Quantikine®

Mouse/Rat Epo Immunoassay

Catalog Number MEP00

For the quantitative determination of mouse/rat Erythropoietin (Epo) concentrations in cell culture supernates, tissue homogenates, serum, and plasma.

This package insert must be read in its entirety before using this product.

FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES.

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INTRODUCTION

Erythropoietin (Epo) is a 34 - 39 kDa secreted glycoprotein that is a member of the type I cytokine superfamily. The mouse and rat Epo genes both encode 192 amino acid (aa) residue precursors that contain 26 aa signal peptides and 166 aa mature proteins containing three potential N-linked glycosylation sites (1 - 4). Both mouse and rat Epo lack the O-linked glycosylation site found in human Epo. Although carbohydrate chains are not required for *in vitro* receptor binding, they are required for *in vivo* Epo bioactivity. Depending on the cell source, different Epo isoforms are produced that differ in their glycan compositions and sialic acid contents (5 - 8). Mature mouse and rat Epo share 94% aa sequence identity. They also share from 80% - 82% aa identity with mature human, porcine, rhesus monkey and feline Epo (2, 3). Epo is primarily produced by cells in the kidney (interstitial peritubular renal fibroblasts) and liver (hepatocytes and Ito cells), where its production is up-regulated by hypoxia. Other tissues and cells, including neural tissues (astrocytes and neurons), testis (Sertoli cells), uterus, placenta, and erythroid progenitors, have also been shown to produce Epo (9 - 14).

Epo is best known for its role in red blood cell formation. While Epo is not a lineage commitment factor, it inhibits apoptosis and induces burst forming unit-erythroid (BFU-E) differentiation into colony forming unit-erythroid (CFU-E), and the subsequent proliferation and maturation of CFU-E into early normoblasts (10, 15, 16). Apart from its role in erythropoiesis, Epo also acts on various non-hematopoietic cells to function as a viability and proliferation factor. Epo can stimulate myoblast proliferation while suppressing its differentiation, resulting in the expansion of the progenitor cell population (17). Epo is a tissue-protective factor that protects against ischemic and toxic injuries to neuronal, cardiovascular and renal tissues (18, 19). Epo has also been shown to promote angiogenesis in various physiologic and pathologic conditions (20, 21).

Epo binds and signals via the high-affinity preformed homodimeric Epo receptor (Epo R) that is composed of two Epo R subunits. Each Epo R subunit is a type I transmembrane glycoprotein that belongs to the type I cytokine receptor superfamily (18, 22 - 24). Its extracellular domain contains the characteristic two fibronectin type III domains and a WSxWS motif near the plasma membrane (24, 25). Binding of Epo to the Epo R homodimer results in conformational change and phosphorylation and activation of the non-receptor protein kinase JAK2, which activates the downstream signaling cascade (26). An alternative Epo heteromeric receptor complex that transduces cell-protective signals and containing the β common receptor (βCR) subunit in addition to the Epo R subunit has been described. βCR also belongs to the type I cytokine receptor superfamily and is a subunit that is shared by the heteromeric IL-3, IL-5 and GM-CSF receptor complexes. Epo binds with lower affinity to the heteromeric receptor consisting of a Epo R subunit and a βCR homodimer (18).

The Quantikine Mouse/Rat Epo Immunoassay is a 4.5 hour solid-phase ELISA designed to measure mouse and rat Epo in cell culture supernates, tissue homogenates, serum, and plasma. It contains antibodies raised against recombinant Epo and has been shown to accurately quantitate the recombinant factor. Results obtained using natural mouse or rat Epo showed linear curves that were parallel to the standard curves obtained using the Quantikine kit standards. These results indicate that the Quantikine Mouse/Rat Epo kit can be used to determine relative mass values for naturally occurring mouse and rat Epo.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for mouse/rat Epo has been pre-coated onto a microplate. Standards, control, and samples are pipetted into the wells and any mouse or rat Epo present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for mouse/rat Epo is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. The enzyme reaction yields a blue product that turns yellow when the Stop Solution is added. The intensity of the color measured is in proportion to the amount of mouse or rat Epo bound in the initial step. The sample values are then read off the standard curve.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, further dilute the samples with the Calibrator Diluent and repeat the assay.
- Any variation in operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- This assay is designed to eliminate interference by soluble receptors, binding proteins and other factors present in biological samples. Until all factors have been tested, the possibility of interference cannot be excluded.

