Multiplex Immunoassay Panels for Human and Rodent Hypoxia Markers

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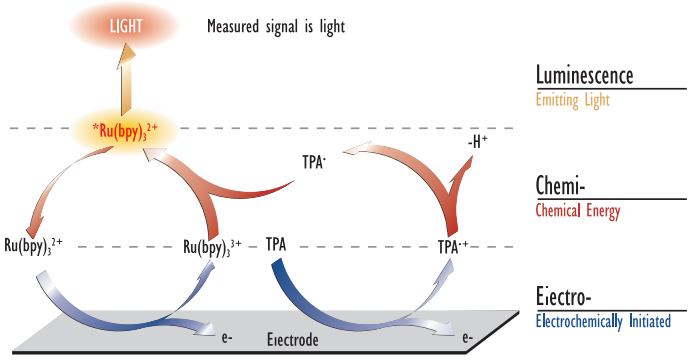
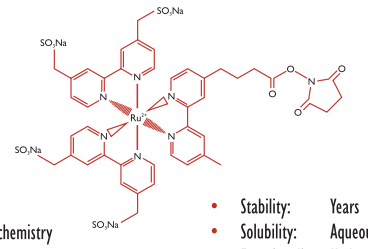
Vascular Endothelial Growth Factor (VEGF) regulates angiogenesis; Erythropoietin (EPO) regulates erythropoiesis, and Insulin-like Growth Factor Binding Protein-1 (IGFBP-1) modulates the metabolic and mitogenic effects of IGFs. If the tissue supply of oxygen is low, these factors are upregulated by the transcription factor HIF-1α (Hypoxia Inducible Factor). Electrochemiluminescence-based immunoassay panels were developed which allow simultaneous measurement of human VEGF, IGFBP-1, and EPO, of human HIF-1α and VEGF, and of mouse or rat VEGF & EPO in a 96-well or 384 well format (human EPO & VEGF duplex only). For the high-throughput cell culture medium protocol, detection antibody reagent and sample are added to a well, and after a two-hour incubation, the plate is washed, MSD Read Buffer is added, and the plate is read on a MSD SECTOR™ Imager 6000 instrument at a throughput of one plate per minute. A serum and plasma protocol containing one additional incubation and wash step was developed and validated by spike recovery and dilution linearity experiments. Only 25 µL of sample is required. CVs are in the 5-10% range.

Electrochemiluminescence (ECL)



Ruthenium (II) tris-bipyridine-(4-methylsulfonate) NHS ester (MSD SULFO-TAG™ label)



- Selective
- Convenient chemistry
- Robust, stable
- Few interfaces

- Stability: Years
- Solubility: Aqueous, DMSO
- Functionality: Hydrophilic
- Specificity: High

MSD MULTI-ARRAY™ Technology

Instrument Features

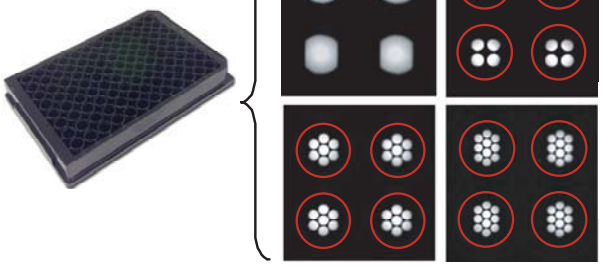
- Cooled CCD camera for low background and large dynamic range
- Fast read time, ~1 minute for all plate types
- Easy to use, no filters or end-user adjustments
- On board data analysis tools
- Integrated stackers for walk-away operation

