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Total Iron-Binding Capacity (TIBC) and Serum Iron Assay Kit

Item No. 702230

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	1 vial/20 ml	RT
400491	UIBC Tris Buffer	2 vials/10 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 ea	RT
400012	96-Well Cover Sheet	2 covers	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 Total Iron-Binding Capacity (TIBC) and Serum Iron Assay Kit. This kit may not perform as described if any reagent or procedure is replaced or modified.

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. Interval timer
5. Microcentrifuge tubes
6. 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).*

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 concentrations can be determined using chromogens, such as bathophenanthroline or ferrozine. At a low pH, ferric iron is released from transferrin and reduced to ferrous iron by hydroxylamine.[1,4] Ferrous iron then forms a stable complex with the chromogen and can be measured *via* colorimetric detection. Transferrin levels can be quantified in terms of total iron-binding capacity (TIBC), which can be calculated after measuring the unsaturated iron-binding capacity (UIBC) and summing it with the serum iron concentration.[1] TIBC can also be used to calculate transferrin saturation, the percentage of serum iron divided by the iron-binding capacity of available transferrin.

Both serum iron levels and transferrin saturation 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 and TIBC in serum.

About This Assay

Cayman's Total Iron-Binding Capacity (TIBC) and Serum Iron Assay Kit provides a convenient colorimetric method of determining iron levels and UIBC in serum. Measurement of both serum iron and UIBC allows for the calculation of TIBC, which is an estimation of transferrin levels in the 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).

Principle Of This Assay

Cayman's Total Iron-Binding Capacity (TIBC) and 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+}, which has a violet color and a maximum absorbance peak at 570 nm. This kit can also be used to determine TIBC by measuring UIBC, alongside serum iron levels. To determine UIBC, serum transferrin is saturated with a known concentration of iron (100 µM) at a basic pH in the presence of carbonate ions. Then, iron that remains unbound in solution is detected through the addition of a ferrozine-containing color reagent. Because ferrozine can also form a complex with copper ions, neocuproine, a copper chelator, is included in the color reagent solution to prevent interference from copper.

Definition of Key Terms

LLOD (Lower Limit of Detection): the smallest measure that can be detected with reasonable certainty for a given analytical procedure. The LLOD is defined as a concentration two standard deviations higher than the mean zero value.

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

PRE-ASSAY PREPARATION

Reagent Preparation

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

   This vial contains 20 ml of Serum Iron Buffer containing acetate, pH 4.5, guanidine hydrochloride and hydroxylamine HCl. This reagent is ready to use as supplied. This reagent will be stable for at least six months when stored at room temperature.

2. **UIBC Tris Buffer – (Item No. 400491)**

   Each vial contains 10 ml of UIBC Tris Buffer containing sodium bicarbonate, pH 8.4. This reagent is ready to use as supplied. This reagent will be stable for at least six months when stored at room temperature.

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

   This vial contains 200 µl of 100 mM ferric chloride in 0.1 M hydrochloric acid. Prepare iron standard solutions as indicated on pages 14-15. This reagent will be stable for at least six months when stored at room temperature.

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

   Each vial contains 5 ml of the Iron Color Reagent, which contains acetate buffer, ferrozine, and neocuproine in 10% DMSO. This reagent is ready to use as supplied. This reagent will be stable for at least six months when stored at -20°C and protected from light. However, we advise to aliquot into smaller batches when not using all at once.

Sample Preparation

Serum

It is highly preferable that fasting serum be used in this assay kit. Typically, normal human serum has iron concentrations in the range of 10-40 µM and TIBC values between 40-80 µM.[5] However, it is recommended that each laboratory 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 serum as these interfere with the assay.

1. Collect blood without using an anticoagulant.
2. Allow blood to clot for 30 minutes at 25°C.
3. Centrifuge the blood at 2,000 x g for 15 minutes at 4°C. Pipette off the top yellow serum layer 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 use in the assay.

Sample Matrix Properties

Parallelism

To assess parallelism, human and animal serum samples were serially diluted with Serum Iron Buffer and evaluated using the Total Iron-Binding Capacity (TIBC) and Serum Iron Assay Kit. The results are shown in the graph below.

