Version 22-04/24

User's Manual

EPO(Erythropoietin) ELISA Kit

REF **DEIA1837**

Σ **96T**

RUO

This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

Creative Diagnostics

Address: 45-1 Ramsey Road, Shirley, NY 11967, USA

Tel: 1-631-624-4882 (USA) 44-161-818-6441 (Europe) **Fax: 1-631-938-8221**

Email: info@creative-diagnostics.com **Web: www.creative-diagnostics.com**

PRODUCT INFORMATION

Intended Use

The EPO ELISA is intended for the quantitative determination of Erythropoietin (EPO) in human serum.

Principles of Testing

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 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 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).

Reagents And Materials Provided

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 – F Extra Low Calibrators: G & H; *refer to vial labels for exact concentrations*	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 = Control 1 & 2 Refer to vial labels 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

Materials Required But Not Supplied

1. Microplate reader capable of reading at 450nm and 405nm.
2. Microplate washer [if washer is unavailable, manual washing is acceptable].
3. Precision Pipettors to deliver 25, 200, 100 and 150 µL.
4. (Optional): A multi-channel dispenser or a repeating dispenser for 25, 100 and 150 µL.
5. Timer capable of ± 2 minute accuracy.
6. Distilled or deionized water.
7. Orbital rotator or shaker

Storage

Store all kit components at 2-8°C except the Wash Concentrate.

Specimen Collection And Preparation

1. The determination of EPO should be performed on human serum.
2. To assay the specimen in duplicate, 400 µL of human serum is required.
3. 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[1,2].
4. 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 (22-28°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.
5. 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.
6. Prior to use, allow all specimens to come to room temperature (22-28°C) and mix by gentle inversion or swirling. Avoid grossly hemolyzed or grossly lipemic samples.

Reagent Preparation

1. All reagents except the calibrators, kit controls and the Wash Concentrate are ready-to-use. Store all reagents at 2-8°C, except the Wash Concentrate, which should be kept at room temperature (22-28°C) until dilution to avoid precipitation.
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 H) 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 insure 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.

Assay Procedure

For enhanced low end sensitivity include Calibrator G and Calibrator H in assay.

1. Place sufficient **Streptavidin Coated Strips** in a holder to run all six (6) calibrators, A - F of the EPO CALIBRATORS [Exact concentration is stated on the vial label], Controls and samples.
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.**

NOTE: For enhanced low end sensitivity pipet in the following sequence: Calibrator A, Calibrator G, Calibrator H, Calibrator B, Calibrator C, etc. until Calibrator F.

3. Add or dispense **25 µL** of Reagent 1 (Biotinylated Antibody) into each of the wells which already contain the calibrators, controls and samples.
4. Add or dispense **25 µL** of Reagent 2 (Enzyme Labeled Antibody) into each of the same wells. 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 an **orbital shaker or rotator** set at 170±10 rpm 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 **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 an **orbital shaker or rotator** set at 170 ± 10 rpm for 30 ± 5 minutes at room temperature (22-28°C).
8. Add or dispense **100 µL** of the Stopping Solution into each of the wells. Tap the microplate as described in Step 4. Be careful to avoid spillage.
9. 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 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.

10. 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.

PROCEDURAL NOTES

1. Samples that have values below the limit of detection (1.2 mIU/mL) should be reported as " < 1.2 mIU/mL" if Calibrator B is the lowest calibrator.
2. Samples that have values below the limit of detection (0.6 mIU/mL) should be reported as " < 0.6 mIU/mL " if calibration curve includes extra low calibrator G and H.
3. It is recommended that all calibrators, controls, and 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 2nd non-zero lowest standard] and the ability to obtain results for the kit controls within the suggested acceptable ranges).
4. The samples should be pipetted into the well with minimum amount of air-bubble.
5. 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".
6. Reagents from different lot numbers must not be interchanged.
7. 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 into each well. This alternative method should replace Step (3) and (4), to be followed with the incubation.
8. When mixing avoid splashing of reagents from wells. This will affect assay precision and accuracy.

Quality Control

Control samples or serum pools should be analyzed with each run of calibrators and 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 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.

Calculation

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.

FOR ENHANCED LOW END SENSITIVITY INCLUDE CALIBRATOR G AND CALIBRATOR H FOR CALCULATION OF RESULTS.

Construct a dose response curve (calibration curve) using Calibrators A, G, H, B, C, D and E.

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 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.

FOR ENHANCED LOW END SENSITIVITY INCLUDE CALIBRATOR G AND CALIBRATOR H FOR CALCULATION OF RESULTS.

Construct a dose response curve (calibration curve) using Calibrators A, G, H, B, C, D and E.

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 G	0.025	0.024	0.025	2.5
Calibrator H	0.049	0.050	0.05	5
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 are> than the concentration of Calibrator E, e.g. 156 mIU/mL, it is recommended to use the data obtained at 405 nm 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 < than the concentration of Calibrator E, e.g. 156 mIU/mL, it is recommended to use the data obtained at 450 nm 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.

Performance Characteristics

Accuracy

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

CD ELISA = 0.94 ELISA Kit – 0.41 mIU/mL r = 0.989 N = 85

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.

Precision

The Intra-assay precision of the EPO ELISA Test 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 Test 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

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 1.2 mIU/mL when using Calibrator B as the lowest calibrator. Hence, sample results below 1.2 mIU/mL should be reported as "Less than 1.2 mIU/mL".

The EPO ELISA with Calibrator G and Calibrator H has a calculated sensitivity of 0.6 mIU/mL Hence, sample results below 0.6 mIU/mL should be reported as "Less than 0.6 mIU/mL".

Specificity

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

Crossreactant	Amount of Crossreactant 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 intraassay variation.

Linearity

Three serum samples were diluted with Calibrator A (Zero Calibrator). Results in mIU/mL are shown below:

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

Recovery

Various amounts of EPO were added to four different 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 5.5	-- 50.0 150.0	-- 57.1 155.5	-- 52.8 150.0	-- 92.5% 96.5%
B	6.0 5.4 4.2	-- 50.0 150.0	-- 55.4 154.2	-- 57.2 168.0	-- 103.2% 108.9%
C	53.6 48.2 37.5	-- 50.0 150.0	-- 98.2 187.5	-- 105.0 202.0	-- 106.9% 107.7%
D	0 0 0	-- 50.0 150.0	-- 50.0 150.0	-- 50.2 145.0	-- 100% 96.7%

Precautions

1. For research use only.
2. Potential Biohazardous Material Caution

1) Stopping 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 and eye protection and appropriate protective clothing. Any spill should be wiped immediately with copious quantities of water. Do not breathe vapor and avoid inhalation.
2) ELISA Reagent 1. 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.
3) ELISA Reagent A, Wash Concentrate, and EPO Calibrators and Controls, all contain ciprofloxacin hydrochloride as a preservative. Keep from personnel who have demonstrated sensitivity to Quinoline based drug products. Females who are, or may be pregnant should avoid any contact with Ciprofloxacin.

References

1. Wide L., Bengtsson C., Birgegard G. Circadian Rhythm of Erythropoietin in Human Serum. Br J Haematol 1989; 72: 85-90.
2. 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.
3. Goldwasser E. and Sherwood J.B. Annotation, Radioimmunoassay of Erythropoietin. Br J Haematol 1981; 48: 359-63.