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Serum Iron Assay Kit

Item No. 702870

www.caymanchem.com
Customer Service 800.364.9897
Technical Support 888.526.5351
1180 E. Ellsworth Rd · Ann Arbor, MI · USA

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	Quantity/Size	Storage
400490	Serum Iron Buffer	2 vials/20 ml	RT
400492	$FeCl_3$ Standard	1 vial/200 µl	RT
400493	Iron Color Reagent	2 vials/5 ml	-20°C
400014	96-Well Solid Plate (Colorimetric Assay)	2 plates	RT
400012	96-Well Cover Sheet	2 ea	RT

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

The reagents in this kit have been tested and formulated to work exclusively with Cayman Chemical's Serum Iron Assay Kit. This kit may not perform as described if any reagent or procedure is replaced or modified.

It is recommended to take appropriate precautions when using the kit reagents (*i.e.*, lab coat, gloves, eye goggles, *etc.*) as some may be harmful. Hydrochloric acid is corrosive and harmful if swallowed. Contact with skin may cause burns. In case of contact with skin or eyes, rinse immediately with plenty of water for 15 minutes.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Email: techserv@caymanchem.com

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed in the **Materials Supplied** section (see page 3) and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader capable of measuring absorbance at 570 nm
2. Adjustable pipettes; multichannel or repeating pipettor recommended
3. A 37°C incubator
4. Microcentrifuge tubes
5. A source of iron-free water; glass-distilled water or pure water is acceptable. *NOTE: UltraPure Water is available for purchase from Cayman (Item No. 400000).*
6. An orbital microplate shaker

INTRODUCTION

Background

Iron (Fe) is one of the most abundant elements and is essential in a variety of organisms.[1] It functions, as ferrous iron (Fe^{2+}) and ferric iron (Fe^{3+}), as both an electron donor and acceptor, respectively, in an oxidation state-dependent manner and is involved in the function of all cells. It is primarily stored in hemoproteins, including hemoglobin and myoglobin, where it is involved in oxygen transport, but is also found in enzymes containing iron-sulfur clusters, which have roles in cellular respiration, DNA synthesis, gene regulation, and steroid synthesis, and in heme-containing cofactors.

Excess levels of non-heme iron can be deleterious because ferrous iron, an electron donor, catalyzes the production of reactive oxidative species (ROS), inducing oxidative stress. To prevent iron overload, non-heme iron is also stored in the iron-storage proteins ferritin and hemosiderin in macrophages and hepatocytes and transported through the bloodstream bound to transferrin, a glycoprotein that binds and transports ferric iron.[1-3] Iron-bound transferrin binds to the transferrin receptor (TfR1) on the surface of iron-requiring cells to form the transferrin/TfR complex, which undergoes clathrin-dependent endocytosis to facilitate intracellular iron release.[2,3] The bioavailability of iron, and its subsequent delivery to different tissues and cells, is dependent on iron cycling by transferrin and TfR1, making transferrin-bound iron the physiological iron source for most cells.[1]

Serum iron levels are decreased in patients with absolute and functional iron deficiencies. They are increased in patients with iron overload diseases, such as hemochromatosis, as well as in patients with acute hepatitis or chronic liver failure. These, together, highlight the importance of measuring iron levels in serum.

About This Assay

Cayman's Serum Iron Assay Kit provides a convenient colorimetric method of determining iron levels in serum. This assay has a range of 0-100 µM (0-560 µg/dl) and a lower limit of detection (LLOD) of 0.68 µM (3.8 µg/dl). *NOTE: Total Iron Binding Capacity and Serum Iron Assay Kit is also available for purchase from Cayman (Item No. 702230).*

Principle Of This Assay

Cayman's Serum Iron Assay Kit is a ferrozine-based, colorimetric assay that detects serum iron concentration with high sensitivity. This method builds upon the procedure developed by Persijn *et al.*[4]; at low pH, transferrin releases bound ferric iron (Fe^{3+}) into solution, where it is reduced by hydroxylamine to ferrous iron (Fe^{2+}). Ferrozine then forms a stable complex with Fe^{2+}. This complex has a violet color and a maximum absorbance peak at 570 nm.

