Researcher's Guide to Erythroferrone Testing

Selecting the Optimal Erythroferrone Test Kit

Method Comparison

Study Goal

Quantify the performance characteristics of available erythroferrone kits to assist researchers in making informed kit selection decisions related to erythroferrone research.

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Introduction

Why test for Erythroferrone?

Therapeutics:
Erythroferrone could be a therapeutic target for iron loading anemias such as hereditary hemochromatosis (1).

Biomarker:
Erythroferrone could be a useful biomarker for ineffective erythropoiesis and distinguish disease sources such as X-linked sideroblastic anemia and β-thalassemia (2).

Drug detection:
Erythroferrone levels may detect the abuse of erythropoiesis stimulating agents (ESAs) in the anti-doping field (3).

Diagnostics in preventative care:
Erythroferrone can predict mortality in patients with chronic kidney disease and heart failure (4).

Erythroferrone testing challenge

The recent discovery of erythroferrone means many immunoassay testing products have little data to support a comprehensive validation.

Erythroferrone Kit Comparison

Kit Summary

Validation of human Erythroferrone ELISA kits from five (5) manufacturers (**Table 1**) was conducted to assess:

accuracy, precision, linearity, lower measurement limits, and physiologic relevance

Method	Manufacturer	Range (ng/ml)	Run Time (hrs)
A	Abbexa (abx250979)	0.156 - 10	2h50m
B	Aviscera (SK00393-19)	1 - 256	5h10m
C	Cusabio (CSB-EL008059HU)	0.015 - 1	4h30m
D	Intrinsic Lifesciences (ERF-001)	0.156 - 10	1h45m
E	MyBioSource (MBS2088219)	0.156 - 10	2h50m

Table 1. Human Erythroferrone ELISA Kits Included in the analysis.

Erythroferrone Kit Comparison

Accuracy – Spike Recovery

Methods	Slope (95%CI)	R	p
A	0.069 (0.066-0.073)	0.688	< 0.001
B	0.134 (0.127-0.141)	0.212	0.016
C	0.001 (0.001-0.001)	0.270	< 0.001
D	0.801 (0.761-0.841)	0.984	<0.001
E	0.047 (0.045-0.049)	0.978	<0.001

Table 2. Correlation statistics of serum Erythroferrone measured by various ELISA kits

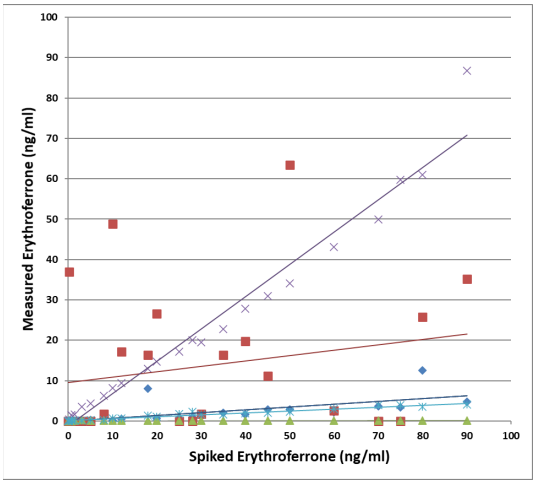

Figure 1. Correlation of Serum Erythroferrone by ELISA Method (Pearson correlation)

NOTE: A TEST WITH PERFECT ACCURACY EXHIBITS A LINEAR REGRESSION SLOPE OF 1.0

Accuracy (**Figure 1**, **Table 2**) was superior using Method D, with a slope closest to 1.0 (0.801), a trait not approached by any other method.

Correlation coefficients (R), an indication of a test's ability to distinguish appropriate and proportional differences (**Table 2**), was satisfactorily high for both Method D and Method E.

Method D demonstrated greatest accuracy for detecting Erythroferrone across the tested spike range (0-90 ng/ml).

Erythroferrone Kit Comparison

Precision

Serum samples spiked with low, medium, and high levels of Erythroferrone (n = 4) were measured over two days. The average coefficient of variation (CV) within run (intra-assay) and between runs (inter-assay) was calculated.

