BioLegend®

Enabling Legendary Discovery™

# LEGEND MAX™

ELISA Kit with Pre-coated Plate

# Human Erythropoietin

Cat. No. 442907

ELISA Kit for Accurate Quantitation of Human Erythropoietin from Cell Culture Supernatant, Serum, Plasma and Other Biological Fluids

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***It is highly recommended that this manual be read in its entirety before using this product. Do not use this kit beyond the expiration date.***

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## Introduction:

Erythropoietin (EPO) is the most important hormone regulating erythropoiesis. It is a glycoprotein also known as hematopoietin. Human EPO is mainly produced by kidney and liver in response to hypoxia to induce red blood cell synthesis. EPO is a secreted protein of 166 amino acids with a predicted molecular mass of ~18 kD. However, it migrates on SDS-PAGE at ~35-55 kD due to heavy glycosylation. Human EPO is closely related to thrombopoietin (TPO), sharing 44% amino acid homology. EPO is highly conserved across species, with human EPO being 80% identical to mouse, and mouse EPO being 95% identical to rat.

EPO functions through binding to its receptor, EPO R, a homodimer, on erythoid precursors, and promotes their differentiation and maturation. As expected, serum levels of EPO are elevated in response to various conditions that result in tissue hypoxia (e.g. blood loss, high altitude, cardiac or pulmonary disease, and anemia). Increased EPO levels can also be due to increased production by cancer cells of the liver and kidney. Decreased levels of EPO are often seen with chronic renal failure and other chronic diseases or chemotherapy, which causes anemia. Human endogenous EPO and recombinant EPO share the same amino acid sequence, with differences in the glycosylation profiles.

The LEGEND MAX™ Human EPO ELISA kit is a Sandwich Enzyme-Linked Immunosorbent Assay (ELISA) with a 96-well strip plate that is pre-coated with a mouse monoclonal anti-human EPO antibody. The Detection Antibody is a biotinylated mouse monoclonal anti-human EPO antibody. This kit is specifically designed for the accurate quantitation of human EPO from cell culture supernatant, serum, plasma (heparin, EDTA and citrate), urine, and other biological fluids. This kit is analytically validated with ready-to-use reagents.

## Materials Provided:

| Description | Quantity | Volume (per bottle) | Part # |
|---|---|---|---|
| Anti-Human EPO Pre-coated 96 well Strip Microplate | 1 plate | | 77925 |
| Human EPO Detection Antibody | 1 bottle | 12 mL | 77926 |
| Human EPO Standard | 1 vial | lyophilized | 77928 |
| Avidin-HRP | 1 bottle | 12 mL | 77897 |
| Assay Buffer A | 1 bottle | 25 mL | 78232 |
| Wash Buffer (20X) | 1 bottle | 50 mL | 78233 |
| Substrate Solution F | 1 bottle | 12 mL | 79132 |
| Stop Solution | 1 bottle | 12 mL | 79133 |
| Plate Sealers | 4 sheets | | 78101 |



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## Materials to be Provided by the End-User:

- Microplate reader able to measure absorbance at 450 nm
- Adjustable pipettes to measure volumes ranging from 1 µL to 1,000 µL
- Deionized water
- Wash bottle or automated microplate washer
- Log-Log graph paper or software for data analysis
- Tubes to prepare standard dilutions
- Timer
- Plate Shaker
- Polypropylene vials

### Storage Information:

Store unopened kit components between 2°C and 8°C. Do not use this kit beyond its expiration date.

| Opened or Reconstituted Components | |
|---|---|
| Microplate wells | If not all microplate strips are used, remove the excess strips by pressing up from underneath each strip. Place excess strips back in the foil pouch with the included desiccant pack and reseal. Store between 2°C and 8°C for up to one month. |
| Standard | The remaining reconstituted standard stock solution can be aliquoted into polypropylene vials and stored at -70°C for up to one month. Avoid repeated freeze-thaw cycles. |
| Detection Antibody | Store opened reagents between 2°C and 8°C and use within one month. |
| Avidin-HRP | |
| Assay Buffer A | |
| Wash Buffer (20X) | |
| Substrate Solution F | |
| Stop Solution | |

### Health Hazard Warnings:

1. Reagents that contain preservatives may be harmful if ingested, inhaled or absorbed through the skin. Refer to the MSDS online at BioLegend's website for details (www.biolegend.com/msds).
2. Substrate Solution F is harmful if inhaled or ingested. Avoid skin, eye and clothing contact.
3. To reduce the likelihood of blood-borne transmission of infectious agents, handle all serum, plasma and other biological fluids in accordance with

NCCLS regulations.

