



LEGEND MAX[™]
ELISA Kit



Mouse Erythropoietin

Cat. No. 442707

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

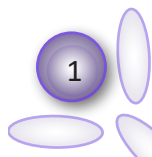
BioLegend, Inc.
biolegend.com

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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LEGEND MAX™ Mouse EPO ELISA Kit

Introduction:

Erythropoietin (EPO) is the most important hormone regulating erythropoiesis. It is a glycoprotein also known as hematopoietin. EPO is mainly produced by kidney and liver in response to hypoxia to induce red blood cell production. Mouse EPO is a secreted protein of 166 amino acids with a predicted molecular mass of ~18.6 kD. However, it migrates on SDS-PAGE at ~33-48 kD due to heavy glycosylation. EPO is closely related to thrombopoietin (TPO) and highly conserved across species, with mouse EPO being 95% and 80% identical to rat and human, respectively.

EPO functions through binding to its receptor, EPO R, a homodimer, on erythroid 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. Mouse endogenous EPO and recombinant EPO share the same amino acid sequence, with differences in the glycosylation profiles.

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

Materials Provided:

Description	Quantity	Volume (per bottle)	Part #
Anti-Mouse EPO Pre-coated 96 well Strip Microplate	1 plate		77888
Mouse EPO Detection Antibody	1 bottle	12 mL	77889
Mouse EPO Standard	1 vial	lyophilized	77891
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	1 pack		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

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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-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.*

Tissue Homogenates: Using an appropriately sized homogenizer, homogenate mouse liver and kidneys into 1 mL per organ of cold 1XPBS containing protease inhibitors. After two rounds of freeze-thaw cycles, centrifuge tissue homogenates, collect supernatant, and store at < -70°C. *Once sample is ready for assay avoid repeated freeze-thaw cycles.*

Reagent and Sample Preparation:

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

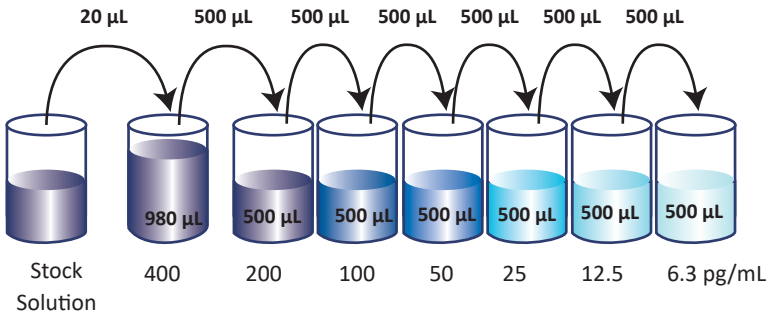
1. Dilute the 20X Wash Buffer to 1X with deionized water. For example, make 1 liter of 1X Wash Buffer by adding 50 mL of 20X Wash Buffer to 950 mL of deionized water. If crystals have formed in the 20X Wash Buffer, bring to room temperature and mix until dissolved.
2. Reconstitute the lyophilized Mouse EPO Standard by adding the volume of Assay Buffer A to make the 20 ng/mL standard stock solution (refer to LEGEND MAX™ Kit Protocol). Allow the reconstituted standard to sit at room temperature for 15-20 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 with 1:2 dilution. To make dilutions, samples should be diluted with Assay Buffer A.

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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.

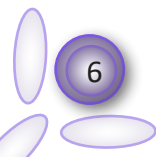
1. Bring all reagents to room temperature prior to use. It is strongly recommended that all standards and samples be run in duplicate. A standard curve is required for each assay.
2. If not all microplate strips will be used, remove the excess strips by pressing up from underneath each strip. Place excess strips back in the foil pouch with the included desiccant pack and reseal.
3. Prepare 1,000 μL of the 400 pg/mL top standard by diluting 20 μL of the standard stock solution in 980 μL of Assay Buffer A. Perform six two-fold serial dilutions of the 400 pg/mL top standard in separate tubes, using Assay Buffer A as the diluent. Thus, the mouse EPO standard concentrations in the tubes are 400 pg/mL , 200 pg/mL , 100 pg/mL , 50 pg/mL , 25 pg/mL , 12.5 pg/mL and 6.3 pg/mL , respectively. Assay Buffer A serves as the zero standard (0 pg/mL).



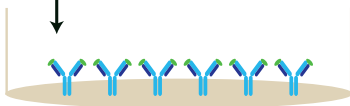
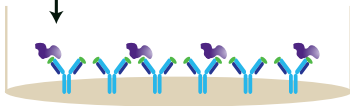
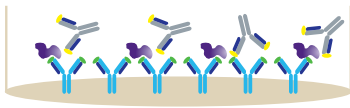
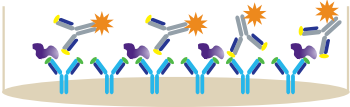
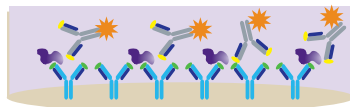
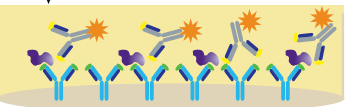
4. For measuring samples:
 - a) Add 50 μL of Assay Buffer A to each well that will contain either standards or samples.
 - b) Add 50 μL of standard dilutions to the wells for standards. Add 50 μL of samples to the wells for samples.
5. Seal the plate with a Plate Sealer included in the kit and incubate 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 Mouse EPO Detection Antibody solution to each well, seal the plate and incubate at room temperature for 1 hour while shaking.