SECTOR PR 400

SECTOR Imager 6000

Plate Features

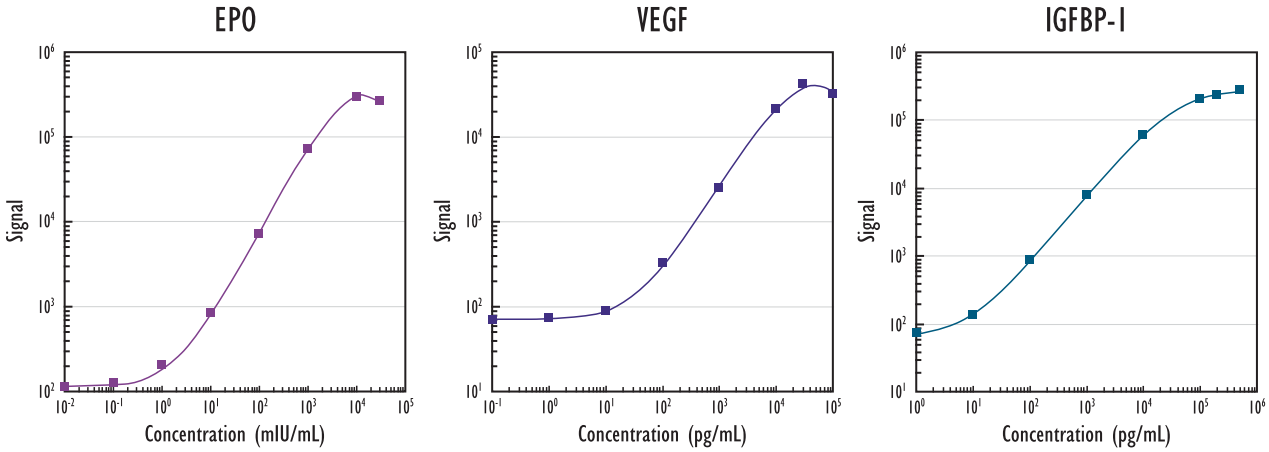
- Disposable plates
- Patterned arrays for maximum flexibility
- Multiplexing in 96- and 384-well formats
- Compatible with automation for high throughput



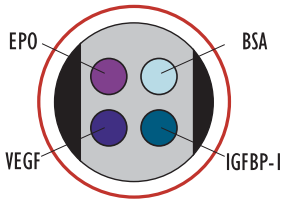
Assay Format

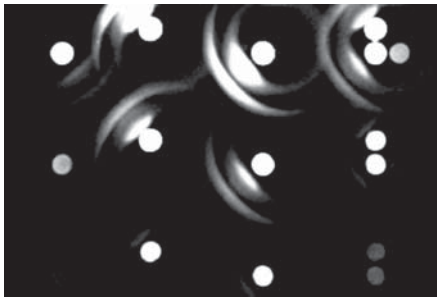
- **I. High-throughput format (cell culture supernatants)**
  - Block plates for one hour at RT or overnight at 4 °C and wash (optional)
  - Add 25 µL each of detection AB reagent and sample to each well
  - Incubate for two hours on a shaker
  - Wash; add MSD Read Buffer; read
- **II. Serum & Plasma Protocol**
  - Block plates for one hour at RT or overnight at 4 °C and wash
  - Add 25 µL each of assay diluent and sample to each well.
  - Incubate for two hours on a shaker; wash
  - Add 25 µL of detection antibody reagent to each well
  - Incubate for two hours on a shaker
  - Wash; add MSD Read Buffer; read

Human Hypoxia Triplex

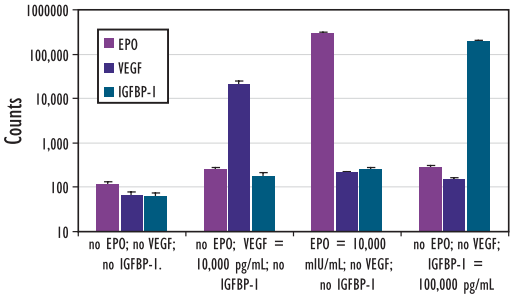


Human EPO - VEGF - IGFBP-1 Multiplexed





EPO, VEGF, and IGFBP-1 in Same Well



% Cross-reactivity

	EPO	VEGF	IGFBP-1
EPO	X	0.64%	0.08%
VEGF	0.05%	X	0.05%
IGFBP-1	0.07%	0.57%	X

% Cross-reactivity: (signal in presence of interferent - signal in absence of interferent)/interferent signal

Serum and Plasma Protocol for Human EPO and VEGF Duplex

Sample	VEGF			EPO		
	Measured (pg/mL)	Expected (pg/mL)	Spike Recovery	Measured (pg/mL)	Expected (pg/mL)	Spike Recovery
S1	45			11		
S1 + 40	77	85	81%	49	51	96%
S1 + 200	248	245	102%	220	211	105%
S1 + 1,000	1,061	1,045	102%	1,034	1,011	102%
S2	22			16		
S2 + 40	73	62	127%	62	56	115%
S2 + 200	259	222	118%	275	216	129%
S2 + 1,000	1,117	1,022	109%	1,185	1,016	117%
P1	18			25		
P1 + 40	62	58	108%	76	65	128%
P1 + 200	245	218	113%	277	225	126%
P1 + 1,000	1,057	1,018	104%	1,155	1,025	113%
P2	20			21		
P2 + 40	69	60	122%	74	61	133%
P2 + 200	242	220	111%	258	221	118%
P2 + 1,000	1,055	1,020	103%	1,133	1,021	111%
Average Spike Recovery			108%			116%