$$4Fe^{3+} + 2NH_2OH \longrightarrow 4Fe^{2+} + N_2O + H_2O + 4H^+$$

$$Fe^{2+} + \text{Ferrozine} \longrightarrow \text{Ferrozine}\bullet Fe^{2+}$$

Abs at 570 nm

Figure 1. Assay scheme

Definition of Key Terms

LLOD (Lower Limit of Detection): defined as the concentration at which the absorbance is two standard deviations higher than the mean zero value.

LLOQ (Lower Limit of Quantification): the lowest standard concentration in which mean Abs - (1.645 x S.D.) is higher than the mean Abs + (1.645 x S.D.) of the blank (zero point).

PRE-ASSAY PREPARATION

Reagent Preparation

1. **Serum Iron Buffer – (Item No. 400490)**

   Each vial contains 20 ml of Serum Iron Buffer. This reagent is ready to use as supplied.

2. **$FeCl_3$ Standard – (Item No. 400492)**

   This vial contains 200 µl of 100 mM ferric chloride in 0.1 M hydrochloric acid.

   Prior to each assay:

   1. Dilute 10 µl of $FeCl_3$ Standard with 990 µl of water to make a 1 mM solution. It is important to use iron-free water to dilute out the hydrochloric acid. It is recommended to prepare the 1 mM standard solution fresh each time and to proceed with the next dilution immediately.
   2. Further dilute 100 µl of the 1 mM standard solution with 900 µl of Serum Iron Buffer to make the bulk standard. The concentration of this bulk standard is 100 µM and it will be used to prepare the standard curves. The 100 µM bulk standard will be stable for one hour at room temperature.

3. **Iron Color Reagent – (Item No. 400493)**

   Each vial contains 5 ml of the Iron Color Reagent and is ready to use as supplied. If not using all at once, aliquot and store protected from light at -20°C.

Sample Preparation

Serum

Typically, normal human sera have iron concentrations in the range of 10-40 µM.[5] However, each laboratory should establish its own reference intervals as these may vary substantially due to differences in methodology, equipment, and sample population. Avoid using hemolytic, icteric (jaundice), or lipemic sera as these interfere with the assay.[6] To minimize lipemia, samples should be collected after a fast.

1. Collect blood in vacutainers without an anticoagulant.
2. Allow blood to clot undisturbed for 30 minutes at room temperature.
3. Centrifuge the blood at 2,000 x g for 15 minutes at 4°C. Transfer the **top** yellow serum layer into a clean test tube without disturbing the white buffy layer. Store serum on ice. If not assaying the same day, store at -80°C. Avoid repeated freeze/thaw cycles.
4. Serum does not need to be diluted prior to the assay.

Sample Matrix Properties

Parallelism

To assess parallelism, serum samples were serially diluted with Serum Iron Buffer and evaluated using the Serum Iron Assay Kit. The results are shown in the graph below.