4. Stop Solution contains strong acid. *Wear eye, hand, and face protection.*

5. Before disposing of the plate, rinse it with an excess amount of tap water.

## Specimen Collection and Handling:

Specimens should be clear and non-hemolyzed. If possible, unknown samples should be run at a number of dilutions to determine the optimal dilution factor that will ensure accurate quantitation.

Serum: Use a serum separator tube and allow clotting for at least 30 minutes, then centrifuge for 10 minutes at 1,000 x *g*. Remove serum layer. *Assay immediately or store serum samples at < -70°C. Avoid repeated freeze-thaw cycles.*

Plasma: Collect blood samples in heparin, citrate or EDTA-containing tubes. Centrifuge for 10 minutes at 1,000 x *g* within 30 minutes of collection. *Assay immediately or store plasma samples at < -70°C. Avoid repeated freeze-thaw cycles*.

Urine: Aseptically collect the first urine of the day (mid-stream) directly into a sterile container. centrifuge to remove particulate matter, assay immediately or aliquot and store at < -70*°C. Avoid repeated freeze-thaw cycles.*

### Reagent and Sample Preparation:

Note: All reagents should be diluted immediately prior to use.

1. Dilute the 20X Wash Buffer to 1X with deionized water. For example, make 1 liter of 1X Wash Buffer by adding 50 mL of 20X Wash Buffer to 950 mL of deionized water. If crystals have formed in the 20X Wash Buffer, bring to room temperature and mix until dissolved.

2. Reconstitute the lyophilized Human EPO Standard by adding the volume of Assay Buffer A to make the 5000 mIU/mL standard stock solution (Refer to LEGEND MAX Kit Lot-Specific Certificate of Analysis/LEGEND MAX Kit Protocol). Allow the reconstituted standard to sit at room temperature for 15 minutes, then briefly vortex to mix completely.

3. For cell culture supernatant samples, the end user may need to determine the dilution factors in a preliminary experiment. If dilutions are necessary, samples should be diluted in the corresponding cell culture medium.

4. It is recommended that serum or plasma samples be analyzed directly without dilution. If dilutions are necessary, samples should be diluted with Assay Buffer A.

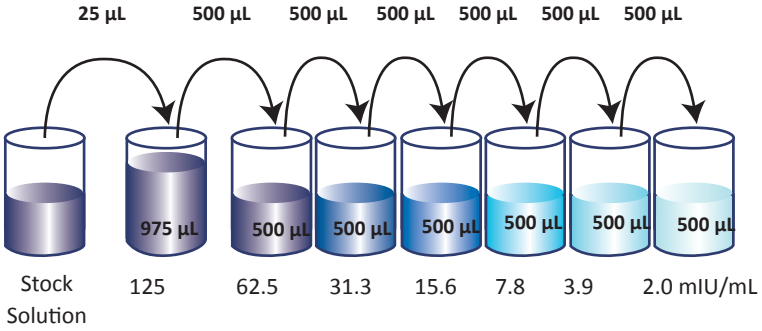
**Assay Procedure:**

Note: Do not mix reagents from different kits or lots. Reagents and/or antibodies from different manufacturers should not be used with this kit.

1. Bring all reagents to room temperature prior to use. It is strongly recommended that all standards and samples be run in duplicate. A standard curve is required for each assay.

2. If not all microplate strips will be used, remove the excess strips by pressing up from underneath each strip. Place excess strips back in the foil pouch with the included desiccant pack and reseal.

