vial of Biotinylated EPO Antibody containing ProClin 300 as a preservative. Ready to use.

Enzyme Antibody - 1 vial of Peroxidase labeled EPO Antibody (mouse anti-human mAb). Ready to use.

Wash Buffer Concentrate - 1 vial of 20-fold concentrated saline solution with surfactant. Contains ciprofloxacin hydrochloride as a preservative.

Substrate - 1 vial of TMB Substrate. Ready to use.

Stop Solution - 1 vial of 1 N Sulfuric Acid. Ready to use.

STORAGE

<table>
<tr><td>Unopened Kit</td><td colspan="2">Store at 2 - 8 ºC. Do not use past the kit expiration date.</td></tr>
<tr><td rowspan="8">Opened Reagents</td><td>Calibrators</td><td rowspan="2">Store unused calibrators and controls at -20 ºC for up to 6 weeks.</td></tr>
<tr><td>Controls</td></tr>
<tr><td>Biotinylated EPO Antibody</td><td rowspan="3">Store at 2 - 8 ºC.</td></tr>
<tr><td>Enzyme Antibody</td></tr>
<tr><td>Substrate</td></tr>
<tr><td>Stop</td><td rowspan="2">Store at room temperature.</td></tr>
<tr><td>Wash Buffer</td></tr>
<tr><td>Microplate wells</td><td>Return unused wells to the foil pouch containing the desiccant and seal. Store at 2 - 8 ºC.</td></tr>
</table>

SUPPLIES REQUIRED BUT NOT PROVIDED

- Microplate Reader
- Microplate Washer
- Pipettes or pipetting equipment with disposable polypropylene tips
- Multi-channel pipette
- Disposable polypropylene test tubes
- Glass measuring cylinders
- Distilled or deionised water
- Orbital rotator or shaker

PRECAUTIONS

The Biotinylated EPO Antibody contains ProClin 300 as a preservative. Avoid contact and wear gloves while handling with 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.

The 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.

The Stop Solution provided with this kit 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 and eye protection, with appropriate protective clothing. Any spill should be wiped immediately with copious quantities of water.Do not breath vapor and avoid inhalation.

CRITICAL PARAMETERS

- Allow samples and all reagents to equilibrate to room temperature (22–25 °C) prior to performing the assay. This is especially important for the TMB Substrate.
- Adhere to recommended incubation temperatures in the protocol as variations may cause inconsistent or poor assay results.
- It is essential that all wells are washed thoroughly and uniformly. When washing is done by hand, use a squeeze bottle and ensure that all wells are completely filled and emptied at each step.
- Use only reagents from the same lot for each assay. This is especially important when running more than one plate per sample group.
- A separate standard curve must be run on each plate.
- Reagents can easily be trapped in the caps of small microfuge tubes. It is recommended to briefly centrifuge these reagents before use.
- Mix all reagents thoroughly prior to use, but avoid foaming!
- Keep the wells sealed with the plate sealer except when adding reagents and during reading.
- Any variation in the protocol can cause variation in binding!
- The kit should not be used beyond the expiration date on the kit label.
- The values obtained by the samples should be within the standard range. If this is not the case, dilute the sample and repeat the assay.
- We take great care to ensure that this product is suitable for all validated sample types, as designated in this manual. Other sample types may be tested and validated by the user.

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 between 2 - 8 °C, if possible. It has been reported that serum samples clotted at room temperature (22–25 °C) 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 -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 and mix by gentle inversion or swirling. Avoid grossly hemolyzed or grossly lipemic samples.

REAGENT PREPARATION

Note: All reagents should be stored at the recommended temperatures. Bring all reagents to room temperature (22 - 25 °C) before use. Reagents can easily be trapped in the caps of small microfuge tubes. It is recommended to briefly centrifuge these reagents before use.

Wash Buffer Concentrate - Bring to room temperature (22–25 °C) before use. Wash Buffer may exhibit precipitation when stored at cold temperatures. Mix thoroughly before use. Add 30 mL of Wash Buffer Concentrate to 570 mL deionized or distilled water and mix.

Calibrators - For Calibrator A, reconstitute the vial with 4 mL of distilled or deionized water and mix. For Calibrators B through F, 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 ensure complete reconstitution. **Use immediately following reconstitution.** Store unused calibrators at -20 °C for up to 6 weeks. Avoid repeated freeze-thaw cycles.

