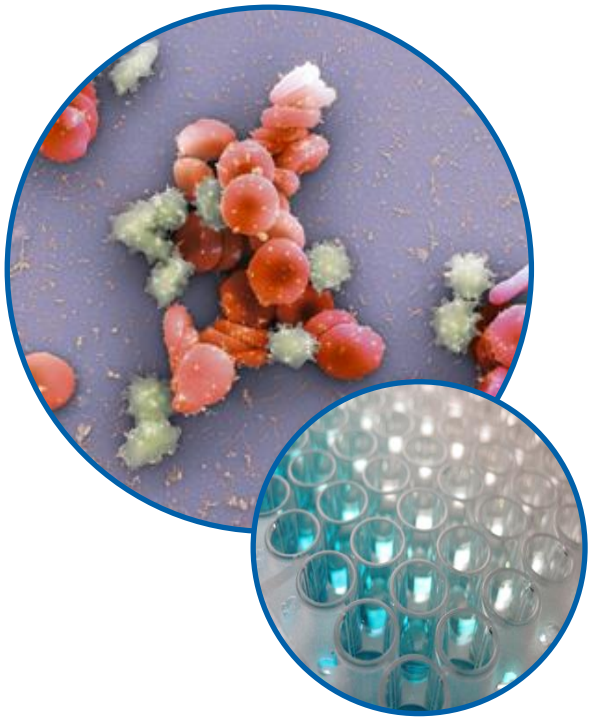


AdipoGen® LIFE SCIENCES

MANUAL

Erythroferrone (human) ELISA Kit

[Myonectin (human) ELISA Kit; CTRP15 (human) ELISA Kit]

For research use only. Not for diagnostic use

Version November-21-2019

Cat. No. AG-45B-0014-KI01

Table of Contents

1. Intended Use

The Erythroferrone (human) ELISA Kit is to be used for the *in vitro* quantitative determination of human Erythroferrone in cell culture supernatants, serum and plasma. This ELISA Kit is for research use only.

2. Introduction

Erythroferrone (ERFE) is a secreted glycoprotein, also known as CTRP15 (C1q (complement component 1q) TNF (tumor necrosis factor)-related protein 15), Myonectin or Fam132b. Erythroferrone is a member of the CTRP family of proteins (C1q/TNF-related proteins), which are the conserved paralogs of adiponectin containing collagen-like and globular C1q-like domains (1). Erythroferrone plays two different regulatory roles:

i) Erythroferrone (called Myonectin for this role) is a myokine abundantly expressed in skeletal muscle tissue, upregulated by voluntary exercise that is suppressed by fasting and induced by refeeding; it participates in the regulation of systemic lipid metabolism by promoting the clearance of nonesterifed fatty acids (NEFA) from circulation (2). As a myokine, Erythroferrone acts as an endurance exercise-driven myokine, which protects the heart from ischemic injury by reducing cardiomyocyte apoptosis and macrophage inflammatory response (3);

ii) Erythroferrone is produced by erythroblasts after bleeding or EPO treatment (4) and acts on hepatocytes to suppress hepcidin expression, resulting in increased release of iron from cellular iron stores. Erythroferrone acts on hepcidin by inhibiting members of the BMP5, BMP6 and BMP7 subgroup of BMPs (5). Erythroferrone functions as erythroid modulator of iron metabolism and hemoglobin synthesis (6).

Levels of erythroferrone measured by this ELISA kit in serum and plasma correspond to measured mouse protein levels (~ 500ng/ml). Erythroferrone is a potential biomarker for different types of anemia, for cardiovascular and metabolic diseases.

3. General References

(1) Is erythroferrone finally the long sought-after systemic erythroid regulator of iron? A. Lawen; World. J. Biol. Chem. **6,** 78 (2015)

(2) Myonectin (CTRP15), a novel myokine that links skeletal muscle to systemic lipid homeostasis: M.M. Seldin, et al.; J. Biol. Chem. **287,** 11968 (2012)

(3) Myonectin Is an Exercise-Induced Myokine That Protects the Heart from Ischemia-Reperfusion Injury: N. Otaka, et al.; Circ. Res. **123,** 1326 (2018)

(4) Identification of Erythroferrone as an Erythroid Regulator of Iron Metabolism: L. Kautz, et al; Nat. Genet. **46,** 678 (2014)