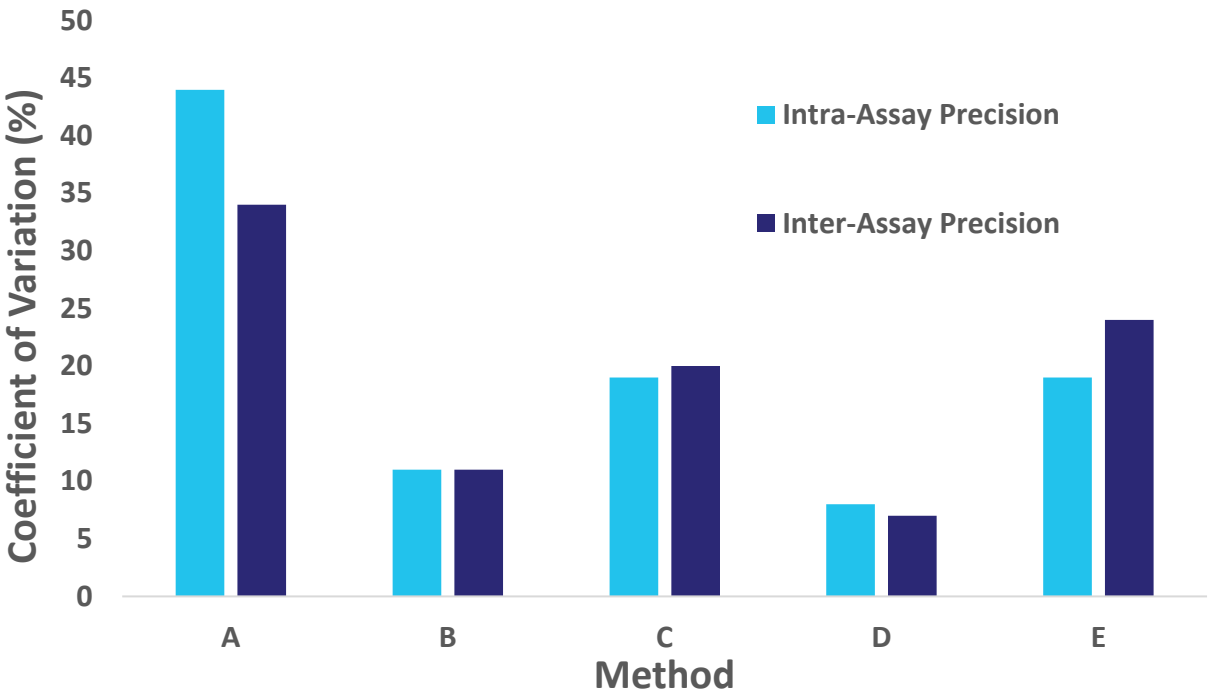

Figure 2. Intra- and Inter-assay Precision

Methods B and D were the most precise with D having CVs < 10%.

Erythroferrone Kit Comparison

Linearity

Method	1:2	1:4	1:8	1:16
A	-27%	-18%	108%	-62%
B	-60%	214%	476%	1668%
C	-58%	-100%	-100%	-100%
D	-19%	-17%	1%	2%
E	-16%	6%	-15%	124%

Table 3. Linearity across dilutions, relative error.

Linearity was assessed using a high Erythroferrone sample diluted 1:2, 1:4, 1:8, and 1:16. The average CV of concentrations corrected for dilution factor across all dilutions was calculated for each method. (**Figure 3**).

Method D demonstrated the lowest CVs and lowest total relative error. Method E showed reasonably low CVs and relative error up to 1:8 dilution while Method A remained serviceable up to a 1:4 dilution (Table 3).

