ab83366 - Iron Assay kit (Colorimetric)

For the rapid, sensitive and accurate measurement of Ferrous and/or Ferric ions in various samples.
View kit datasheet: www.abcam.com/ab83366 (use www.abcam.co.jp/ab83366 for Japan)
This product is for research use only and is not intended for diagnostic use.
To get Chinese protocol, please scan QR code via WeChat or visit Protocols section at www.abcam.cn/ab83366

Overview: Iron Assay Kit ab83366 provides a simple convenient means of measuring ferrous (Fe^{2+}) and/or ferric (Fe^{3+}) iron in biological samples. Ferric carrier protein will dissociate ferric into solution in the presence of the acid assay buffer. If the assay buffer has a neutral pH, iron will bind tightly to the iron carrier protein. However, under acidic conditions (pH less than 5.5), iron no longer has a binding affinity for the carrier protein and will be dissociated and released into the solution as iron/ferric ions, whose concentration you can measure using this kit. The Iron Assay Buffer in this kit has an acidic pH that enables this release of iron/ferric ions into the solution.

Free ferrous iron (Fe^{2+}) reacts with Iron Probe to produce a stable colored complex with absorbance at 593 nm. Ferric iron (Fe^{3+}) can be reduced to form Fe^{2+} enabling the measurement of total iron (Fe^{2+} and Fe^{3+}). The level of ferric iron (Fe^{3+}) is calculated by subtracting ferrous iron from total iron.

A specific chelate chemical is included in the buffer to block copper ion (Cu^{2+}) interference. The kit measures iron in the linear range of 0.4 to 10 nmol or 8 µM to 200 µM.

Storage and Stability: Store kit at -20°C in the dark immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted. Reconstituted components are stable for 2 months. Mix the Iron Reducer to dissolve any precipitate that may have formed during freezing.

Materials Supplied

- **Iron Assay Buffer -** 25ml
- **Iron Probe -** 12 mL
- **Iron reducer -** 700 µL
- **Iron Standard (100 mM)** - 100 µL

Storage conditions for all items: -20°C – before and after preparation

Materials Required, Not Supplied

- Microplate reader capable of measuring absorbance at OD 593 nm.
- MilliQ water or other type of double distilled water (ddH2O)
- Pipettes and pipette tips, including multi-channel pipette
- Assorted glassware for the preparation of reagents and buffer solutions
- Tubes for the preparation of reagents and buffer solutions
- 96 well plate with clear flat bottom
- Dounce homogenizer (if using tissue)
- (Optional) Protease inhibitors: we recommend Protease Inhibitor Cocktail II (www.abcam.com/ab201116) [AEBSF, aprotinin, E-64, EDTA, leupeptin] as a general use cocktail.

Reagent Preparation: Briefly centrifuge small vials at low speed prior to opening

- **Iron Standard and Reducer:**
  Ready to use as supplied. Equilibrate to room temperature before use. Aliquot standard so that you have enough volume to perform the desired number of assays. Store at - 20°C.
- **Iron Assay Buffer:**
  Ready to use as supplied. Equilibrate to room temperature before use. Store at - 20°C.
- **Iron Probe:**
  Ready to use as supplied. Keep on ice during the assay. Aliquot probe so that you have enough volume to perform the desired number of assays. Store at - 20°C. Once the probe is thawed, use within two months. If any precipitate is observed, do not warm the vial at 37°C. Instead, centrifuge and take the top clear supernatant for the assay.

Standard Preparation

Always prepare a fresh set of standards for every use.
Discard the working standard solutions after use as they do not store well.

1. Prepare a 1 mM standard by diluting 10 µL Iron Standard in 990 µL of ddH2O.
2. Using 1mM standard, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes:

Standard #	Volume of Iron 1mM Standard (µL)	Assay Buffer (µL)	Final Volume standard in well (µL)	End Conc Iron in well (nmol/well)
1	0	300	100	0
2	6	294	100	2
3	12	288	100	4
4	18	282	100	6
5	24	276	100	8
6	30	270	100	10

Each dilution has enough amount of standard to set up duplicate reading (2 x 100 µL).

Sample Preparation

General Sample Information

- We recommend performing several dilutions of your sample to ensure the readings are within the standard value range.
- We recommend that you use fresh samples. If you cannot perform the assay at the same time, we suggest that you complete the Sample Preparation step before storing the samples. Alternatively, if that is not possible, we suggest that you snap freeze samples in liquid nitrogen upon extraction and store the samples immediately at -80°C. When you are ready to test your samples, thaw them on ice. Be aware however that this might affect the stability of your samples and the readings can be lower than expected.