PRECAUTION

The Stop Solution provided with this kit is an acid solution. Wear eye, hand, face and clothing protection when using this material.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- For best results, pipette reagents and samples into the center of each well.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution.
- Stop Solution should be added to the plate in the same order as the Substrate Solution.

MATERIALS PROVIDED

Mouse/Rat Epo Microplate (Part 892567) - One 96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody against mouse/rat Epo.

Mouse/Rat Epo Conjugate (Part 892568) - 12.0 mL of a monoclonal antibody against mouse/rat Epo conjugated to horseradish peroxidase with preservatives.

Mouse/Rat Epo Standard (Part 892569) - 15 ng of recombinant Epo in a buffered protein base with preservatives; lyophilized.

Mouse/Rat Epo Control (Part 892570) - 1 vial of recombinant Epo in a buffered protein base with preservatives; lyophilized. The concentration ranges of mouse/rat Epo after reconstitution are shown on the vial label. The assay value of the Control should be within the range specified on the label.

Assay Diluent RD1W (*for mouse samples*) (Part 895038) - 12.0 mL of a buffered protein solution with preservatives.

Assay Diluent RD1-38 (*for rat samples*) (Part 895301) - 12.0 mL of a buffered protein solution with preservatives.

Calibrator Diluent RD6Z (Part 895466) - 21 mL of a diluted animal serum with preservatives.

Wash Buffer Concentrate (Part 895024) - 50 mL of a 25-fold concentrated solution of buffered surfactant with preservatives.

Color Reagent A (Part 895000) - 12.0 mL of stabilized hydrogen peroxide.

Color Reagent B (Part 895001) - 12.0 mL of stabilized chromogen (tetramethylbenzidine).

Stop Solution (Part 895174) - 23 mL of a diluted hydrochloric acid.

Plate Covers (Part 640197) - 4 adhesive strips.

STORAGE

<table>
<tr><td>Unopened Kit</td><td colspan="2">Store at 2 - 8° C. Do not use past kit expiration date.</td></tr>
<tr><td rowspan="11">Opened/ Reconstituted Reagents</td><td>Mouse/Rat Epo Conjugate</td><td rowspan="8">May be stored for up to 1 month at 2 - 8° C.*</td></tr>
<tr><td>Diluted Wash Buffer</td></tr>
<tr><td>Stop Solution</td></tr>
<tr><td>Assay Diluent RD1W</td></tr>
<tr><td>Assay Diluent RD1-38</td></tr>
<tr><td>Calibrator Diluent RD6Z</td></tr>
<tr><td>Unmixed Color Reagent A</td></tr>
<tr><td>Unmixed Color Reagent B</td></tr>
<tr><td>Mouse/Rat Epo Standard (3000 pg/mL)</td><td rowspan="2">Aliquot and store for up to 1 month at ≤ -20° C in a manual defrost freezer.* Avoid repeated freeze-thaw cycles.</td></tr>
<tr><td>Mouse/Rat Epo Control</td></tr>
<tr><td>Microplate Wells</td><td>Return unused wells to the foil pouch containing the desiccant pack, reseal along entire edge of zip-seal. May be stored for up to 1 month at 2 - 8° C.*</td></tr>
</table>

*Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 100 mL and 1000 mL graduated cylinders.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.

SAMPLE COLLECTION AND STORAGE

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20° C. Avoid repeated freeze-thaw cycles.