(5) Erythroferrone inhibits the induction of hepcidin by BMP6: J. Arezes, et al.; Blood **132,** 1473 (2018)

(6) Erythropoietic regulators of iron metabolism: T. Ganz; Free Radic. Biol. Med. **133,** 69 (2019)

4. Assay Principle

This assay is a sandwich Enzyme Linked-Immunosorbent Assay (ELISA) for quantitative determination of human Erythroferrone in cell culture supernatants, serum and plasma. A polyclonal antibody specific for Erythroferrone has been precoated onto the 96-well microtiter plate. Standards (STD) and samples are pipetted into the wells for binding to the coated antibody. After extensive washing to remove unbound compounds, Erythroferrone is recognized by the addition of a biotinylated polyclonal antibody specific for Erythroferrone (DET). After removal of excess biotinylated antibody, streptavidin-peroxidase (STREP-HRP) is added. Following a final washing, peroxidase activity is quantified using the substrate 3,3',5,5'-tetramethylbenzidine (TMB). The intensity of the color reaction is measured at 450nm after acidification and is directly proportional to the concentration of Erythroferrone in the samples.

5. Handling & Storage

- Reagent must be stored at 2-8°C when not in use.
- Plate and reagents should be at room temperature before use.
- Do not expose reagents to temperatures greater than 25°C.

6. Kit Components

• 1 vial human ERFE Standard (lyophilized)	(100 ng)	(STD)
• 1 vial Detection Antibody	(30 µl)	(DET)
• 1 vial HRP Labeled Streptavidin (lyophilized)	(2 µg)	(STREP-HRP)
• 2 bottles Wash Buffer 10X	(2 x 30 ml)	(Wash Buffer 10X)
• 2 bottles ELISA Buffer 10X	(2 x 30 ml)	(ELISA Buffer 10X)
• 1 bottle TMB Substrate Solution	(12 ml)	(TMB)
• 1 bottle Stop Solution	(12 ml)	(STOP)
• 1 plate coated with Erythroferrone Antibody	(6 x 16-well strips)	
• 2 plate Covers (plastic film)		
• 2 silica Gel Minibags		

7. Materials Required but *Not* Supplied

- Microtiterplate reader at 450nm
- Calibrated precision pipettes. Disposable pipette tips
- Deionized water
- Microtubes or equivalent for preparing dilutions
- Disposable plastic containers for preparing working buffers
- Plate washer: automated or manual
- Glass or plastic tubes for diluting and aliquoting standard

8. General ELISA Protocol

8.1. Preparation and Storage of Reagents

NOTE: *Prepare just the appropriate amount of the buffers necessary for the assay.*

- **Wash Buffer 10X** has to be diluted with deionized water 1:10 before use (e.g. 30 ml Wash Buffer 10X + 270 ml water) to obtain Wash Buffer 1X.
- **ELISA Buffer 10X** has to be diluted with deionized water 1:10 before use (e.g. 10 ml ELISA Buffer 10X + 90 ml water) to obtain ELISA Buffer 1X.
- **Detection Antibody (DET)** has to be diluted to 1:500 in ELISA Buffer 1X (20 µl DET + 10 ml ELISA Buffer 1X).

  NOTE: *The diluted Detection Antibody is not stable and cannot be stored!*

- **HRP Labeled Streptavidin (STREP-HRP)** has to be reconstituted with 100 µl of ELISA Buffer 1X.
  - After reconstitution of STREP-HRP, prepare aliquots and store them at -20°C. **Avoid freeze/thaw cycles**.
  - Dilute the reconstituted STREP-HRP to the working concentration by adding 50 µl in 10 ml of ELISA Buffer 1X (1:200).

  NOTE: *The diluted STREP-HRP is not stable and cannot be stored!*

- **Human Erythroferrone Standard (STD)** has to be reconstituted with 100 µl of ELISA Buffer 1X.
  - This reconstitution produces a stock solution of **1 µg/ml**. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes **at room temperature.** Mix well prior to making dilutions.

  NOTE: *The reconstituted standard is aliquoted and stored at -20°C!*

  - Dilute the standard protein concentrate (STD) (**1 µg/ml**) in ELISA Buffer 1X. A seven-point standard curve using 2-fold serial dilutions in ELISA Buffer 1X is recommended.
  - Suggested standard points are:

    20, 10, 5, 2.5, 1.25, 0.625, 0.3125 and 0 ng/ml.