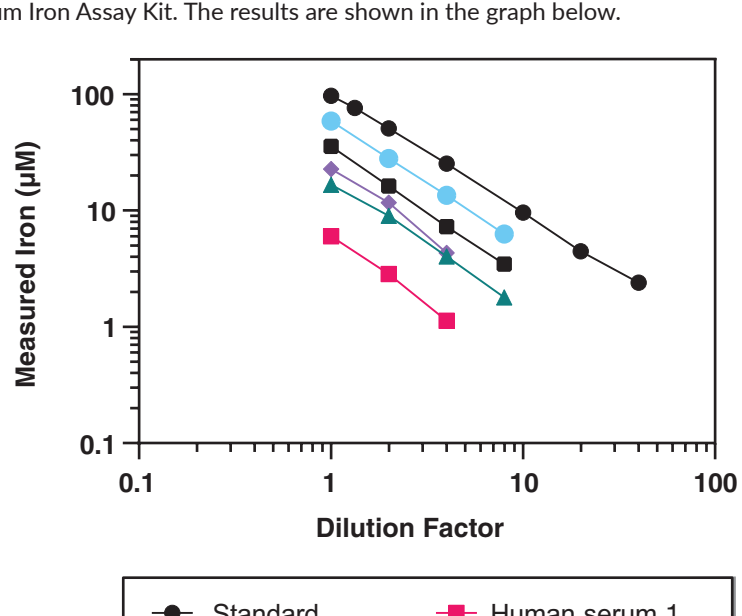


Figure 1. Parallelism of serum samples in the Total Iron-Binding Capacity (TIBC) and Serum Iron Assay Kit

Linearity

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

Dilution	Measured Concentration (µM)	% Recovery
1:1	108.18	100
1:2	114.19	106
1:4	113.78	105
1:8	112.97	104

Table 1. Dilutional linearity in 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 samples were spiked with different amounts of iron, diluted with Serum Iron Buffer, and analyzed using the Total Iron-Binding Capacity (TIBC) and Serum Iron Assay Kit. The results are shown below.

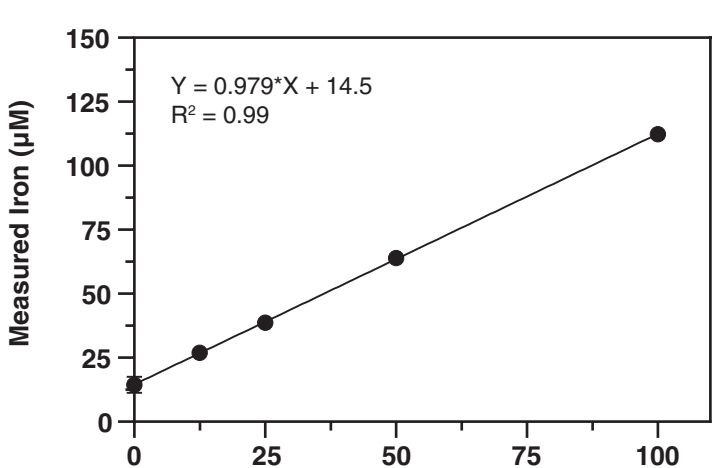


Figure 2. Spike and recovery in human serum

ASSAY PROTOCOL

Preparation of Assay-Specific Reagents

1. $FeCl_3$ Standard Preparation

The vial contains 100 mM $FeCl_3$ µl in 0.1 M HCl. Mix 10 µl of $FeCl_3$ Standard (Item No. 400492) with 990 µl of water to make a 1 mM iron standard solution. It is important to use iron-free water in order to dilute out the hydrochloric acid. Then, prepare a 100 µM iron standard by diluting the 1 mM iron standard solution 10-fold: mix 100 µl of the 1 mM iron standard solution with 900 µl of either Serum Iron Buffer or UIBC Tris Buffer. Prepare the rest of the iron standard dilutions according to Table 2 (see page 15).

Well	Volume of 100 µM Iron Standard (µl)	Volume of Diluent (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

2. UIBC Working Reagent Preparation

Prepare the UIBC Working Reagent by diluting freshly prepared 1 mM iron standard solution (see page 14) 1:30 with UIBC Tris Buffer (one part of 1 mM iron standard solution and 29 parts of buffer). Each well requires 150 µl of UIBC Working Reagent. Scale the volume up or down as needed using the formula:

(# sample unknowns) x (# replicates) x (150 µl) x 1.1 = volume of UIBC Working Reagent needed

Below is an example for 8 samples and 2 replicates of each sample:

(8 sample unknowns) x (2 replicates) x (150 µl) x 1.1 = 2.64 ml of UIBC Working Reagent needed
Mix 88 µl of 1 mM iron standard solution with 2.552 ml of buffer to prepare 2.64 ml of UIBC Working Reagent

NOTE: An extra 10% extra volume is added to the calculated volume of UIBC Working Reagent to ensure that a sufficient amount is prepared. Always use freshly prepared UIBC Working Reagent and Iron Standard Curve Solutions.

Plate Set Up

There is no specific pattern for using the wells on the plate. A typical layout of standards and samples to be measured in duplicate is given below in Figure 3. 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 28).