Mouse Tissue Homogenates - The preparation of tissue homogenates will vary depending upon the tissue type. For this assay, heart, lung, and spleen tissue from three mice was rinsed with 1X PBS to remove excess blood, homogenized in 5 - 10 mL of 1X PBS, and stored overnight at ≤ -20° C. After two freeze-thaw cycles to break up the cell membranes, the homogenates were centrifuged for 5 minutes at 5000 x g. Homogenates should be assayed immediately or aliquotted and stored at ≤ -20° C. Avoid repeated freeze-thaw cycles.

Serum - Allow blood samples to clot for 2 hours at room temperature or overnight at 2 - 8° C before centrifuging for 20 minutes at 2000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20° C. Avoid repeated freeze-thaw cycles.

Mouse Plasma - Collect plasma using heparin as an anticoagulant. Centrifuge for 20 minutes at 2000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20° C. Avoid repeated freeze-thaw cycles.

Note: *Mouse EDTA plasma samples have not been validated for use in this assay.*

Rat Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 20 minutes at 2000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20° C. Avoid repeated freeze-thaw cycles.

Note: *Grossly hemolyzed or lipemic samples may not be suitable for use in this assay.*

SAMPLE PREPARATION

Mouse serum and plasma samples require a 2-fold dilution into Calibrator Diluent RD6Z prior to assay. A suggested 2-fold dilution is 70 µL sample + 70 µL Calibrator Diluent RD6Z.

Rat serum and plasma samples do not require dilution.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

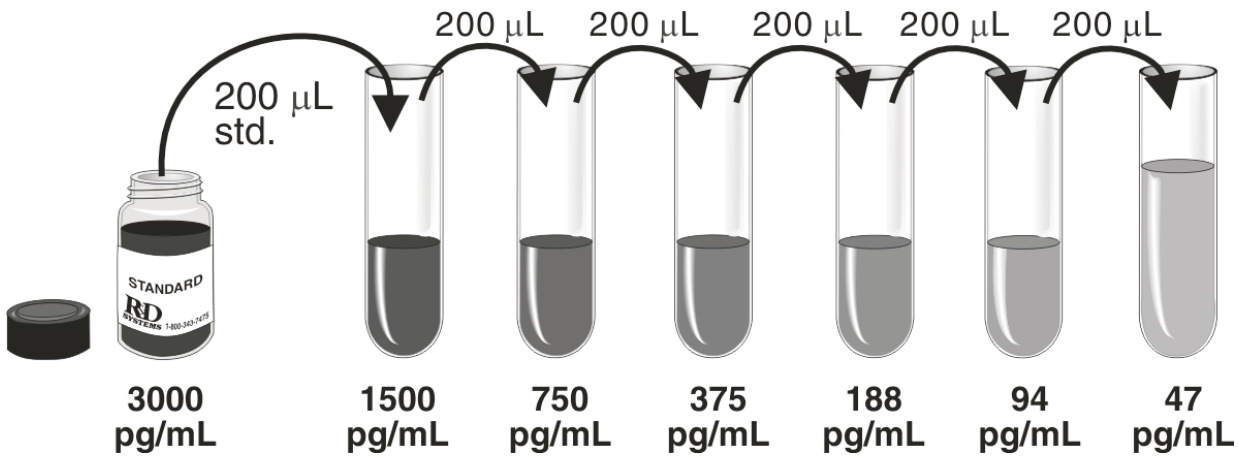
Mouse/Rat Epo Kit Control - Reconstitute the Kit Control with 1.0 mL deionized or distilled water. Assay the Control undiluted.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. To prepare enough Wash Buffer for one plate, add 25 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 625 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 100 µL of the resultant mixture is required per well.

Mouse/Rat Epo Standard - Reconstitute the Standard with 5.0 mL of Calibrator Diluent RD6Z. Do not substitute other diluents. This reconstitution produces a stock solution of 3000 pg/mL. Allow the stock solution to sit for a minimum of 5 minutes with gentle mixing prior to making dilutions.

