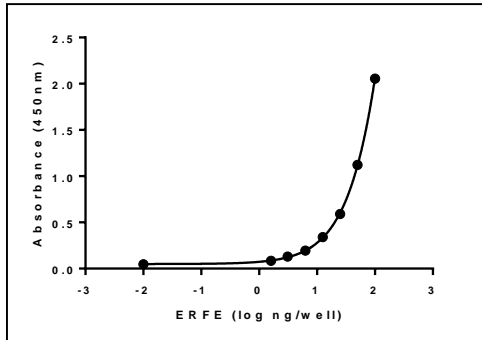


Standard Curve:

Eight point standard curve, including a zero, are used for each run. A typical standard curve is shown below:



ERFE (ng/ml)	OD 450nm
10.0	2.056
5.0	1.122
2.5	0.591
1.25	0.340
0.63	0.194
0.31	0.130
0.16	0.084
0.001	0.047



Intrinsic Erythroferrone IE™ ELISA

SKU# ERF-001

Intended Use:

The Intrinsic Erythroferrone IE™ ELISA is designed for quantification of human Erythroferrone (ERFE) in serum. Quantification of ERFE from other species or from plasma or other body fluids has not been evaluated. **For Research Use Only.**

This kit does not contain any azide- or mercury-based preservatives.

Summary and Explanation:

Erythroferrone (ERFE) is a recently discovered hormone produced by erythroblasts in the bone marrow in response to erythropoietin¹. Recent animal studies have shown that rather than being involved in regulation of baseline erythropoiesis, ERFE acts as a stress erythropoiesis-specific regulator of hepcidin expression. By suppressing hepcidin expression in the liver, ERFE contributes to increased dietary iron absorption and recycling of stored iron necessary for recovery of blood loss due to hemorrhage or phlebotomy.² In addition, ERFE was found to be involved in hepcidin regulation in inherited iron loading anemias, such as β -Thalassemia.³ ERFE has potential as a clinical marker for assessing erythropoiesis in patients with blood disorders.⁴

Principle of the Test:

Detection of human erythroferrone is based on the double monoclonal antibody sandwich ELISA method. The 96-well microplate assay plate is pre-coated with mouse anti-human erythroferrone monoclonal antibody. Human serum samples, standards, or controls are mixed with buffer and incubated in the assay plate. Human erythroferrone present in the sample binds to the immobilized antibody on the assay plate. The plate is then washed to remove unbound human erythroferrone. A 2nd monoclonal anti-erythroferrone antibody conjugated to horseradish peroxidase (HRP) is added. This detection antibody-HRP conjugate binds to the immobilized ERFE and is quantified by the addition of TMB. The reaction produces a blue color and halted with the addition of the stop solution. The absorbance is read at 450nm on a microplate reader and a standard curve is prepared by plotting the log concentration of the standard curve versus the absorbance. The intensity of the color is proportional to the concentration of ERFE in the sample. Total assay run time is less than 3 hours.

Calculating Results:

Create a standard curve by using computer software capable of generating a four parameter logistic (4-PL) curve-fit (Recommended: Graphpad Prism Software, www.graphpad.com). There is no need to correct the OD values for background.

Calculate the concentration of human ERFE in the sample from the mean absorbance of the standard curve. Since the sample was diluted 1/10, the final ERFE concentration must be multiplied by 10, the corresponding dilution factor.

Quality Control:

It is recommended that each laboratory establish human erythroferrone serum controls and repeatedly measure the ERFE concentration of the controls to monitor kit performance.

Citations:

1. Kautz L, Jung G, Valore E, Rivella S, Nemeth E, Ganz T. 2014. Identification of erythroferrone as an erythroid regulator of iron metabolism. *Nature Genetics* 46: 678-684.
2. Kautz L, Jung G, Nemeth E, Ganz T. 2014. Erythroferrone contributes to recovery from anemia of inflammation. *Blood* 124: 2569-2574.
3. Kautz L, Jung G, Du X, Gabayan G, Chapman J, Nasoff M, Nemeth E, Ganz T. 2015. Erythroferrone contributes to hepcidin expression and iron overload in a mouse model of β -thalassemia. *Blood* 126: 2031-2037.
4. Kim A, Nemeth E. 2015. New insights into iron regulation and erythropoiesis. *Current Opinions Hematology* 22: 199-205.

Patents Pending - US & Abroad

SKU# ERF-001

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Materials and Storage:

Store unopened kit at 2-8°C. Do not use kit after the expiration date. Do not mix component lots between kits.