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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 10 minutes in the dark. Wells containing mouse 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
A diagram of a well containing immobilized antibodies (blue Y-shapes) on a surface.
2. Add 50 μL prepared standards or samples
Incubate 2 hrs, RT, shaking
A diagram of a well where purple Y-shaped molecules (sample/standard) have bound to the immobilized antibodies.
3. Wash 4 times
Add 100 μL Detection Antibody solution
Incubate 1 hr, RT, shaking
A diagram of a well where grey Y-shaped molecules (detection antibody) have bound to the purple Y-shaped molecules.
4. Wash 4 times
Add 100 μL Avidin-HRP solution
Incubate 30 min, RT, shaking
A diagram of a well where orange star-shaped molecules (Avidin-HRP) have bound to the grey Y-shaped molecules.
5. Wash 5 times
Add 100 μL Substrate Solution F
Incubate 10 min, RT, in the dark
A diagram of a well where the orange star-shaped molecules are reacting with the substrate, creating a yellow color.
6. Add 100 μL Stop Solution
A diagram of a well where the reaction is stopped, resulting in a uniform yellow color.
7. Read absorbance at 450 nm and 570 nm

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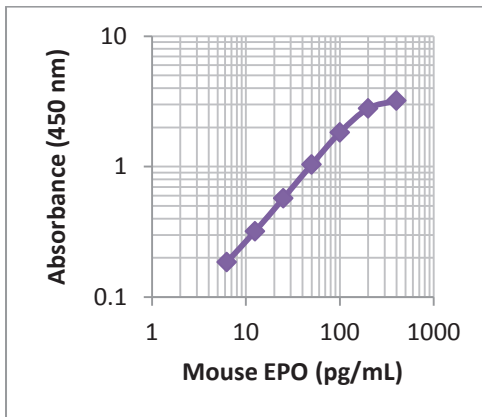
Calculation of Results:

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.



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Performance Characteristics:

Specificity: No cross-reactivity was observed when this kit was used to analyze the following recombinant proteins, each at 50 ng/mL. This kit has high cross-reactivity with human EPO and rat EPO.

Mouse	TPO, EPO R, CCL1, CCL8, CCL9, CXCL3, CXCL9, CXCL13, GM-CSF, IFN γ , IL-10, IL-1 β , IL-27, IL-3, IL-33, IL-4, Isthmin, MMP-9, TNF- α
Human	EPO R, TPO

Sensitivity: The average minimum detectable concentration of mouse EPO is 1.0 pg/mL.

Recovery: Three levels of recombinant mouse EPO (238 pg/mL, 59 pg/mL and 15 pg/mL) were spiked into four mouse serum and four plasma samples (each serum or plasma was pooled from minimum ten mice of the same strain, four strains) and analyzed with the LEGEND MAX™ Mouse EPO ELISA Kit. On average, 96% and 86% of the protein was recovered from serum and plasma, respectively.

Linearity: Four mouse serum and four 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, 101% and 113% of the expected level was detected from serum and plasma, respectively.

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

	Sample 1	Sample 2
Number of Replicates	16	16
Mean Concentration (pg/mL)	213.5	18.5
Standard Deviation	12.7	0.7
% CV	5.9	3.7

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

	Sample 1	Sample 2
Number of Assays	4	4
Mean Concentration (pg/mL)	218.5	20.0
Standard Deviation	9.8	1.1
% CV	4.5	5.6



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Biological Samples:

Serum and Plasma

Serum or Heparin plasma pooled from minimum ten mice of each strain (four strains) were tested for endogenous EPO. The concentrations (pg/mL) measured are shown below:

Strain	Serum	Heparin Plasma
BALB/c	67.5	28.6
C57BL/6	43.4	72.3
Swiss-Webster	127.7	135.3
CD-1	176.9	47.2

Tissue Homogenates from mouse anemia model

BALB/c mouse (female, four months old) was bled, 500 µL blood via facial mandibular vein. The mouse was sacrificed 15 hours later and blood, liver and kidney were isolated. Plasma and tissue homogenates were prepared following instructions in “Specimen Collection and Handling” section. Total protein concentrations were determined by Bradford Protein Assay (mg/mL). Samples were tested with the LEGEND MAX™ Mouse EPO ELISA Kit. The concentrations of mouse EPO in pre-anemic and post-anemic plasma and tissue homogenates are shown below.

	Pre-anemia	Post-anemia
Plasma (pg/mL)	21.8	401.5
Kidney Homogenates (pg/mg)	ND	7.3
Liver Homogenates (pg/mg)	ND	0.8

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Troubleshooting Guide:

Problem	Probable Cause	Solution
High Background	Background wells were contaminated	Avoid cross-well contamination by using the provided plate sealers. Use multichannel pipettes and change tips between pipetting samples and reagents.
	Insufficient washes	Increase number of washes. 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. 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 (NaN ₃)	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. Avoid repeated freeze-thaw cycles.
	Reagents added to wells with incorrect concentrations	Check for pipetting errors and the correct reagent volume.

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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. 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. 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												



LEGEND MAX™ Kits are manufactured by **BioLegend Inc.**

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