Tissue samples:

1. **Harvest the amount of tissue necessary for each assay (initial recommendation = 10 mg tissue).**
2. **Wash tissue in cold PBS.**
   a. **Homogenize tissue in 4-10 volumes of Iron Assay Buffer using a Dounce homogenizer sitting on ice, with 10-15 passes.**
3. **Centrifuge at 16,000 x g for 10 minutes to remove insoluble materials.**
4. **Collect supernatant and transfer to a clean tube. Keep on ice.**

Serum, Urine and other biological fluids:
Urine can be tested directly.
Serum:
1. For serum preparation, collect whole blood in a covered test tube on ice.
2. After collection of the whole blood, allow the blood to clot by leaving it undisturbed at room temperature (typically 15 – 30 minutes).
3. Remove the clot by centrifuging samples at 1000 – 2000 x g for 10 minutes in a cold microcentrifuge.
4. Following centrifugation, it is important to immediately transfer the serum into a clean polypropylene tube using a Pasteur pipette.
5. If samples are not analyzed immediately, serum should be aliquoted into 0.5 mL aliquots. Store at - 80°C.
6. Normal serum iron concentration ~ 10 – 40 µM.

Plasma*: This kit is not compatible with EDTA plasma due to the presence of chelating substance such as EDTA and citrate as EDTA, which can directly bind to iron causing interference with the assay. This kit is also not suitable for use with plasma samples in general due to the presence of iron binding transferrin in plasma leading to inaccurate measurements of free iron.*
NOTE: *We suggest using different volumes of sample to ensure readings are within the Standard Curve range.*

Assay Procedure

- Equilibrate all materials and prepared reagents to correct temperature prior to use.
- We recommended to assay all standards, controls and samples in duplicate.
- Prepare all reagents, working standards, and samples as directed in the previous sections. Samples can be tested for FERROUS (Fe2+) ion, FERRIC (Fe3+) ion or TOTAL Fe (II + III).

1. Set up Reaction wells (see table below):
   - Standard wells = 100 µL Standard dilutions.
   - Sample wells = 2 – 50 µL samples (adjust volume to 100 µL/well with Iron Assay Buffer).
2. Add 5 µL Iron Reducer to each Standard well.
3. For iron (II) assay: add 5 µL of Assay Buffer to each sample.
4. For total iron (II+III) assay: add 5 µL of Iron Reducer to each sample.
5. Mix and incubate standards and samples at 37°C for 30 minutes.
6. Add 100 µL Iron Probe to each well containing the Iron Standard and test samples.
7. Mix and incubate at 37°C for 60 minutes protected from light.
8. Measure output immediately on a colorimetric microplate reader. (OD 593 nm).

# order in reaction	Component	Standard (µL)	Iron (II) (µL)	Total Iron (II + III) (µL)
1	Standard	100	-	-
2	Sample	-	100	100
3	Iron Reducer	5	-	5
4	Iron Buffer	-	5	-
5	Iron Probe	100	100	100

Calculations

Samples producing signals greater than that of the highest standard should be further diluted in appropriate buffer and reanalyzed, then multiply the concentration found by the appropriate dilution factor.

1. Average the duplicate reading for each standard and sample.
2. Subtract the mean absorbance value of the blank (Standard #1) from all standard and sample readings. This is the corrected absorbance.
3. Plot the corrected absorbance values for each standard as a function of the final concentration of Iron.
4. Draw the best smooth curve through these points to construct the standard curve. Most plate reader software or Excel can plot these values and curve fit. Calculate the trendline equation based on your standard curve data (use the equation that provides the most accurate fit).
5. Iron (II) and Total Iron (II+III) contents of the test samples can be acquired directly from the standard curve.
6. Iron (III) content of the test samples can be calculated as:
   Iron (III) =Total Iron (II+III) – Iron (II)
7. Concentration of Iron (Iron(II), Iron(III) and Total Iron) in the test samples is calculated as:

$$Iron\ Concentration = \left(\frac{Sa}{Sv}\right) * D$$

Where:
Sa = content of iron in sample well calculated from standard curve (nmol).
Sv = volume of sample added into the reaction wells (µL).
D = Sample dilution factor.

Iron ion molecular weight: is 55.845 g/mol

<u>Typical Data</u> typical standard curve – Data provided for demonstration purposes only. A new standard curve must be generated for each assay performed.

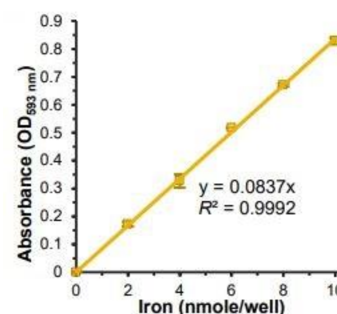

Figure 1: Typical Iron standard calibration curve using colorimetric reading.

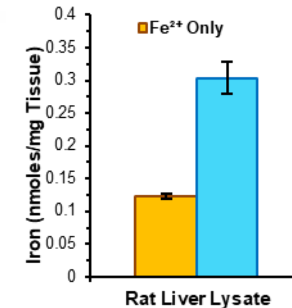

Figure 2: Assay of soluble free iron and total iron in perfused rat liver homogenate, 8 mg of wet tissue was used per well.

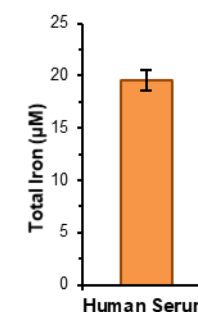

Figure 3: Assay of total iron in the off-the-clot human serum, 50 µl of serum was used per well.