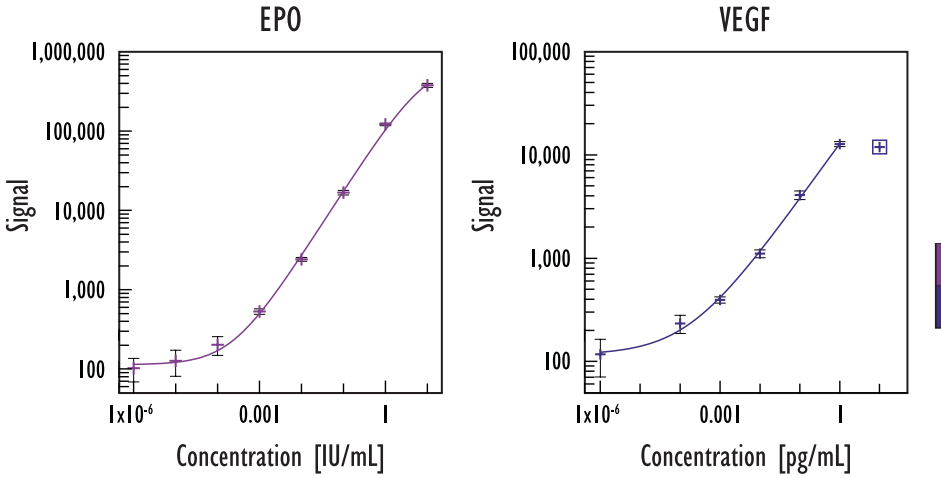
Assay Range for the Serum & Plasma Protocol:	0.5 - >10,000 mIU/mL EPO
Assay Range for the Serum & Plasma Protocol:	5 - >10,000 pg/mL VEGF

The serum and plasma protocol was validated by spike recovery experiments. Two human serum pools and an EDTA and Heparin plasma pool were spike with three levels (see table).

Calibrators: Anchoring to International Reference Materials

- The MSD calibrator for the human EPO assay has been anchored to NIBSC recombinant EPO reference material 88/574, which is also the 2nd WHO Standard (2004).
- The MSD calibrator for the human VEGF assay has been anchored to NIBSC recombinant VEGF-165 Research Reagent 01/424.
- No reference material is currently available for mouse or rat EPO and VEGF, or for IGFBP-1 and HIF-1α.

Hypoxia Markers in 384-Well 4 Spot Format: Human EPO/VEGF Duplex

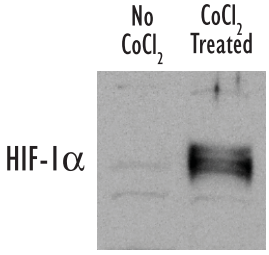


	Detection Limit
EPO	0.002 IU/mL
VEGF	2 pg/mL

Detection limit is 2.5 StdDev above background

Multiplexed serum biomarker assays also can be performed on MULTI-SPOT 384-Well 4 Spot plates. The above example demonstrates the measurement of the human hypoxia markers erythropoetin (EPO) and vascular endothelial growth factor (VEGF) in tissue culture medium. Performance in the 384-Well 4 Spot format is similar to the 96-Well 4 Spot format with dynamic range, sensitivity, specificity, and variability all being comparable. Titration curves indicate dynamic range windows of 3-4 logs and detection limits of 0.002 IU/mL for EPO and 2 pg/mL VEGF. The above titration curves are determined from 12 replicates per point; variability is low, and the average CV above the detection limit is $\leq$ 7%. Measurement of the signal on both spots when individual calibrators are used as samples allows for an assessment of assay specificity. Using spikes of 10 ng/mL VEGF and 100 mIU/mL of EPO, the cross reactivity between spots is less than 0.5%. Recommended protocols for 384-Well 4 Spot serum biomarker assays are similar to those suggested for 96-Well 4 Spot assays except less sample (10 µL) is required.