Start with the dilution of the concentrate (STD):

To obtain	Add	Into
20 ng/ml	20 µl of Erythroferrone (STD) (1 µg/ml)	980 µl of ELISA Buffer 1X
10 ng/ml	300 µl of Erythroferrone (STD) (20 ng/ml)	300 µl of ELISA Buffer 1X
5 ng/ml	300 µl of Erythroferrone (STD) (10 ng/ml)	300 µl of ELISA Buffer 1X
2.5 ng/ml	300 µl of Erythroferrone (5 ng/ml)	300 µl of ELISA Buffer 1X
1.25 ng/ml	300 µl of Erythroferrone (2.5 ng/ml)	300 µl of ELISA Buffer 1X
0.625 ng/ml	300 µl of Erythroferrone (1.25 ng/ml)	300 µl of ELISA Buffer 1X
0.3125 ng/ml	300 µl of Erythroferrone (0.625 ng/ml)	300 µl of ELISA Buffer 1X
0 ng/ml	300 µl of ELISA Buffer 1X	Empty tube

8.2. Sample collection, storage and dilution

Serum : Use a serum separator tube. Let samples clot at room temperature for 30 minutes before centrifugation for 20 minutes at 1,000xg. Assay freshly prepared serum or store serum in aliquot at ≤ -20°C for later use. Avoid repeated freeze/thaw cycles.

Plasma : Collect plasma using heparin, citrate or EDTA as an anticoagulant. Centrifuge for 15 minutes at 1000xg within 30 minutes of collection. Assay freshly prepared plasma or store plasma sample in aliquot at ≤ -80°C for later use. Avoid repeated freeze/ thaw cycles.

Serum, Plasma and Cell Culture Supernatant have to be diluted in ELISA Buffer 1X. Samples containing visible precipitates must be clarified before use.

NOTE: *As a starting point, 1/150 dilution of serum or plasma is recommended! If sample values fall outside the detection range of the assay, a lower or higher dilution may be required!*

8.3. Assay Procedure (Checklist)

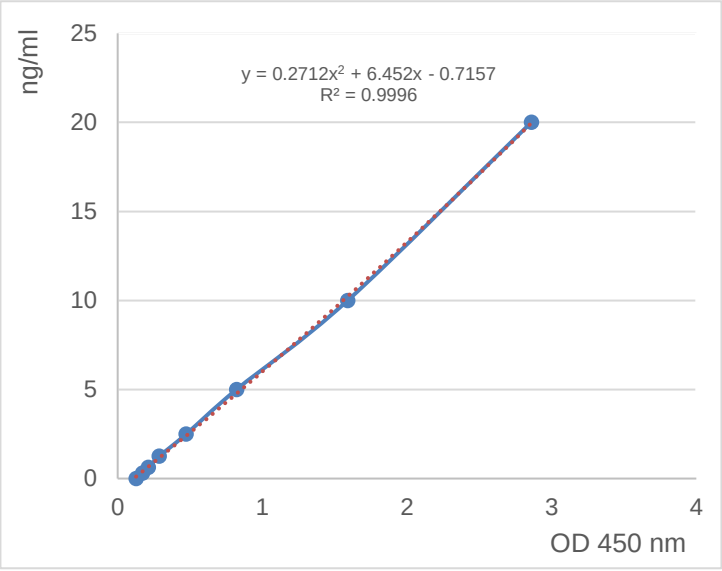
☐	1. Determine the number of 16-well strips needed for the assay and insert them in the frame for current use. The extra strips are left in the bag with 2 silica gel minibags and stored at 4°C. **NOTE:** *Remaining 16-well strips coated with* Erythroferrone *antibody when opened can be stored in the presence of 2 silica gel minibags at 4°C for up to 1 month.*
☐	2. Add 100 µl of the different standards into the appropriate wells in duplicate! At the same time, add 100 µl of diluted serum, plasma or cell culture supernatant samples in duplicate to the wells **(see 8.1. Preparation and Storage of Reagents and 8.2 Preparation of Samples).**
☐	3. Cover the plate with plastic film and incubate for **2 h at RT.**
☐	4. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multichannel pipette or auto-washer. Repeat the process for a total of five washes. After the last wash, complete removal of liquid is essential for good performance.
☐	5. Add 100 µl to each well of the diluted Detection Antibody **(DET) (see 8.1 Preparation and Storage of Reagents)**.
☐	6. Cover the plate with plastic film and incubate for **1 h at RT**.
☐	7. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multichannel pipette or auto-washer. Repeat the process for a total of five washes. After the last wash, complete removal of liquid is essential for good performance.
☐	8. Add 100 µl to each well of the diluted HRP Labeled Streptavidin **(STREP-HRP) (see 8.1. Preparation and Storage of Reagents)**.
☐	9. Cover the plate with plastic film and incubate for **30 min at RT**.
☐	10. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multichannel pipette or auto-washer. Repeat the process for a total of five washes. After the last wash, complete removal of liquid is essential for good performance.
☐	11. Add 100 µl to each well of TMB substrate solution **(TMB)**.
☐	12. Allow the color reaction to develop **at RT in the dark for 10 minutes**. Do not cover the plate.
☐	13. Stop the reaction by adding 100 µl of Stop Solution (**STOP**). Tap the plate gently to ensure thorough mixing. The substrate reaction yields a blue solution that turns yellow when Stop Solution (**STOP**) is added.
	! CAUTION: CORROSIVE SOLUTION !
☐	14. Measure the OD at 450 nm in an ELISA reader.