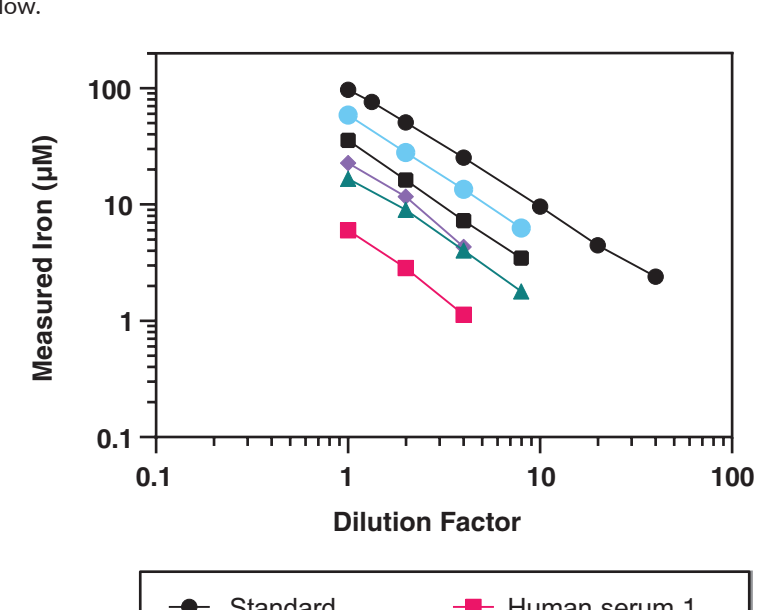


Figure 2. Parallelism of serum samples in the Serum Iron Assay Kit

Linearity

Human serum was spiked with iron to a final concentration of 100 µM, serially diluted with Serum Iron Buffer, and evaluated using the Serum Iron Assay Kit. The results are shown in the table below.

Dilution Factor	Measured Concentration (µM)	% Linearity
Neat	108	100
2	114	106
4	114	105
8	113	104

Table 1. Dilutional linearity in a human serum sample
NOTE: Linearity has been calculated using the following formula:
%Linearity = (Observed concentration value, dilution adjusted / First observed concentration value in the dilution series, dilution adjusted)*100

Spike and Recovery

Human serum was spiked with iron, diluted with Serum Iron Buffer, and analyzed using the Serum Iron Assay Kit. The results are shown below. The error bars represent standard deviations obtained from different dilutions of each spike.

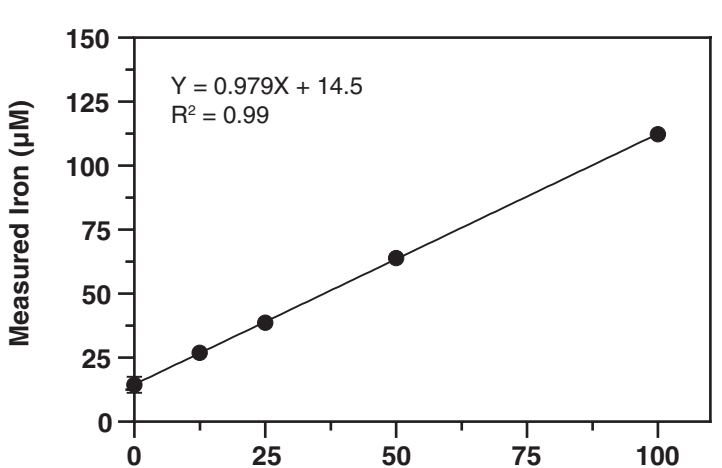


Figure 3. Spike and recovery in human serum

ASSAY PROTOCOL

Preparation of Assay-Specific Reagents

1. $FeCl_3$ Standard Preparation

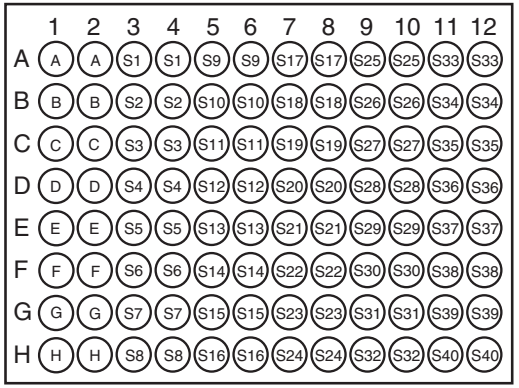
Use the 100 µM bulk standard (see Reagent Preparation, page 9) to prepare the assay standards according to Table 2 (see page 15). The standards will be stable for one hour at room temperature.