HIF-1α Assay: ECL Signal for Nuclear Extracts and Cell Lysates from $CoCl_2$-Treated and Untreated Cells

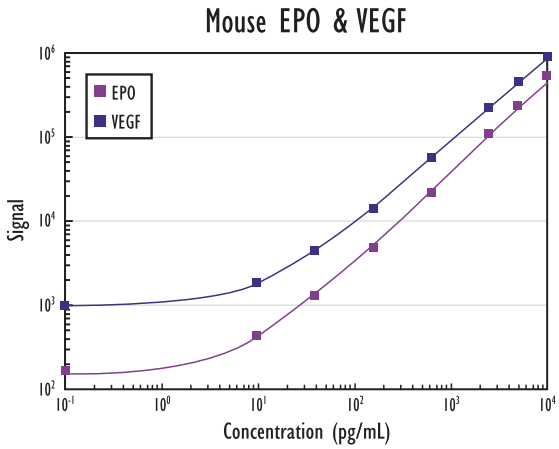


Western Blot of nuclear extracts from untreated and $CoCl_2$-treated HeLa cells

µg Nuclear Extract per Well	COS Nuclear Extracts, Treated	HeLa Nuclear Extracts, Untreated	HeLa Nuclear Extracts, Treated	µg Lysate Protein per Well	HeLa Lysates, Untreated	HeLa Lysates, Untreated
0	77	77	77	0	77	77
0.00035	78	71	79	0.13	147	270
0.0035	124	71	105	0.40	341	1,006
0.035	692	109	436	1.2	654	2,387
0.35	7,709	232	4,109	3.6	1,729	4,841
3.5	88,433	1,232	37,574	11	3,895	12,128
35	320,266	7,300	357,792	32	7,023	29,212
Detection Limit (ng/well)	3		3			20

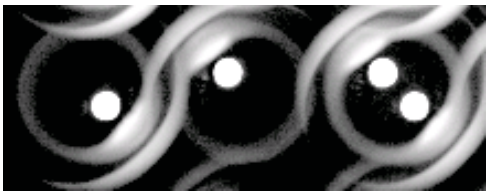
Detection limits estimated as 2.5 StdDev above zero calibrator

Mouse Hypoxia Assays: Calibration Curve, Assay Range & Cross-Reactivity

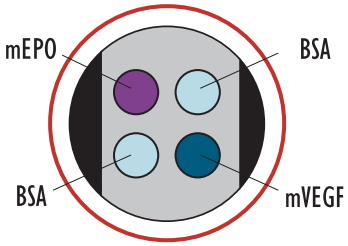


mEPO Detection Limit:	2 pg/mL
mEPO Assay Range:	2-10,000 pg/mL

mVEGF Detection Limit:	3 pg/mL
mVEGF Assay Range:	3-10,000 pg/mL



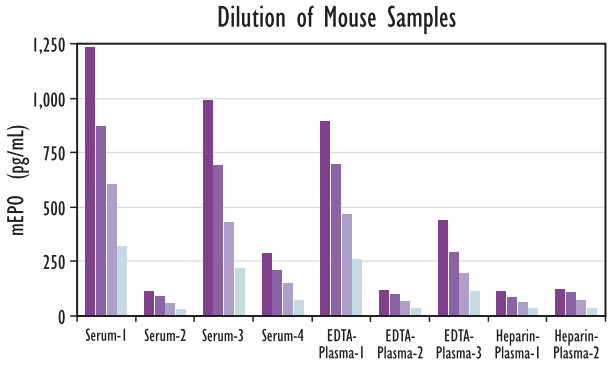
10 ng/mL mVEGF | 10 ng/mL mEPO | 10 ng/mL mEPO + 10 ng/mL mVEGF



% Cross-Reactivity:	< 0.2%

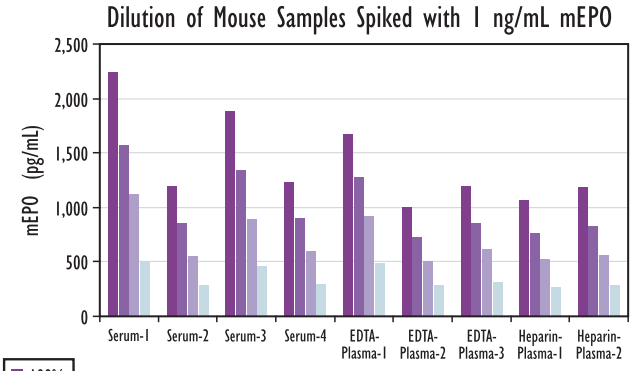
% Cross-reactivity: (signal-background on "wrong" spot)/signal on correct spot