9. Calculation of Results

- Average the duplicate readings for each standard, control and sample and subtract the average blank value (obtained with the 0 ng/ml point).
- Generate the standard curve by plotting the average absorbance obtained for each standard concentration on the horizontal (X) axis vs. the corresponding Erythroferrone concentration (ng/ml) on the vertical axis (see **10.** TYPICAL DATA).
- Calculate the Erythroferrone concentrations of samples by interpolation of the regression curve formula in a form of a quadratic equation.
- If the test sample was diluted, multiply the interpolated value by the dilution factor to calculate the concentration of human Erythroferrone in the sample.

10. Typical Data

The following data are obtained using the different concentrations of standard as described in this protocol:



Standard Erythroferrone (ng/ml)	Optical Density (mean)
20	2.859
10	1.59
5	0.821
2.5	0.474
1.25	0.288
0.625	0.212
0.3125	0.171
0	0.127

Figure: Standard curve

11. Performance Characteristics

A. Sensitivity (Limit of detection):

The lowest level of Erythroferrone that can be detected by this assay is 270 pg/ml. **NOTE:** *The Limit of detection was measured by adding three standard deviations to the mean value of 50 zero standard.*

B. Assay range: 0.3125 ng/ml – 20 ng/ml

C. Specificity:

This ELISA is specific for the measurement of natural and recombinant human Erythroferrone [CTRP15; Myonectin].

D. Intra-assay precision:

Four samples of known concentrations of human Erythroferrone were assayed in replicates 6 times to test precision within an assay.

Samples	Means (ng/ml)	SD	CV (%)	n
A1	548.42	20.46	3.50	6
A2	497.07	22.73	4.57	6
A3	522.52	15.38	2.94	6
A4	794.90	22.26	2.80	6

E. Inter-assay precision:

Three samples of known concentrations of human Erythroferrone were assayed in 4 separate assays to test precision between assays.

Samples	Means (ng/ml)	SD	CV (%)	n
B1	496.52	5.14	1.03	4
B2	625.77	15.98	2.55	4
B3	525.39	19.37	3.69	4
B4	618.85	28.10	4.54	4

F. Recovery:

When samples are spiked with known concentrations of human Erythroferrone in serum or plasma, the recovery averages range from 84% to 105%.

G. Linearity:

Different samples containing human Erythroferrone were diluted several fold (1/150 to 1/600) and the measured recoveries ranged from 82% to 110%.

H. Expected values:

Human Erythroferrone levels range in serum or plasma from 300 ng/ml to >1'500 ng/ml.

I. Erythroferrone (ERFE) / Myonectin Levels and Dilution of Samples:

Erythroferrone/Myonectin has been shown to be expressed in muscle (called Myonectin/CTRP15) or in erythroblasts (called Erythroferrone/ERFE). Based on extensive in-house validation and in accordance with the values detected in undiluted samples by K. Ramirez Cuevas, *et al.;* Drug Test. Anal. (2019), we provide the following recommendations for the usage of the Erythroferrone (human) ELISA Kit.

