Validation of an ELISA Method for the Determination of Recombinant Human Erythropoietin (rHu-EPO) in Dog Serum

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Introduction

Erythropoietin (Epo), a glycoprotein (~30,400 Daltons), is produced by the kidney and responsible for regulating red blood cell production (erythropoiesis) in mammals. The production of Epo is highly regulated by changes in oxygen availability. The level of Epo has been shown to increase in circulation under conditions such as hypoxia, renal disease, pulmonary disease, heart disease, or smoking; leading to increased production of red blood cells. On the other hand, the level of Epo has been shown to decrease in circulation under conditions such as anemia, renal disease, AIDS, and chronic infections[1].

Recombinant Human Erythropoietin was validated using a commercial ELISA kit[1] over a range of 10-2500 mIU/mL. Assay parameters were evaluated and optimized to improve the performance for the method.

Materials and methods

Specific reagents used in the assay and procedure are summarized in Table 1. The assay quantitates rhu-Epo in dog serum by double-antibody sandwich enzyme linked immunosorbent assay (ELISA). Epo binds to plates coated with mouse monoclonal capture antibody specific for Epo and the detection is accomplished using a polyclonal antibody conjugated to horseradish peroxidase (HRP). Tetramethy-benzidine (TMB) is the substrate. Prior to preparing the standards and QCs in dog serum, it was necessary to screen the matrix in order to identify the matrices with below lower limit of quantitation (BQ) levels of endogenous analyte.

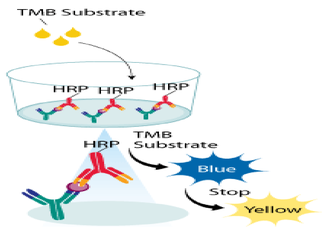

Figure 1. ELISA assay design

Reagent	96-well coated plate		
	standards	Quality Controls	Validation samples
Assay Diluent (µL)	50	50	50
Sample (µL)	50	50	50
Cover plate and place on a plate shaker set at 450 rpm for 1 hour			
Aspirate content			
EPO HRP Conjugate (µL)	200		
Cover plate and place on a plate shaker set at 450 rpm for 1 hour			
Wash plate 4x with wash buffer			
Substrate (µL)	200		
Cover plate and incubate at room temperature for 10 minutes protected from light			
2N H_2SO_4 stop solution (µL)	100		
Read the plate using absorance reader at 450 nm			

Table 1. Assay procedure

Results

The validation parameters of accuracy, precision, robustness, freeze-thaw stability, long-term stability, and bench-top stability were evaluated for each compound. The intra-assay and inter-assay (pooled) precision (%CV) and accuracy (%RE) for each validation sample concentration was ≤ 20% (≤ 25% for LLOQ and ULOQ) for the four compounds. The inter-assay total error (|%CV| + |%RE|) was < 30% (< 40% for LLOQ).

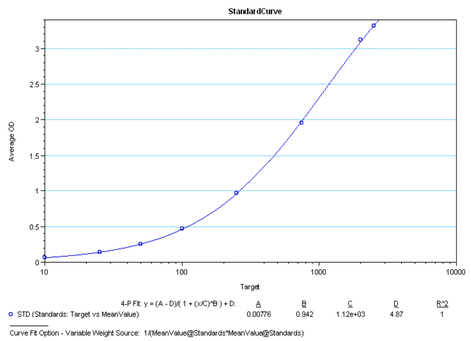

Figure 2. Representative Calibration Curve

Characteristic	Statistic	Nominal concentration (mIU/mL)				
		LLOQ	QCL	QCM	QCH	ULOQ
		10.0	30.0	250	1800	2500
# Results	N	35	35	36	36	36
Accuracy	Mean Bias (%RE)	14.3	10.9	3.5	8.9	4.7
Precision	Interbatch (%CV)	8.4	6.9	5.9	8.0	7.9
Total Error	Mean| + Interbatch|	22.792	17.871	9.376	16.913	12.626

Table 2. Total Error for Precision and Accuracy

Sample	Unspiked Matrix	Matrix spiked at QCL 30.0 mIU/mL	%RE	Matrix spiked at QCH 1800 mIU/mL	%RE
1	<LLOQ	27.3	-9.00	1680	-6.67
2	<LLOQ	31.8	6.00	1730	-3.89
3	<LLOQ	31.8	6.00	1940	7.78
4	<LLOQ	30.6	2.00	1760	-2.22
5	<LLOQ	32.8	9.33	1840	2.22
6	<LLOQ	32.0	6.67	1780	-1.11
7	<LLOQ	29.6	-1.33	1840	2.22
8	<LLOQ	33.4	11.3	2050	13.9
9	<LLOQ	35.6	18.7	2150	19.4
10	<LLOQ	31.9	6.33	1850	2.78

Table 3. Selectivity evaluation of rHu-Epo in dog serum

(mIU/mL)		%RE
Nominal	Concentration	
1800	2130	18.3
900	1040	15.6
450	494	9.8
150	122	-18.7
15.0	13.6	-9.3

Dilution of sample prepared from high stock waswas shown to be linear down to 15.0 mIU/mL, which represent 2700-fold

Table 4. Dilution linearity of rHu-Epo in dog serum

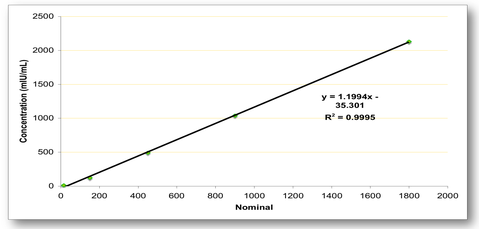

Figure 3. Dilution linearity of rHu-Epo in dog serum

Validation Experiment	Result
Short Term Stability (Bench)	4 Hours
Short Term Stability (4°C)	24 Hours
Freeze Thaw Stability (-80°C)	3 Cycles
Selectivity	No interference observed
Dilution (fold)	2700
Long-Term Freezer Stability (-80°C)	56 Days

Table 5. Validation summary of rHu-Epo in dog serum

Conclusions

A sensitive assay for the detection of rHu-Epo was developed, optimized, and validated over a range of 10-2500 mIU/mL. The method were reliable and robust, and considered suitable for the analysis of rHu-Epo in dog serum.

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Literature cited

1. R&D Systems. Quantikine® IVD® ELISA-Human Erythropoietin Immunoassay- (Product# DEP00).

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