Mouse EPO Assay: Dilution Linearity



Four mouse serum pools and five mouse plasma pools (~20 animals per pool) were diluted with zero calibrator to 75%, 50%, and 25%.

mEPO Average Measured vs. Expected Concentration:	104%
Range:	87% - 124%

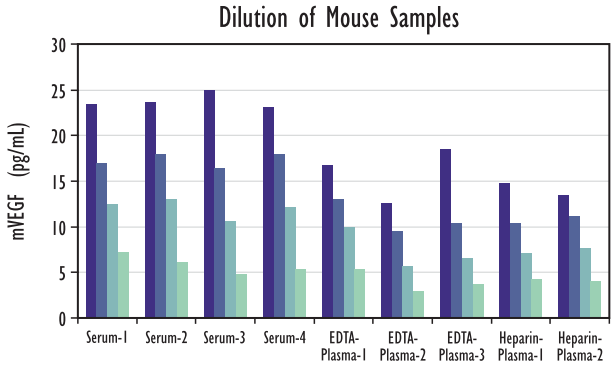


100%
75%
50%
25%

Pools were spiked with mEPO and subsequently diluted.

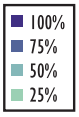
mEPO Average Measured vs. Expected Concentration:	99%
Range:	90% - 115%

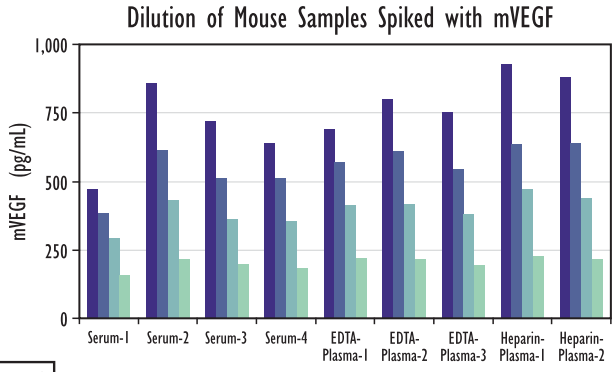
Mouse VEGF Assay: Dilution Linearity



Four mouse serum pools and five mouse plasma pools (~20 animals per pool) were diluted with zero calibrator to 75%, 50%, and 25%.

mVEGF Average Measured vs. Expected Concentration:	100%
Range:	70% - 127%

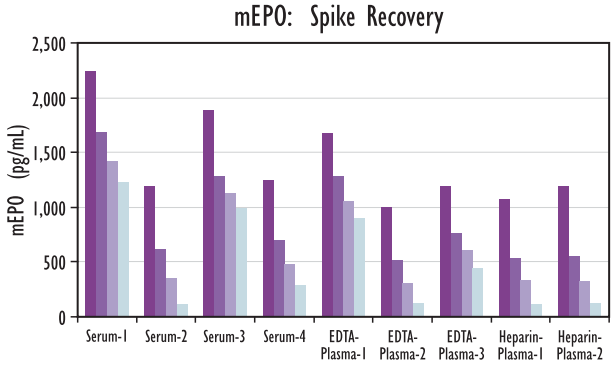




Pools were spiked with mEPO and subsequently diluted.

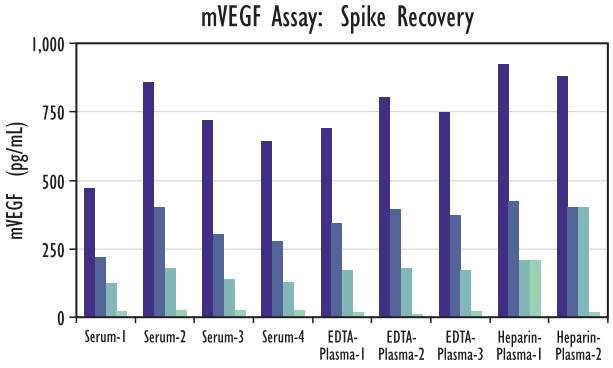
mVEGF Average Measured vs. Expected Concentration:	106%
Range:	92% - 132%