1. To detect ERFE increase upon Erythropoietin (EPO) treatment or blood transfusion, use undiluted or low diluted serum/plasma (about 1/10):

   > Values measured will range from 2-4 ng/ml to 15-25 ng/ml.

2. To detect steady state levels of ERFE, use diluted serum (>1/150).

   > Values will range from 300 ng/ml to >1200 ng/ml

For a possible scientific explanation of these observations and further information related to this recommendation, please visit https://adipogen.com/erythroferrone-elisa-iron-metabolism-cardiovascular-protection.

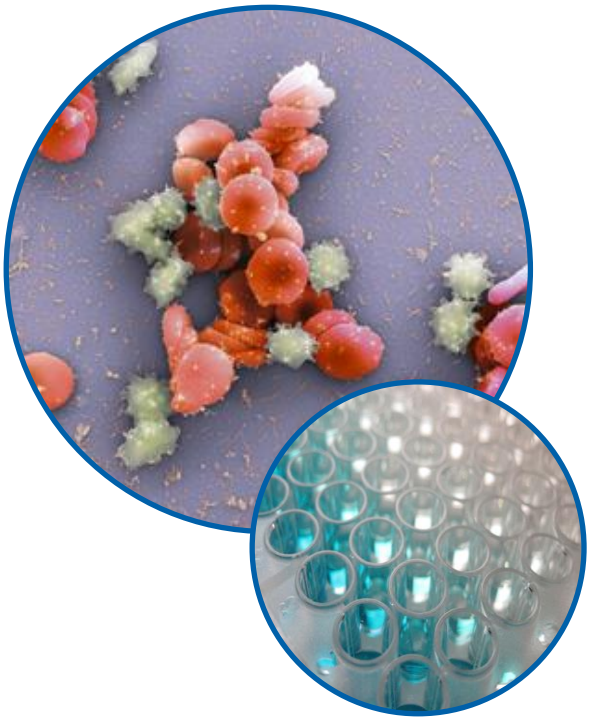
12. Technical Hints and Limitations

- It is recommended that all standards and samples be run in duplicate.
- Do not combine leftover reagents with those reserved for additional wells.
- Reagents from the kit with a volume less than 100µl should be centrifuged.
- Residual wash liquid should be drained from the wells after last wash by tapping the plate on absorbent paper.
- Crystals could appear in the 10X solution due to high salt concentration in the stock solutions. Crystals are readily dissolved at room temperature or at 37°C before dilution of the buffer solutions.
- Once reagents have been added to the 16-well strips, DO NOT let the strips DRY at any time during the assay.
- Keep TMB Solution protected from light.
- The Stop Solution (STOP) consists of sulfuric acid. Although diluted, the Stop Solution should be handled with gloves, eye protection and protective clothing.

13. Troubleshooting

PROBLEM	POSSIBLE CAUSES	SOLUTIONS
No signal or weak signal	Omission of key reagent	Check that all reagents have been added in the correct order.
	Washes too stringent	Use an automated plate washer if possible.
	Incubation times inadequate	Incubation times should be followed as indicated in the manual.
	Plate reader settings not optimal	Verify the wavelength and filter setting in the plate reader.
	Incorrect assay temperature	Use recommended incubation temperature. Bring substrates to room temperature before use.
High background	Concentration of STREP-HRP too high	Use recommended dilution factor.
	Inadequate washing	Ensure all wells are filling wash buffer and are aspirated completely.
Poor standard curve	Wells not completely aspirated	Completely aspirate wells between steps.
	Reagents poorly mixed	Be sure that reagents are thoroughly mixed.
Unexpected results	Omission of reagents	Be sure that reagents were prepared correctly and added in the correct order.
	Dilution error	Check pipetting technique and double-check calculations.

14. Notes



Product Specific References:

1. K. Ramirez Cuevas, et al.; Drug Test. Anal. (2019)

For more References please visit www.adipogen.com!

Adipogen Life Sciences
Schützenstrasse 12
CH-1410 Liestal
Switzerland
TEL: +41-61-926-60-40
FAX: +41-61-926-60-49
Email: info@adipogen.com

AdipoGen®
LIFE SCIENCES