	1	2	3	4	5	6	7	8	9	10	11	12
A	A	A	Blk	Blk	S8	S8	S16	S16	S24	S24	S32	S32
B	B	B	S1	S1	S9	S9	S17	S17	S25	S25	S33	S33
C	C	C	S2	S2	S10	S10	S18	S18	S26	S26	S34	S34
D	D	D	S3	S3	S11	S11	S19	S19	S27	S27	S35	S35
E	E	E	S4	S4	S12	S12	S20	S20	S28	S28	S36	S36
F	F	F	S5	S5	S13	S13	S21	S21	S29	S29	S37	S37
G	G	G	S6	S6	S14	S14	S22	S22	S30	S30	S38	S38
H	H	H	S7	S7	S15	S15	S23	S23	S31	S31	S39	S39

A-H - Standard wells
Blk - Blank wells
S1-S39 - Sample wells

Figure 3. 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).
- Thirty-nine samples can be assayed in duplicate.
- Monitor the absorbance at 570 nm.
- The assay is set up at room temperature with sample incubation at 37°C. Absorbance values can be read at room temperature or at 37°C as long as incubation was done at 37°C for 30 minutes and the signal has reached a plateau.
- Signal is stable up to an hour after the addition of Iron Color Reagent.
- UIBC and Serum Iron assays can be run simultaneously on the same plate as the incubation times are the same.

Performing the Assay

Serum Iron Assay

1. **Standard and Sample Wells:** 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 3, on page 17).
2. **Blank Wells:** Add 200 µl of Serum Iron Buffer to the designated wells on the plate.
3. Cover the plate and mix briefly on a platform shaker.
4. Incubate at 37°C for 10 minutes.
5. Remove cover, read, and record absorbance at 570 nm. This absorbance value is referred to as Abs1.
6. Add 50 µl of Iron Color Reagent to all wells.
7. Cover the plate and mix briefly on a platform shaker.
8. Incubate at 37°C for 30 minutes in the dark.
9. Remove cover, read, and record absorbance at 570 nm. This absorbance value is referred to as Abs2.

UIBC Assay

1. **Standard Wells:** Add 150 µl of UIBC Tris Buffer and 50 µl of standard to the designated wells on the plate. (See **Sample plate format**, Figure 3, on page 17).
2. **Sample Wells:** Add 150 µl of UIBC Working Reagent and 50 µl of sample to the designated wells on the plate. (See **Sample plate format**, Figure 3, on page 17).
3. **Blank Wells:** Add 200 µl of UIBC Tris Buffer to the designated wells on the plate.
4. Cover the plate and mix briefly on a platform shaker.
5. Incubate at 37°C for 10 minutes.
6. Remove cover, read, and record absorbance at 570 nm. This absorbance value is referred to as Abs1.
7. Add 50 µl of Iron Color Reagent to all wells.
8. Cover the plate and mix briefly on a platform shaker.
9. Incubate at 37°C for 30 minutes in the dark.
10. Remove cover, read, and record absorbance at 570 nm. This absorbance value is referred to as Abs2.

ANALYSIS

Calculations

Many plate readers come with data reduction software that plot data automatically. Alternatively, a spreadsheet program can be used. We recommend using a linear curve fit (see Figure 4 on page 23).

1. Determine the change in absorbance (ΔAbs_{570}) by subtracting Abs1 from Abs2.
2. Calculate the average ΔAbs_{570} of the each standard, sample and blank wells.
3. Substract the average ΔAbs_{570} of standard H (see Table 2 on page 15) from itself and all other standards. This is referred to as the corrected ΔAbs_{570} of the standards below (step 5).
4. Substract the average ΔAbs_{570} of the blank wells (see Table 2 on page 15) from the samples. This is referred to as the corrected ΔAbs_{570} of the samples below (step 6).
5. Plot the average corrected ΔAbs_{570} values from step 3 above *versus* the final standard iron concentration (see Table 2 on page 15). See Figure 4, on page 23, for a typical standard curve.

6. For each sample, use the equation obtained from the linear regression of the standard curve to determine the serum iron concentration and to derive UIBC and TIBC. Use the corrected ΔAbs_{570} values from step 4.