Mouse EPO & VEGF Assays: Spike Recoveries



Four mouse serum pools and five mouse plasma pools (~20 animals per pool) were spiked with 1,000, 500, and 250 pg/mL mEPO.

mEPO Average Spike Recovery:	83%
StdDev:	14%
Range:	52% - 108%

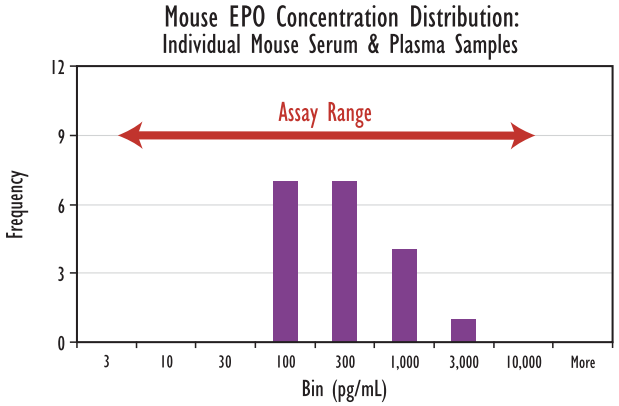


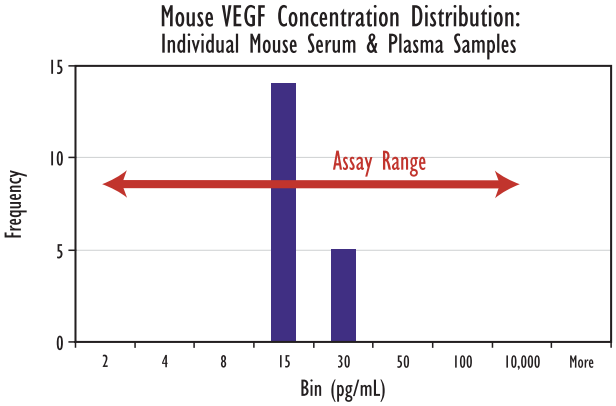
Four mouse serum pools and five mouse plasma pools (~20 animals per pool) were spiked with 1,000, 500, and 250 pg/mL mVEGF.

mVEGF Average Spike Recovery:	65%
StdDev:	15%
Range:	39% - 91%

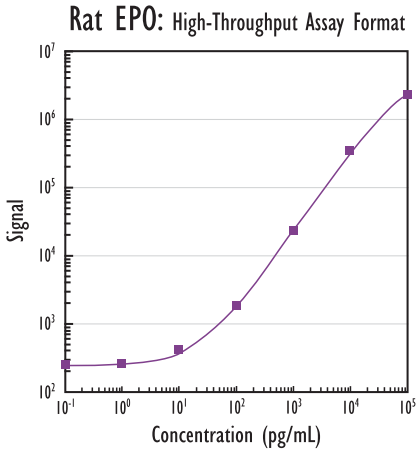
Presumably, some of the spiked VEGF is bound (for example by soluble VEGF receptors) and no longer recognized by the assay.

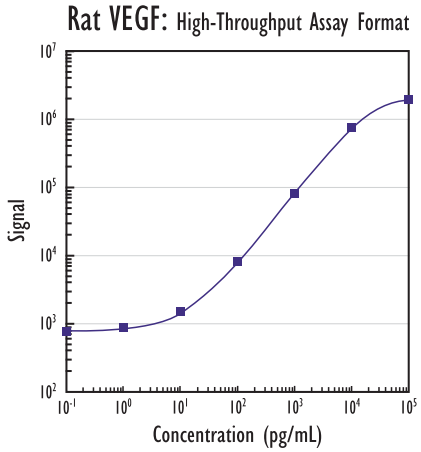
Mouse EPO & VEGF Assays: Assay Range & Expected Levels in Serum & Plasma