Troubleshooting

Problem	Cause	Solution
Assay not working	Use of ice-cold buffer	Buffers must be at room temperature
	Plate read at incorrect wavelength	Check the wavelength and filter settings of instrument
	Use of a different 96-well plate	Colorimetric: Clear plates Fluorometric: black wells/clear bottom plate

Sample with erratic readings	Samples not deproteinized (if indicated on protocol)	Use provided protocol for deproteinization
	Cells/tissue samples not homogenized completely	Use Dounce homogenizer, increase number of strokes
	Samples used after multiple free/ thaw cycles	Aliquot and freeze samples if needed to use multiple times
	Use of old or inappropriately stored samples	Use fresh samples or store at - 80°C (after snap freeze in liquid nitrogen) till use
	Presence of interfering substance in the sample	Check protocol for interfering substances; deproteinize samples
Standard readings do not follow a linear pattern	Pipetting errors in standard or reaction mix	Avoid pipetting small volumes (< 5 µL) and prepare a master mix whenever possible
	Air bubbles formed in well	Pipette gently against the wall of the tubes
	Standard stock is at incorrect concentration	Always refer to dilutions described in the protocol
Unanticipated results	Measured at incorrect wavelength	Check equipment and filter setting
	Samples contain interfering substances	Troubleshoot if it interferes with the kit
	Sample readings above/ below the linear range	Concentrate/ Dilute sample so it is within the linear range
Lower/ Higher readings in samples and standards	Improperly thawed components	Thaw all components completely and mix gently before use
	Allowing reagents to sit for extended times on ice	Always thaw and prepare fresh reaction mix before use
	Incorrect incubation times or temperatures	Verify correct incubation times and temperatures in protocol
	Standard stock is at incorrect concentration	Always refer to dilutions described in the protocol

Interferences

These chemicals or biological materials will cause interference in this assay causing compromised results or complete failure:

- Chromium (III).
- Copper (II): A specific chelate chemical is included in the buffer to block copper ion (Cu^{2+}) interference
- Transferrin – iron binding protein present in plasma

FAQs

What are normal iron levels?

1) Normal serum Iron ~10-40 µM.
2) Cells such as HeLa or cervical cells typically contain 1-0.9 pg iron/cell so 2×10^6 cells lysed in ~ 250 µL of assay buffer (Fe MW 55.85) should have about 4 – 8 nmol Fe per 50 µL test sample- possibly less if iron tightly bound and not released well be acid buffer (assay buffer)

On addition of Iron Probe (provided in kit) I could see an immediate development of color in wells containing the standards. However, even after incubation for longer period (over 1 hour and more) the wells containing our samples did not show any change in color at all.

It is possible that you are using too dilute a sample to get readings within in the linear range of the standard curve. Try using higher volumes or more concentrated of the samples.

I am seeing the samples turn cloudy but not blue with addition of the probe (despite following the protocol). Is it common for the sample/probe to do this?

This is a common problem seen in liver and serum samples. Lipoproteins in the sample are the main culprits behind this turbidity. For this purpose, we would recommend adding 5 µl/well of 1 M SDS (28.8% or 288 mg/mL of SDS) to all the sample wells after step 4 of the assay procedure. Incubate for 30 min at 37°C as stated in step 5 and then follow the protocol. This SDS will clear up the turbidity (by dissolving any lipoproteins in the samples).

When I added Iron Probe (NM), samples turned cloudy. If I added acid solution to my samples (ex: HCL), it turned clear. However, when I added NaOH, it turned cloudier. Can you explain what is happening?

This assay works in an acidic environment. That is why when HCl is added, the samples are turning clear. One of the reasons that the samples are not changing color is because they are very dilute. Please use more of the samples. Ideally the Ferene S to iron ratio should be large than 5. However, there may be some interference being generated by some components in the samples leading to the cloudiness. Copper (II) and Chromium (III) will interfere with the result, Copper (II) can be block by the thiourea.

Do you have any recommended preparation steps for using this kit with serum?

It is important to avoid freeze-thaw cycles because this is detrimental to many serum components. Samples which are hemolyzed, icteric or lipemic can invalidate certain tests. Do not leave it for a long length of time at room temperature after preparation.

I have used this kit with serum samples successfully, but have issues using it with plasma. Why?

This kit is not suitable for use with plasma samples in general due to the presence of iron binding transferrin in plasma leading to inaccurate measurements of free iron.

Could you provide your cell preparation protocol?

We have not tested cells with this kit. However, the kit should work with a variety of sample types.

We suggest gently washing the cells with ice-cold PBS and scraping off the cells with a cell scraper into ice-cold PBS. Pellet the cells at 1,000g for 5 minutes before resuspending in Iron assay

buffer. Please use 1x10^6 cells in 100 µl of Iron assay buffer, homogenize on ice using a dounce homogenizer or gentle sonication on ice with a few short pulses. Centrifuge at 16,000g for 10 minutes at 4 °C to remove insoluble materials and use the supernatant for the assay.

Note: If you use trypsin, please use without EDTA and wash the cell pellet properly with ice-cold PBS.

Technical Support

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