Well	Volume of Bulk Standard (µl)	Volume of Serum Iron Buffer (µl)	Final Concentration (µM)
A	200	0	100
B	150	50	75
C	100	100	50
D	50	150	25
E	20	180	10
F	10	190	5
G	5	195	2.5
H	0	200	0

Table 2. Preparation of the iron standards

Plate Set Up

A typical layout of standards and samples to be measured in duplicate is given below in Figure 4. The user may vary the location of wells as needed for the number of samples being assayed. It is suggested that the contents of each well are recorded on the template sheet provided (see page 24).



A-H = Standard wells
S1-S40 = Sample wells

Figure 4. Sample plate format

Pipetting Hints

- It is recommended that a multichannel pipette be used to deliver reagents to the wells. This saves time and helps maintain more precise incubation times.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

General Information

- The final volume of the assay is 250 µl in all of the wells.
- All reagents must be equilibrated to room temperature before beginning the assay.
- It is not necessary to use all the wells on the plate at one time.
- It is recommended that the standards and samples be assayed at least in duplicate (triplicate is recommended).
- Eighty samples can be assayed in duplicate and forty-eight in triplicate.
- Monitor the absorbance at 570 nm.
- The assay is performed at 37°C.

Performing the Assay

1. Add 150 µl of Serum Iron Buffer and 50 µl of standard/sample to the designated wells on the plate. (See **Sample plate format**, Figure 4, on page 16).
2. Cover the plate and mix briefly on a platform shaker.
3. Incubate at 37°C for 10 minutes in the dark.
4. Remove cover, read, and record absorbance at 570 nm. This absorbance value is referred to as Abs1.
5. Add 50 µl of Iron Color Reagent to all wells.
6. Cover the plate and mix briefly on a platform shaker.
7. Incubate at 37°C for 30 minutes in the dark.
8. Remove cover, read, and record absorbance at 570 nm. This absorbance value is referred to as Abs2.

ANALYSIS

Calculations

1. Determine the change in absorbance (ΔAbs_{570}) by subtracting Abs1 from Abs2 for all wells.
2. Subtract the ΔAbs_{570} of standard H (see Table 2 on page 15) from itself and all other standards and samples. This is referred to as the corrected ΔAbs_{570}.
3. Plot the corrected ΔAbs_{570} values of the standards versus the final standard iron concentration (see Table 2 on page 15). See Figure 5 on page 20 for a typical standard curve.
4. Calculate the iron concentration of the samples using the equation obtained from the linear regression of the standard curve substituting the corrected ΔAbs_{570} value for each sample.

$$\text{Sample Iron, µM} = \frac{(\text{Corrected } \Delta Abs_{570} - \text{y-intercept})}{\text{Slope}}$$

NOTE: To convert to µg/dl, divide the concentration in µM by 0.179.

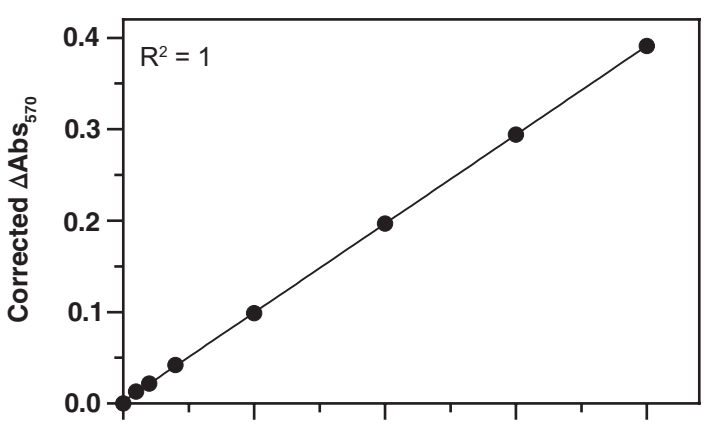


Figure 5. Typical standard curve

Performance Characteristics

Sensitivity:

The LLOD is 0.68 µM (3.8 µg/dl).

The LLOQ is 2.5 µM (14 µg/dl).