3. Prepare 1,000 µL of the 125 mIU/mL top standard by diluting 25 µL of the standard stock solution in 975 µL of Assay Buffer A. Perform six two-fold serial dilutions of the 125 mIU/mL top standard in separate tubes using Assay Buffer A as the diluent. Thus, the human EPO standard concentrations in the tubes are 125 mIU/mL, 62.5 mIU/mL, 31.3 mIU/mL, 15.6 mIU/mL, 7.8 mIU/mL, 3.9 mIU/mL and 2.0 mIU/mL, respectively. Assay Buffer A serves as the zero standard (0 mIU/mL).




4. For measuring samples:
   a) Add 50 µL of Assay Buffer A to each well that will contain either standards or samples.
   b) Add 50 µL of standard dilutions to the wells for standards. Add 50 µL of samples to the wells for samples.

5. Seal the plate with a Plate Sealer included in the kit and incubate the plate at room temperature for 2 hours while shaking at 200 rpm.

6. Discard the contents of the plate into a sink, then wash the plate 4 times with 1X Wash Buffer. Wash the plate with at least 300 µL of 1X Wash Buffer per well and blot any residual buffer by firmly tapping plate upside down on absorbent paper. All subsequent washes should be performed similarly.

7. Add 100 µL of Human EPO Detection Antibody solution to each well, seal the plate and incubate at room temperature for 2 hours while shaking.

8. Discard the contents of the plate into a sink, then wash the plate 4 times with 1X Wash Buffer as in step 6.

9. Add 100 µL of Avidin-HRP solution to each well, seal the plate and incubate at room temperature for 30 minutes while shaking.

10. Discard the contents of the plate into a sink, then wash the plate 5 times with 1X Wash Buffer as in step 6. For this final wash, soak wells in 1X Wash Buffer for 30 seconds to 1 minute for each wash. This will help minimize background.

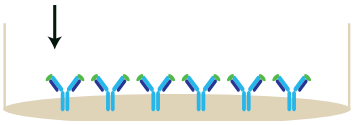
11. Add 100 µL of Substrate Solution F to each well and incubate for 15 minutes in the dark. Wells containing human EPO should turn blue in color with intensity proportional to concentration. It is not necessary to seal the plate during this step.

12. Stop the reaction by adding 100 µL of Stop Solution to each well. The well color should change from blue to yellow.

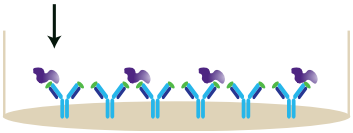
13. Read absorbance at 450 nm within 20 minutes. If the reader is capable of reading at 570 nm, the absorbance at 570 nm can be subtracted from the absorbance at 450 nm.

## Assay Procedure Summary

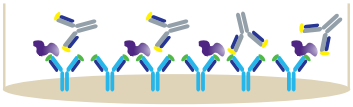
1. Add 50 µL Assay Buffer A



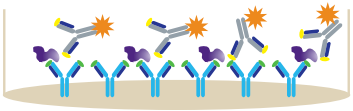
2. Add 50 µL prepared standards or samples
Incubate 2 hrs, RT, shaking



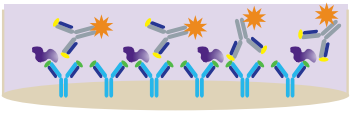
3. Wash 4 times
Add 100 µL Detection Antibody solution
Incubate 2 hrs, RT, shaking



4. Wash 4 times
Add 100 µL Avidin-HRP solution
Incubate 30 min, RT, shaking



5. Wash 5 times
Add 100 µL Substrate Solution F
Incubate 15 min, RT, in the dark



6. Add 100 µL Stop Solution

7. Read absorbance at 450 nm and 570 nm

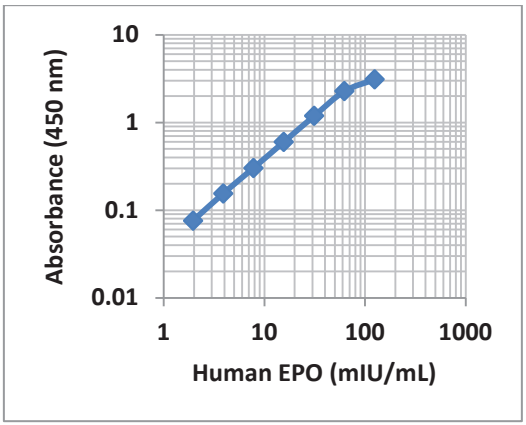
## Calculation of Results:

The data can be best calculated with computer-based curve-fitting software using a 5- or 4-parameter logistics curve-fitting algorithm. If appropriate software is not available, use log-log graph paper to determine sample concentrations. Determine the mean absorbance for each set of duplicate or triplicate standards, controls, and samples. Plot the standard curve on log-log graph paper with antigen concentration on the X-axis and absorbance on the Y-axis. Draw a best fit line through the standard points. To determine the unknown antigen concentrations, find the mean absorbance value of the unknown concentration on the Y-axis and draw a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the X-axis and read the sample concentration.

If samples were diluted, multiply the concentration by the appropriate dilution factor. If a test sample's absorbance value falls outside the linear portion of the standard curve, the test sample needs to be re-analyzed at a higher (or lower) dilution as appropriate.

## Typical Data:

This standard curve was generated at BioLegend for demonstration purposes only. A standard curve must be run with each assay.



## Performance Characteristics:

Specificity: No cross-reactivity was observed when this kit was used to analyze the following recombinant cytokines/chemokines, each at 50 ng/mL. This kit has high cross-reactivity with mouse EPO and rat EPO. There was no cross-species reactivity found with Cynomolgus monkey, Rhesus monkey, bovine, or rat serum, while medium detectable levels in mouse serum and very low level of cross-species reactivity detected in porcine and equine serum.

| | |
|---|---|
| Human | CCL2, CCL5, CXCL9, CXCL16, sFasL, IL-17A, IL-2, IL-22, IL-4, IL-5, IL-8, NGAL, SCF, TGF-β1, TNF-α1 |
| Mouse | EPO R, TPO |

*Sensitivity:* The average minimum detectable concentration of human EPO is 0.25 mIU/mL.

*Recovery:* Three levels of recombinant Human EPO (62.5 mIU/mL, 15.6 mIU/mL and 3.9 mIU/mL) were spiked into 5 human serum, 6 plasma samples (2 of each heparin, EDTA and citrate plasma) and 4 urine samples, and analyzed with the LEGEND MAX™ Human EPO ELISA Kit. On average, 92% and 95% of the protein were recovered from serum and plasma, respectively. The spike recovery of urine samples was low, at 69%.

*Linearity:* Ten human serum and 6 plasma samples containing high concentrations of EPO were diluted with Assay Buffer A to produce sample concentrations within the dynamic range of the assay. On average, 90 and 103% of the expected level were detected from serum and plasma, respectively.

*Intra-Assay Precision*: Two samples with different concentrations of human EPO were tested with 16 replicates in one assay.

| | Sample 1 | Sample 2 |
|---|---|---|
| Number of Replicates | 16 | 16 |
| Mean Concentration (mIU/mL) | 60.2 | 6.8 |
| Standard Deviation | 1.22 | 0.22 |
| % CV | 2.0 | 3.3 |

*Inter-Assay Precision*: Two samples with different concentrations of human EPO were assayed in four independent assays.

| | Sample 1 | Sample 2 |
|---|---|---|
| Number of Assays | 4 | 4 |
| Mean Concentration (mIU/mL) | 60.9 | 6.9 |
| Standard Deviation | 3.56 | 0.36 |
| % CV | 5.8 | 5.2 |

*Biological Samples*:

*Serum and Plasma*

Normal human serum (n=55) and plasma (n=15) samples were tested for endogenous EPO. The concentrations measured are shown below:

| | Serum (n=55) | Plasma (n=15) | Urine (n=4) |
|---|---|---|---|
| Detectable % | 100 | 100 | 0 |
| Mean (mIU/mL) | 18.7 | 15.0 | ND* |
| Maximum (mIU/mL) | 74.2 | 58.2 | ND |
| Minimum (mIU/mL) | 3.4 | 3.4 | ND |