Materials Provided	1 Kit	SKU#
1. Microwell plate coated anti-erythroferrone antibody	1x96-well	-
2. Human Erythroferrone Standard (1 plastic vial, clear)	50 µl	XES-200
3. Anti-ERFE-HRP Conjugate (100X, 1 plastic vial, amber)	150 µl	HEC-001
4. Sample Diluent, 1 bottle (conc., 10X)	4 ml	CSD-800
5. Wash Solution, 1 bottle (conc., 25X)	25 ml	CWB-008
6. TMB Substrate, 1 bottle (ready to use)	12 ml	CTM-001
7. Stop Solution, 1 bottle (ready to use)	12 ml	CST-001
8. Microplate Sealing Film	2	-
9. 96-well Polypropylene (PP) Sample Set-up Plate	1	-

Materials Not Provided:

1. Precision pipettes and tips.
2. Squirt bottle, manifold dispenser, or automated microplate washer.
3. Deionized or distilled water .
4. Horizontal orbital microplate shaker.
5. Microplate reader (450nm).
6. Computer software capable of 4 parameter logistic curve fitting for data analysis

Warnings and Precautions:

This kit does not contain any azide- or mercury-based preservatives.

Kit is for research use only.

1. Use separate pipette tips for each sample, standard, and reagent to avoid cross-contamination.
2. Use separate reservoirs for each reagent, especially the TMB Substrate.
3. The Stop Solution contains 0.5M sulfuric acid. Use appropriate protection.
4. Hemolyzed, hyperlipemic, heat-treated or contaminated samples may give erroneous results.
5. Do not dilute samples directly in the antibody coated microwell plate.
6. Do not touch or scrape the bottom or sides of the wells in the antibody coated microwell plate.
7. Incubation times and temperatures other than those specified may give erroneous results.
8. Do not allow the wells to dry once the assay has begun.
9. Do not reuse microwell plate coated with antibody or pour reagents back into their bottles once dispensed.

Specimen Collection and Handling:

Serum samples are required for the assay. Use of plasma or other types of body fluids require further investigation. Collect serum samples according to standard techniques. Samples must be centrifuged to remove lipids and cellular debris.

For long term sample storage, aliquot in small volumes and freeze at -80°C. Avoid repeated freeze-thaw cycles. Samples should be thawed and allowed to equilibrate to room temperature 30 min before use. Mix samples completely before analysis.

Samples should first be tested at 1:10 dilution (10µl sample + 90µl sample diluent, for final 100µl assay volume per well). Therefore, to begin quantitation of human ERFE in duplicate requires 25µl of serum. If the ERFE concentration is excessively high, further dilute the samples and re-run them.

Preparation of Reagents:

This kit has sufficient reagents and is designed to run a duplicate 8-point standard curve and 40 samples in **duplicate**.

Bring all samples and reagents to room temperature (20-25°C) before use.

1. 1X Wash Solution: Transfer contents of concentrated **Wash Solution** bottle (25ml) to 600ml of deionized or distilled water.
2. 1X Sample Diluent: Vortex to mix, transfer contents of the concentrated **Sample Diluent** bottle (4ml) to 36ml of deionized or distilled water.
3. Antibody-HRP Conjugate: Briefly vortex and centrifuge prior to transferring 120µl of the concentrated **HRP Conjugate** into 12ml of the 1X Sample Diluent.

Preparation of Standard and Samples:Standard:

1. **Vortex ERFE Standard** provided in the kit. Briefly centrifuge the standard prior to dispensing. Dilute 25µl of ERFE standard (concentration = 200ng/ml) in 475µl of Sample Diluent. Vortex to mix completely.
2. Into the PP 96-well sample setup plate, transfer 450µl of diluted ERFE Standard to well A1 and 220µl Sample Diluent to wells B1-H1. Perform a 1:2 serial dilution (220µl to 220µl) from well A1 to G1; Leave well H1 undiluted.

Samples:

3. Transfer 225µl of sample diluent to the sample set-up plate and pipette 25µl sample to achieve a final volume of 250µl/well (1:10 sample dilution).

Assay Procedure:

1. Transfer 100µl/well of ERFE standard and samples from the PP 96-well sample set-up plate to the microwell assay plate in duplicate.
2. Apply sealing film and incubate on an orbital shaker (350 rpm) at room temperature for **1 hour**.
3. Wash microwell plate three times with 1X Wash Solution (300µl/well).
4. Transfer 100µl/well of the 1X HRP Conjugate solution to the microwell plate and incubate for **30 minutes** at room temperature on the orbital shaker.
5. Wash microwell plate three times with 1X Wash Solution (300µl/well).
6. Develop the microwell ELISA plate by adding 100µl/well TMB Substrate. Incubate for **15 minutes** at room temperature. Protect from ambient light.
7. Stop the reaction by adding 100µl/well Stop Solution **precisely** 15 minutes after the addition of the TMB Substrate.
8. Measure absorbance at 450nm of the microwell plate using a microplate reader.