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

$$\text{UIBC, } \mu M = 100 - \left[\frac{(\Delta Abs_{570} - \text{y-intercept})}{\text{Slope}} \right]$$

$$\text{TIBC, } \mu M = \text{Serum Iron} + \text{UIBC}$$

$$\text{\% saturation of transferrin} = \frac{\text{Serum Iron}}{\text{TIBC}} \times 100$$

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

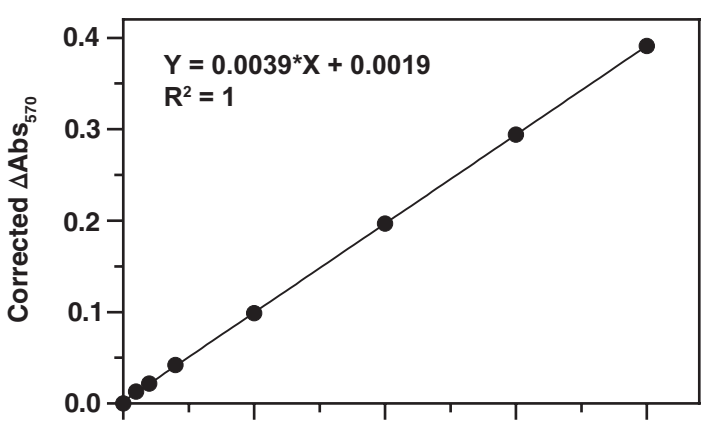


Figure 4. Typical standard curve

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 six series 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] Normal serum bilirubin levels should not interfere.

RESOURCES

Troubleshooting

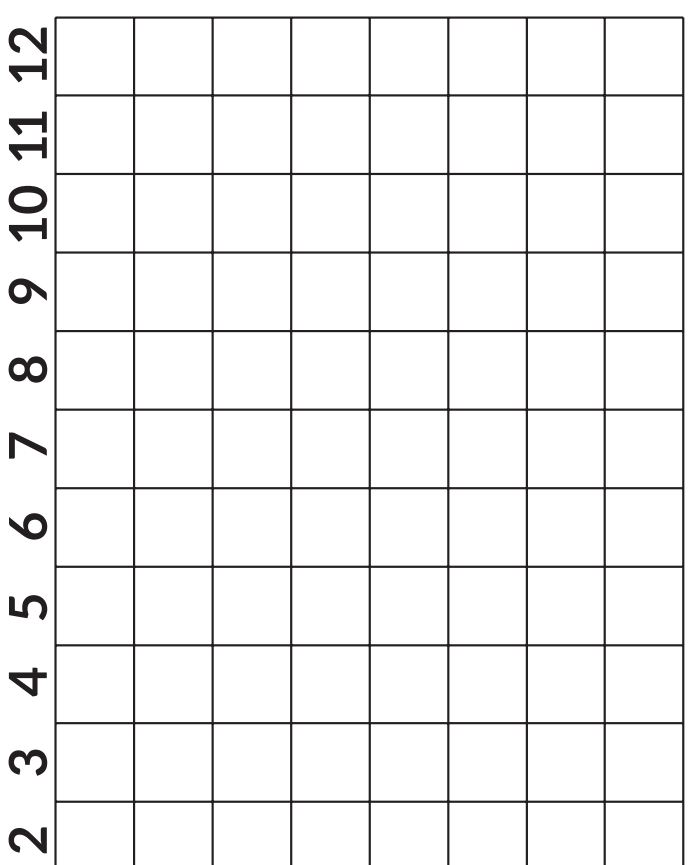
Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates/triplicates	A. Poor pipetting/technique B. Bubble in the well(s)	A. Be careful not to splash the contents of the wells B. Carefully tap the side of the plate with your finger to remove bubbles
Little to no color change observed in wells	A. Iron Color Reagent was not added B. Insufficient incubation time at 37°C C. UIBC Tris Buffer was used instead of Serum Iron Buffer and *vice versa* D. UIBC Working Reagent was not made correctly	A. Make sure to add the appropriate amount of Iron Color Reagent B. Incubate samples at 37°C as indicated on page 19 C. Use the correct buffer D. Make sure to add iron in the UIBC Working Reagent
Unbound iron determined in UIBC assay is greater than 100 µM (*i.e.*, negative UIBC numbers)	Serum Iron Buffer was used to make UIBC Working Reagent instead of the UIBC Tris Buffer	Use the correct buffer

Procedure	Standard/Sample Wells	Blank Wells
Serum Iron Buffer	150 µl	200 µl
Standard/Samples	50 µl	--
Incubation	Seal, shake, and incubate at 37°C for 10 minutes protected from light	
Iron Color Reagent	50 µl	50 µl
Develop	Seal, shake, and develop at 37°C for 30 minutes protected from light	
Read	Read absorbance at 570 nm	

Table 3. Serum Iron Assay summary

Procedure	Standard Wells	Sample Wells	Blank Wells
UIBC Tris Buffer	150 µl	--	200 µl
UIBC Working Reagent	--	150 µl	--
Standard/Samples	50 µl	50 µl	50 µl
Incubation	Seal, shake, and incubate at 37 °C for 10 minutes protected from light		
Iron Color Reagent	50 µl	50 µl	50 µl
Develop	Seal, shake, and develop at 37°C for 30 minutes protected from light		
Read	Read absorbance at 570 nm		

Table 4. UIBC Assay summary



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NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.