Pipette 200 µL of Calibrator Diluent RD6Z into each tube. Use the stock solution to produce a 2-fold dilution series (below). Mix each tube gently but thoroughly before the next transfer. The undiluted mouse/rat Epo Standard serves as the high standard (3000 pg/mL). Calibrator Diluent RD6Z serves as the zero standard (0 pg/mL).



ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all samples, control, and standards be assayed in duplicate.

1. Prepare all reagents, standard dilutions, control, and samples as directed in the previous sections.

2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.

3. Add 50 µL of Assay Diluent RD1W (*for mouse samples*) or Assay Diluent RD1-38 (*for rat samples*) to each well.

4. Add 50 µL of Standard, Control, or sample* per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm. A plate layout is provided to record standards and samples assayed.

5. Aspirate each well and wash, repeating the process four times for a total of five washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

6. Add 100 µL of Mouse/Rat Epo Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature on the shaker.

7. Repeat the aspiration/wash as in step 5.

8. Add 100 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature **on the benchtop. Protect from light.**

9. Add 100 µL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.

10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

*Mouse serum and plasma samples require dilution. See Sample Preparation.

PROCEDURE SUMMARY AND CHECKLIST

1. ☐ Bring all reagents to room temperature.
☐ Prepare reagents and samples as instructed.
☐ Return unused components to storage temperature as indicated in the instructions.

2. ☐ Add 50 µL of the appropriate Assay Diluent to each well.

3. ☐ Add 50 µL Standard, Control, or sample* to each well.
☐ Cover the plate and incubate for 2 hours at room temperature on the shaker.

4. ☐ Aspirate and wash each well five times.

5. ☐ Add 100 µL Conjugate to each well.
☐ Cover the plate and incubate for 2 hours at room temperature on the shaker.

6. ☐ Aspirate and wash each well five times.

7. ☐ Add 100 µL Substrate Solution to each well. Incubate for 30 minutes at room temperature **on the benchtop. Protect from light.**

8. ☐ Add 100 µL Stop Solution to each well.

9. ☐ Read Optical Density at 450 nm (correction wavelength set at 540 nm or 570 nm).

*Mouse serum and plasma samples require dilution. See Sample Preparation.

CALCULATION OF RESULTS

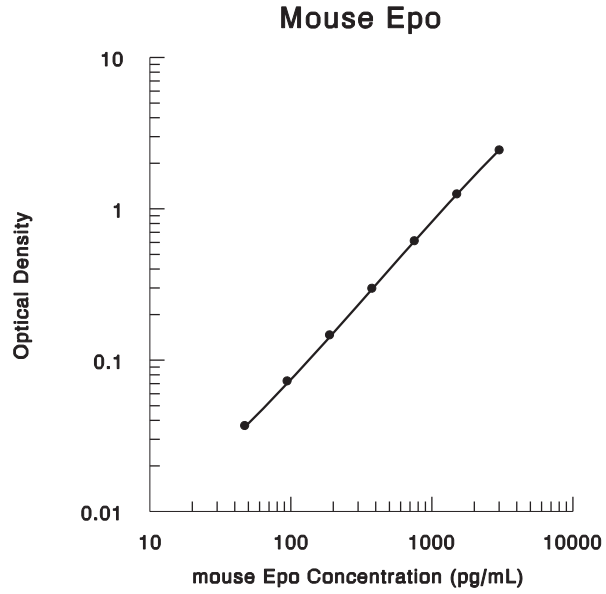
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density.

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the Epo concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

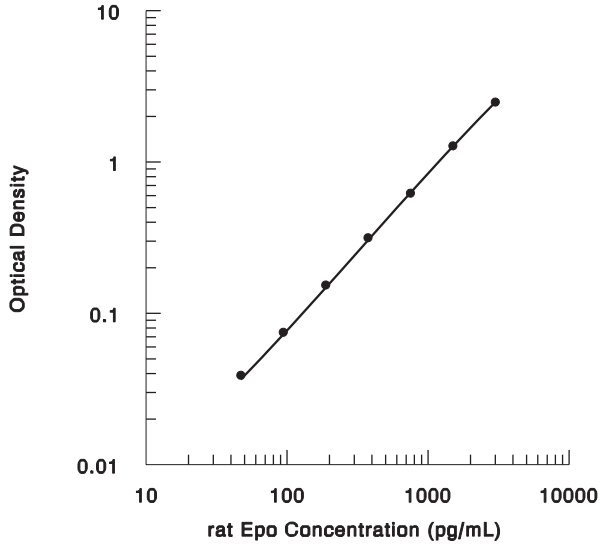
Because mouse serum and plasma samples have been diluted prior to assay, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.