Controls - 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 ensure complete reconstitution. **Use immediately following reconstitution.** Store unused controls at -20 °C for up to 6 weeks. Avoid repeated freeze-thaw cycles.

ASSAY PROTOCOL

Read the entire protocol before beginning the assay. It is recommended that all standards and samples be assayed in dulplicate. For enhanced low end sensitivity, include Calibrators G and H in assay. *Note: Reagents and samples may require specific handling temperatures and need preparation prior to the assay. See the Reagent and Sample Preparation sections before proceeding. Ensure there are a sufficient amount of streptavidin coated strips when including Calibrators G and H.*

1. Prepare all reagents and samples as described in the previous sections.
2. Remove any excess microplate strips from the plate frame and return them to the foil pouch containing the desiccant pack.

Calibrator/Sample/Antibody Incubation

3. Pipet 200 µL of calibrator, control or sample into duplicate wells. Freeze (-20 °C) the remaining calibrators and controls as soon as possible after use. If using a single pipette tip, be sure to load the plate lowest concentration to highest concentration. *Note: To combine steps 4 & 5, see procedural notes on page 8.*
4. Pipette 25 µL of Biotinylated Antibody into each of the wells which already contain the sample.
5. Pipette 25 µL of Enzyme Antibody into each of the same wells. Mix wells by tapping plate frame gently.
6. Cover the microplate(s) with aluminum foil or a tray to avoid exposure to light. Incubate on an orbital shaker or rotator set at 170 ± 10 rpm for 2 hours ± 15 minutes at room temperature (22 - 25 °C).

Wash

7. Aspirate and wash each well five (5) times with the Working Wash Solution, using an automatic microplate washer. Blot dry by inverting the plate on an absorbent material. The wash solution volume should be set to dispense 350 µL into each well.

Substrate Incubation

8. Add or dispense 150 µL of the Substrate Solution into each of the wells. Mix wells by tapping plate frame gently.
9. With appropriate cover to avoid light exposure, place the microplate(s) on an orbital shaker or rotator set at 170 ± 10 rpm for 30 ±5 minutes at room temperature (22–25 °C).

Stop Reaction

10. Add or dispense 100 µL of the Stop Solution into each of the wells. Mix gently.
11. Read the absorbance of the solution in the wells within 10 minutes, using a microplate reader set to 450 nm against 250 µL of distilled or deionized water. Read the plate again with the reader set to 405 nm against distilled or deionized water. *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 on the vial label and will change slightly from one lot to another). Hence, patient samples with EPO > 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. In general, patient and control samples should be read using the 450 nm for EPO concentrations up to Calibrator E. EPO concentrations above Calibrator E should be interpolated using the 405 nm reading.*

SUMMARY

Prepare reagents and samples as previously described.

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Pipette 200 µL calibrator, control or sample in duplicate into the wells.

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Pipette 25 µL Biotinylated Antibody into the wells.

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Pipette 25 µL Enzyme Antibody into the wells.

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Cover and Incubate 2 hrs. at RT (22 - 25 °C) on a shaker.

↓

Aspirate and wash 5 times.

↓

Add 150 µL of Substrate to each well. Cover and Incubate 30 min. at RT on a shaker.

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Add 100 µL of Stop Solution to each well. Read at 450 nm and at 405nm.

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, until the analyst or technician has gained sufficient experience (as evidenced by the coefficient of variation duplicate being less than 10% [except for the values below the 2 nd non-zero lowest standard] and the ability to obtain results for the kit controls within the suggested acceptable ranges).
- The samples should be pipetted into the well with minimum amount of air-bubble.
- 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 the Calibrator F has an assigned EPO value of 494 mIU/mL, the report 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, Biotinylated Antibody and Enzyme 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 into each well. This alternative method should replace Step (4) and (5), to be followed with the incubation.
- When mixing avoid splashing of reagents from wells. This will affect assay precision and accuracy.

CALCULATION OF RESULTS

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.

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 enhanced low sensitivity, include Calibrators G and H in the dose response curve. 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 156 mIU/mL) should be interpolated using the 405 nm reading.

Automated Method

Computer programs using cubic spline or 4 Parameter Logistics (4 PL) 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. Construct a dose response curve (calibration curve) using Calibrators A, B, C, D and E.