*ND = Nondetectable

*Cell Culture Supernatant*

Human hepatocellular carcinoma Hep G2 cells and chronic myelogenous leukemia (CML) K-562 cells were incubated at ~1 x $10^6$ cells/mL in EMEM and IMDM media, respectively. Cells were stimulated with 100 µM $CoCl_2$, and supernatants were removed at one and two days after stimulation and assayed for human EPO. The concentrations (mIU/mL) of human EPO produced in Hep G2 cell culture are shown below. K-562 cells did not secrete any detectable level of human EPO in the culture.

| Hep G2 cells | Day 1 | Day 2 |
|---|---|---|
| (-) Control | 29.3 | 45.0 |
| $CoCl_2$(100 µM ) | 29.4 | 60.1 |

## Troubleshooting Guide:

| Problem | Probable Cause | Solution |
|---|---|---|
| High Background | Background wells were contaminated | Avoid cross-well contamination by using the provided plate sealers.<br>Use multichannel pipettes and change tips between pipetting samples and reagents. |
| | Insufficient washes | Increase number of washes.<br>Increase soaking time between washes prior to addition of substrate solution. |
| | TMB Substrate Solution was contaminated | TMB Substrate Solution should be clear and colorless prior to addition to wells.<br>Use a clean container prior to pipetting substrate solution into wells. |
| No or poor signal | Detection Antibody, Avidin-HRP or Substrate solution were NOT added | Rerun the assay and follow the protocol. |
| | Wrong reagent or reagents were added in wrong sequential order | |
| | Insufficient plate agitation | The plate should be agitated during all incubation steps using a plate shaker at a speed where solutions in wells are within constant motion without splashing. |
| | The wash buffer contains Sodium Azide (NaN3) | Avoid Sodium Azide contamination in the wash buffer as it inhibits HRP activity. |
| | Incubations were done at an inappropriate temperature, timing or without agitation | Rerun the assay and follow the protocol. |
| Low or poor standard curve signal | The standard was incorrectly reconstituted or diluted | Adjust the calculations and follow the protocol. |
| | Standard was inappropriately stored | Store the reconstituted standard stock solution in polypropylene vials at -70°C.<br>Avoid repeated freeze-thaw cycles. |
| | Reagents added to wells with incorrect concentrations | Check for pipetting errors and the correct reagent volume. |

| Problem | Probable Cause | Solution |
|---|---|---|
| Signal is high, standard curves have saturated signal | Standard reconstituted with less volume than required | Reconstitute new lyophilized standard with the correct volume of solution recommended in the protocol. |
| | Standards/samples, detection antibody, Avidin-HRP or substrate solution were incubated for too long | Rerun the assay and follow the protocol. |
| Sample readings are out of range | Samples contain no or below detectable levels of the analyte | If samples are below detectable levels, it may be possible to use a larger sample volume. Contact technical support for appropriate protocol modifications. |
| | Samples contain analyte concentrations greater than highest standard point | Samples may require dilution and analysis. |
| High variation in samples and/or standards | Multichannel pipette errors | Confirm that pipette calibrations are accurate. |
| | Plate washing was not adequate or uniform | Ensure pipette tips are tightly secured.<br>Ensure uniformity in all wash steps. |
| | Non-homogenous samples | Thoroughly mix samples before assaying. |
| | Samples may have high particulate matter | Remove particulate matter by centrifugation. |
| | Cross-well contamination | Do not reuse plate sealers.<br>Always change tips for reagent additions. Ensure that pipette tips do not touch the reagents on the plate. |

| ELISA Plate Template | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | | | | | | | | | | | | |
| B | | | | | | | | | | | | |
| C | | | | | | | | | | | | |
| D | | | | | | | | | | | | |
| E | | | | | | | | | | | | |
| F | | | | | | | | | | | | |
| G | | | | | | | | | | | | |
| H | | | | | | | | | | | | |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