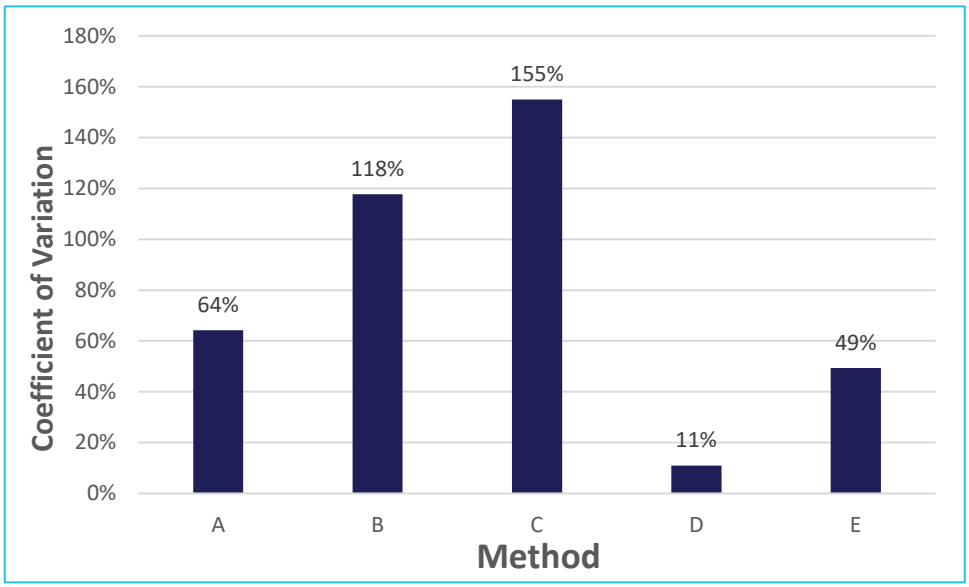

Figure 3. Average coefficient of variation for linearity across all dilutions

Erythroferrone Kit Comparison

Lower Limits of Detection and Quantitation

Method:	A	B	C	D	E
OD1	0.014	0.051	-0.193	0.050	0.009
OD2	0.007	0.025	-0.097	0.049	0.015
OD3	0.015	0.056	-0.015	0.053	-0.001
OD4	0.011	0.046	0.015	0.056	-0.002
OD5	0.007	0.025	-0.164	0.054	0.000
OD6	0.005	0.041	-0.134	0.050	-0.007
OD7	0.023	0.020	0.045	0.058	0.003
OD8	0.018	0.038	0.015	0.069	-0.004
AVE OD	0.012	0.038	-0.066	0.055	0.002
CV	49%	35%	-139%*	12%	394%
LLOD (ng/ml)	**0.25**	**1.92**	**0.02**	**0.01**	**0.19**
LLOQ (ng/ml)	**0.48**	**3.04**	**0.09**	**0.08**	**0.42**

Table 4. Lower limit of detection and quantitation. *a negative CV reflects the average OD for zero erythroferrone buffer was negative. This indicates interference in the assay.

The lower limits of detection (LLOD) and quantitation (LLOQ) were assessed by repeated measurement of assay buffer (zero erythroferrone sample). The LLOD and LLOQ were calculated based on the average OD plus 2 and 5 standard deviations, respectively.

Method D demonstrated the lowest CV across zero erythroferrone sample buffer and had the lowest LLOD and LLOQ. The remaining methods have large CVs and orders of magnitude larger LLODs and LLOQs.

Erythroferrone Kit Comparison

Physiologic Relevance

Erythroferrone is produced by erythroblasts in the bone marrow and during cases of ineffective erythropoiesis erythroblasts are unable to mature and accumulate in number. This leads to an overabundance of erythroferrone in circulation compared to healthy donors. A prime example of ineffective erythropoiesis is β-thalassemia.

Method:	A	B	C	D	E
Healthy Donor	0.15	5.77	0.00	6.48	0.00
β-thalassemia	1.19	112.00	0.14	51.68	1.68
p	0.0029	0.0079	0.0811	0.0098	0.0002

Table 5. Healthy blood donor and β-thalassemia patient erythroferrone concentrations. Average erythroferrone levels (ng/ml) in healthy blood donors (n=10) and β-thalassemia patients (n=10) were compared for each method and the p-value was calculated.

Methods A, B, D, and E were able to distinguish the healthy blood donor group from the β-thalassemia patients based on their erythroferrone levels ($p < 0.05$).

Watch the discovers of erythroferrone, Drs. Ganz and Nemeth, discuss its clinical utility in ineffective erythropoiesis.

Conclusion

At-a-glance

Method	Accuracy	Precision Intra- And Inter-assay	Linearity	Limit Of Quantitation	Physiologic Relevance
A			✓		✓✓
B		✓✓			✓✓
C		✓			
D	✓✓	✓✓	✓✓	✓✓	✓✓
E		✓	✓		✓✓

✓✓ optimal
✓ suitable (depending on intended research)

A, Abbexa (abx250979); B, Aviscera (SA00393-19); C, Cusabio (CSB-EL008059HU); D, Intrinsic LifeSciences (ERF-001); E, MyBioSource (MBS2088219)

Conclusion

Summary Table

	Accuracy	Precision intra- and inter-assay	Linearity	Limit of Quantitation
Optimal Method By Category	**D**	**B, D**	**D**	**D**
Ideal erythroferrone kit properties	Test slope of 1.0 indicates results of two test methods produce the same result.	Precision is an indicator of the assay reproducibility. CVs < 10 are considered acceptable in a clinical setting; < 5 would be considered highly reproducible.	Linearity is the accuracy of a measurement from a diluted sample. Typically, accuracy declines as sample dilution increases.	A low limit of quantitation allows for better sensitivity.
Why important? What is the impact on research/ data?	Adjusting to accommodate a slope that deviates from 1.0. requires time, reagent and samples.	Low within-run (intra-assay) AND between run (inter-assay) CVs help minimize variability of results. An assay with a poor precision can increase variability and widen confidence intervals, weakening the strength of the data and lowering scientific merit. Low intra-assay CVs can be diminished by high inter-assay CVs.	If a sample needs to be diluted, the accuracy of the measurement is dependent on the linearity of the assay. An assay with a narrow range AND poor linearity will hinder accurate measurement and likely affect the relationships between study groups. It is best to minimize large sample dilutions to reduce sources of analytical error.	Erythroferrone is very low in steady state erythropoiesis requiring assays with a low LLOQ to resolve sample concentrations. An assay with a very low LLOQ allows researchers to distinguish patient groups with minimal elevations of erythroferrone.

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Intrinsic Lifesciences Website

Further background on erythroferrone biology

Informational talk with the experts on erythroferrone