SAMPLE DATA

Data obtained at 450 nm (raw absorbance unit, A.U., readout against distilled or deionized water):

Well	1st Reading (A.U.)	2nd Reading (A.U.)	Average A.U.	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
Sample 1	0.012	-	0.012	1.1
Sample 2	0.031	-	0.031	3.2
Sample 3	0.089	-	0.089	9.6
Sample 4	0.508	-	0.508	50.1
Sample 5	3.283	-	3.283	>156*

**Because the concentration is >156 mIU/mL, it is recommended to use the data obtained at 405 nm.*

Data obtained at 405 nm (raw A.U. readout against distilled or deionized water):

Well	1st Reading (A.U.)	2nd Reading (A.U.)	Average A.U.	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
Sample 1	0	-	0	<156**
Sample 2	0.007	-	0.007	<156**
Sample 3	0.023	-	0.023	<156**
Sample 4	0.14	-	0.14	<156**
Sample 5	1.161	-	1.161	302

***Because the concentration is >156 mIU/mL, it is recommended to use the data obtained at 450 nm.*

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

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 nonparallelism 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, and 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).

EXPECTED VALUES

EPO levels were measured in one hundred and twenty (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 on 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 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).

PERFORMANCE CHARACTERISTICS

Accuracy

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

EPO ELISA = 0.94 ICMA 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 when using Calibrator B as the lowest calibrator. Hence, patient sample results below 1.1 mIU/mL should be reported as < 1.1 mIU/mL.

Reproducibility

Intra-assay Precision (Precision within an assay) - The intra-assay precision was measured by assaying two control samples 22 times on one plate.
Inter-assay Precision (Precision between assays) - The inter-assay precison was assessed by repeated measurements of two samples obtained in 22 different assays.

	Intra-assay Precision		Inter-assay Precision	
Control	A	B	A	B
Mean (mIU/mL)	14.4	189	20.4	183
CV (%)	8.4	4.8	8.8	5.1

SPECIFICITY AND CROSS-REACTIVITY

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 a1-Acid Glycoprotein	800 µg/mL
Human a1-Antitrypsin	500 µg/mL
Triglycerides	30 mg/mL
Human Albumin	60 mg/mL
Human Gamma Globulin	60 mg/mL
ACTH (intact molecule: amino acid sequence 1-39)	5000 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 intraassay 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	57.1	52.8	92.5%
	5.5	150	155.5	150	96.5%
B	6.0	-	-	-	-
	5.4	50	55.4	57.2	103.2%
	4.2	150	154.2	168	108.9%
C	53.6	-	-	-	-
	48.2	50	98.2	105	106.9%
	37.5	150	187.5	202	107.7%
D	0	-	-	-	-
	0	50	50	50.2	100%
	0	150	150	145	96.7%

LINEARITY

Three patient serum samples were diluted with Calibrator A (Zero Calibrator).

Sample	Dilution	Expected mIU/mL	Observed mIU/ mL	% Observed Expected
A	Undiluted	-	247	-
	1:2	123.5	119	96%
	1:4	61.8	58.5	95%
	1:8	30.9	28.8	93%
B	Undiluted	-	139	-
	1:2	69.5	74.0	106%
	1:4	34.8	39.9	115%
	1:8	17.4	19.8	114%
C	Undiluted	-	> 500	-
	1:2	-	253	-
	1:4	126.5	116	92%
	1:8	63.3	57	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.

TROUBLESHOOTING

Problem	Recommendation
Low Absorbance	• Check reagents for proper storage. • Control expiration date. • Check preparation of reagents. • Control incubation times and temperature. • Check reader wavelength.
High Absorbance/high zero standard value	• Check preparation of reagents. • Control incubation times and temperature. • Equilibrate ELISA reagents to room temperature (22 - 25 °C). • Ensure that every well of the ELISA plate is completely filled and emptied at every wash step. • Check that plates are blotted on tissue paper after washing.
Flat cure/poor reproduciblity	• Check reagents for proper storage. • Control expiration date. • Check preparation of working standards. • Check incubation times and temperatures. • Use separate reservoirs for pipetting different solutions with multichanned pipettes. Always use new pipette tips. • Check pipette calibration. • Ensure efficient washing procedure.

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