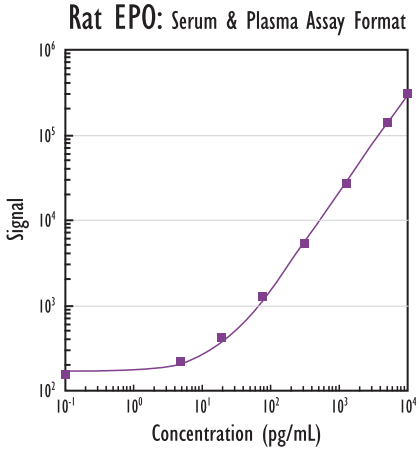


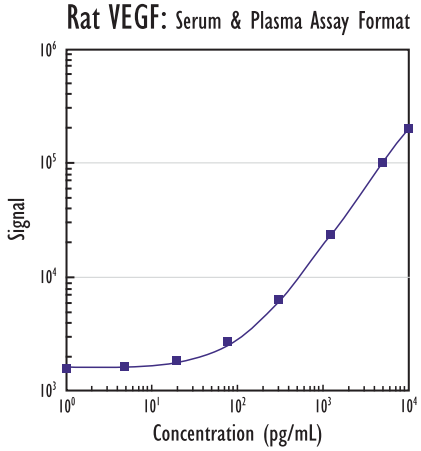


Rat EPO and VEGF Assays: Calibration Curves









Rat EPO and VEGF assays use the same plates, labeled antibodies and diluents as the mouse EPO and VEGF assay, but a different calibrator (i.e. recombinant rat EPO and recombinant rat VEGF).

Rat EPO & VEGF: Spike Recovery and Dilution Linearity

Sample	Rat EPO			Rat VEGF		
	Measured (pg/mL)	Expected (pg/mL)	Spike Recovery	Measured (pg/mL)	Expected (pg/mL)	Spike Recovery
R1	95			181		
R2	31			334		
R3	86			227		
R4	57			161		
R1 + 500	562	595	93%	459	681	56%
R2 + 500	474	531	89%	615	834	56%
R3 + 500	566	586	96%	476	727	50%
R4 + 500	532	557	95%	452	661	58%
R1 + 1,000	1,107	1,095	101%	818	1,181	64%
R2 + 1,000	974	1,031	94%	918	1,334	58%
R3 + 1,000	1,040	1,086	95%	773	1,227	55%
R4 + 1,000	1,015	1,057	96%	873	1,161	71%
Average Spike Recovery			95%			58%

Sample	Rat EPO			Rat VEGF		
	Measured (pg/mL)	Expected (pg/mL)	% of Expected	Measured (pg/mL)	Expected (pg/mL)	% of Expected
R1 50%	51	48	107%	97	90	107%
R2 50%	22	16	143%	196	167	118%
R3 50%	45	43	106%	114	114	100%
R4 50%	33	29	113%	79	81	98%
Average Dilution Linearity			117%			106%

To validate the rat EPO/VEGF serum and plasma protocol, two rat serum pools, one rat EDTA plasma pool and one rat heparin plasma pool were diluted to 50% or spiked with two levels of recombinant rat EPO and VEGF. Diluted samples and EPO spike recovered as expected. Spike recovery of rat VEGF was low, presumably because binding proteins such as soluble VEGF receptors bind to some the spiked VEGF.

Hypoxia Assays: Performance Summary

Sample	Assay Range: One-Step Protocol (Cell Culture Supernatants)	Assay Range: Sequential Protocol (Serum & Plasma Tumor Lysates)	Native Serum or Plasma Levels (Pooled Samples)	Multiplexing
Human EPO	0.4 - 2,000 mIU/mL	0.5 - 10,000 mIU/mL	5 - 30 mIU/mL	VEGF, IGFBP-1
Human VEGF	10 - 10,000 pg/mL	5 - >10,000 pg/mL	20 - 300 pg/mL	EPO, IGFBP-1, HIF-1α
Human IGFBP-1	4 - 40,000 pg/mL	4 - 40,000 pg/mL	300 - 3,000 pg/mL	EPO, VEGF
Human HIF-1α	-	30 - 10,000 ng lysate/well	-	VEGF
Mouse EPO	2 - 10,000 pg/mL	2 - >10,000 pg/mL	100 - 1,200 pg/mL	VEGF
Mouse VEGF	2 - 10,000 pg/mL	3 - >10,000 pg/mL	10 - 30 pg/mL	EPO
Rat EPO	6 - 10,000 pg/mL	6 - >10,000 pg/mL	20 - 100 pg/mL	VEGF
Rat VEGF	8 - 10,000 pg/mL	40 - >10,000 pg/mL	150 - 300 pg/mL	EPO