Precision:

When a series of 24 serum iron measurements were performed on the same day under the same experimental conditions, the intra-assay coefficient of variation was 4.2%. When a series of six of serum iron measurements were performed on different days under the same experimental conditions, the inter-assay coefficient of variation was 7.2%.

Interferences

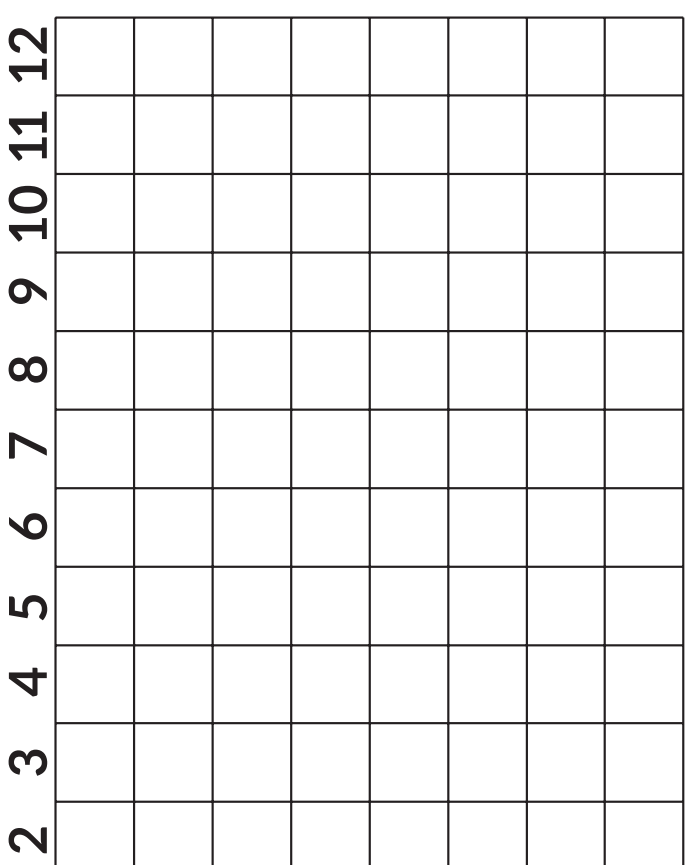
Young, *et al.* has published a list of common interferences which can affect the accuracy of iron levels determined in this assay kit.[6] Avoid using hemolytic, icteric (jaundice), or lipemic sera as these interfere with the assay. Normal serum bilirubin levels should not interfere.

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of replicates	A. Poor pipetting/technique B. Bubble in the well(s)	A. Be careful not to splash the contents of the wells B. Carefully tap the side of the plate with your finger to remove bubbles
Little to no color change observed in wells	A. Iron Color Reagent was not added B. Insufficient incubation time at 37°C	A. Make sure to add the appropriate amount of Iron Color Reagent B. Incubate samples at 37°C as indicated on page 19

Procedure	Standard Wells	Sample Wells
Add Serum Iron Buffer	150 µl	150 µl
Add Standard	50 µl	--
Add Sample	--	50 µl
Incubate	Seal, briefly shake, and incubate at 37°C for 10 minutes, protected from light	
Read	Read absorbance 1 at 570 nm	
Add Iron Color Reagent	50 µl	50 µl
Develop	Seal, briefly shake, and develop at 37°C for 30 minutes, protected from light	
Read	Read absorbance 2 at 570 nm	

Table 3. Assay summary



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2. Luck, A.N. and Mason, A.B. Transferrin-mediated cellular iron delivery. *Curr. Top. Membr.* **69**, 3-35 (2012).
3. Gomme, P.T. and McCann, K.B. Transferrin: Structure, function and potential therapeutic actions. *Drug Discov. Today* **10(4)**, 267-273 (2005).
4. Persijn, J.-P., Van der Slik, W., and Riethorst, A. Determination of serum iron and latent iron-binding capacity (LIBC). *Clinica Chimica Acta* **35**, 91-98 (1971).
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NOTES

Warranty and Limitation of Remedy

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