pg/mL	O.D.	Average	Corrected
0	0.041 0.045	0.043	—
47	0.078 0.082	0.080	0.037
94	0.113 0.118	0.116	0.073
188	0.187 0.192	0.190	0.147
375	0.339 0.345	0.342	0.299
750	0.658 0.660	0.659	0.616
1500	1.284 1.314	1.299	1.256
3000	2.473 2.521	2.497	2.454



pg/mL	O.D.	Average	Corrected
0	0.049 0.058	0.054	—
47	0.090 0.095	0.093	0.039
94	0.125 0.132	0.129	0.075
188	0.205 0.209	0.207	0.154
375	0.367 0.371	0.369	0.316
750	0.667 0.686	0.677	0.623
1500	1.308 1.356	1.332	1.279
3000	2.508 2.579	2.544	2.490

PRECISION

Intra-assay Precision (Precision within an assay)
Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays)
Three samples of known concentration were tested in separate assays to assess inter-assay precision.

Mouse Assay

	Intra-assay Precision			Inter-assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pg/mL)	196	258	704	180	239	700
Standard deviation	7.7	7.8	31.2	17.5	15.3	17.5
CV (%)	3.9	3.0	4.4	9.7	6.4	2.5

Rat Assay

	Intra-assay Precision			Inter-assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	15	27	16
Mean (pg/mL)	114	168	1634	91.7	167	1695
Standard deviation	6.8	6.8	31.6	8.8	14.6	72.4
CV (%)	6.0	4.0	1.9	9.6	8.7	4.3

RECOVERY

The recovery of Epo spiked to three levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Mouse cell culture supernates (n=7)	110	102 - 117%
Mouse tissue homogenates (n=3)	94	80 - 111%
Mouse serum* (n=9)	93	85 - 114%
Mouse heparin plasma* (n=8)	103	90 - 120%

*Mouse serum and plasma samples were spiked and then diluted as described in the Sample Preparation section.

Sample Type	Average % Recovery	Range
Rat cell culture supernates (n=4)	107	96 - 116%
Rat serum (n=14)	98	82 - 111%
Rat heparin plasma (n=9)	93	83 - 113%
Rat EDTA plasma (n=9)	109	104 - 113%

LINEARITY

To assess the linearity of the assay, samples containing or spiked with high concentrations of Epo in each matrix were diluted with Calibrator Diluent RD6Z and then assayed.

		Mouse cell culture supernates (n=9)	Mouse tissue homogenates (n=3)	Mouse serum* (n=6)	Mouse heparin plasma* (n=9)
1:2	Average % of Expected	92	106	95	94
	Range (%)	90 - 96	94 - 120	91 - 98	89 - 97
1:4	Average % of Expected	93	105	96	96
	Range (%)	90 - 102	92 - 119	90 - 100	88 - 102
1:8	Average % of Expected	94	107	98	97
	Range (%)	90 - 100	94 - 120	90 - 105	87 - 106
1:16	Average % of Expected	95	106	106	97
	Range (%)	85 - 103	95 - 113	95 - 117	83 - 108

*Mouse serum and plasma samples were spiked and then diluted as described in the Sample Preparation section.

		Rat cell culture supernates (n=4)	Rat serum (n=10)	Rat heparin plasma (n=9)	Rat EDTA plasma (n=7)
1:2	Average % of Expected	98	97	99	98
	Range (%)	93 - 109	91 - 101	95 - 106	95 - 102
1:4	Average % of Expected	97	96	100	100
	Range (%)	92 - 110	85 - 102	97 - 110	93 - 105
1:8	Average % of Expected	99	100	103	102
	Range (%)	93 - 110	88 - 113	93 - 116	92 - 106
1:16	Average % of Expected	97	101	92	93
	Range (%)	86 - 104	83 - 115	83 - 99	85 - 101

SENSITIVITY

Nineteen assays were evaluated and the minimum detectable dose (MDD) of mouse Epo ranged from 6.5 - 46.9 pg/mL. The mean MDD was 18.0 pg/mL.

Twenty-two assays were evaluated and the minimum detectable dose (MDD) of rat Epo ranged from 3.3 - 21.7 pg/mL. The mean MDD was 12.5 pg/mL.

The minimum detectable dose was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

CALIBRATION

This immunoassay is calibrated against a highly purified mouse myeloma cell-expressed recombinant mouse Epo produced at R&D Systems. The 166 amino acid residue mouse Epo has a calculated molecular mass of approximately 18.5 kDa. As a result of glycosylation, the recombinant mouse Epo migrates as an approximately 36 kDa protein in SDS-PAGE.

The protein concentrations of the recombinant mouse Epo was determined by the method of Bradford (27) using purified bovine serum albumin as a standard.

SAMPLE VALUES

Mouse Serum/Plasma - Twenty individual mouse serum and plasma samples were evaluated for detectable levels of mouse Epo in this assay.

Sample	Mean of Detectable (pg/mL)	% Detectable	Range (pg/mL)
Mouse serum (n=20)	237	65	ND - 683
Mouse heparin plasma (n=20)	151	30	ND - 198

ND = Non-detectable

Rat Serum/Plasma - Twenty individual rat serum and plasma samples were evaluated for detectable levels of rat Epo in this assay. No detectable levels were observed in normal rat samples. One rat injected with phenylhydrazine hydrochloride produced a serum level of Epo measuring 4884 pg/mL (28).

Tissue Homogenates - Lung, heart, and spleen tissues from three adult female mice were prepared as described in the Sample Collection and Storage section on page 5. Supernates were removed, tested for mouse Epo, and measured 61 pg/mL, 111 pg/mL, and 75 pg/mL, respectively.

SPECIFICITY

This assay recognizes both recombinant and natural mouse and rat Epo. The factors listed below were prepared at 50 ng/mL in Calibrator Diluent RD6Z and assayed for cross-reactivity. Preparations of the following factors at 50 ng/mL in a mid-range mouse/rat Epo control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant mouse:

ICAM-1
IFN-γ
IGF-I
IGF-II
IL-1α
IL-1β
IL-1ra
IL-2
IL-3
IL-4
IL-5
IL-6
IL-7
IL-9
IL-10
IL-11
IL-12
IL-12 p40
IL-13
IL-17
IL-18
JE/MCP-1
Leptin
LIF
M-CSF
MIP-1α
MIP-1β
OSM
RANTES
sTNF RII
TNF-α
Tpo
VEGF

Recombinant rat:

CINC-1
CINC-2α
CINC-2β
CINC-3
CNTF
CNTF sRα
EphA5/Fc
EphB1/Fc
E-Selectin/Fc
Fractalkine
GDNF
GDNF sRα1
GM-CSF
IFN-γ
IL-1α
IL-1β
IL-1ra
IL-1 R6
IL-2
IL-4
IL-6
IL-10
IL-18
LIX
MAG/Fc
MIP-3α
Neuropilin-1/Fc
Neuropilin-2/Fc
β-NGF
PDGF-AA
PDGF-BB
TIMP-1
VEGF

Recombinant mouse Epo R/Fc Chimera interferes with this assay at concentrations > 2 ng/mL.

Recombinant human Epo has approximately 9% cross-reactivity in this assay at concentrations ≥ 2 ng/mL.

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PLATE LAYOUT

Use this plate layout as a record of standards and samples assayed.