ab83366
Iron Assay kit (Colorimetric)

Instructions for use:
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.cn/ab83366 for China, or www.abcam.co.jp/ab83366 for Japan)

This product is for research use only and is not intended for diagnostic use.
1. BACKGROUND

Iron Assay Kit (Colorimetric) (ab83366) provides a simple convenient means of measuring Ferrous and/or Ferric ions in samples. The ferric carrier protein will dissociate ferric into solution in the presence of acid buffer. After reduction to the ferrous form (Fe2+), iron reacts with Ferene S (an iron chromogen) to produce a stable colored complex and give absorbance at 593 nm. A specific chelate chemical is included in the buffer to block copper ion (Cu2+) interference.

This kit measures iron in the linear range of 0.4 – 10 nmol, or 8 µM – 200 µM iron concentration in various samples.

Iron is essential to nearly all known organisms. It is generally stored in the centre of metalloproteins, in the heme complex, and in oxygen carrier proteins. Inorganic iron also contributes to redox reactions in the iron-sulfur clusters of many enzymes, such as nitrogenase and hydrogenase.
INTRODUCTION

2. ASSAY SUMMARY

Standard curve preparation

↓

Sample preparation

↓

Add iron probe

↓

Incubate at 37°C for 60 minutes protected from light

↓

Measure absorbance (OD593 nm)
3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

- All kit components have been formulated and quality control tested to function successfully as a kit.

- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.

- Reagents should be treated as possible mutagens and should be handle with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.

- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas.

- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

4. 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.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Materials Supplied section. Reconstituted components are stable for 2 months.

Mix the Iron Reducer to dissolve any precipitate that may have formed during freezing.
5. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

6. MATERIALS SUPPLIED

<table>
<thead>
<tr>
<th>Item</th>
<th>Amount</th>
<th>Storage Condition (Before Preparation)</th>
<th>Storage Condition (After Preparation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron Assay Buffer</td>
<td>25 mL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>Iron Probe</td>
<td>12 mL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>Iron Reducer</td>
<td>700 µL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>Iron Standard (100 mM)</td>
<td>100 µL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
</tbody>
</table>
7. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully perform this assay:

- 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 (ab201116) [AEBSF, aprotinin, E-64, EDTA, leupeptin] as a general use cocktail.
8. TECHNICAL HINTS

- This kit is sold based on number of tests. A ‘test’ simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.

- Selected components in this kit are supplied in surplus amount to account for additional dilutions, evaporation, or instrumentation settings where higher volumes are required. They should be disposed of in accordance with established safety procedures.

- Avoid foaming or bubbles when mixing or reconstituting components.

- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.

- Ensure plates are properly sealed or covered during incubation steps.

- Ensure all reagents and solutions are at the appropriate temperature before starting the assay.

- Samples which generate values that are greater than the most concentrated standard should be further diluted in the appropriate sample dilution buffer.

- Make sure you have the right type of plate for your detection method of choice.

- Make sure all necessary equipment is switched on and set at the appropriate temperature.
9. REAGENT PREPARATION

- Briefly centrifuge small vials at low speed prior to opening

9.1. Iron Standard:
   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.

9.2. Iron Assay Buffer:
   Ready to use as supplied. Equilibrate to room temperature before use. Store at -20°C.

9.3. 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.

9.4. Iron Reducer:
   Ready to use as supplied. Equilibrate to room temperature before use. Aliquot reducer so that you have enough volume to perform the desired number of assays. Store at -20°C.
10. 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.

10.1. Prepare a 1 mM standard by diluting 10 µL Iron Standard in 990 µL of ddH₂O.

10.2. Using 1mM standard, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes:

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

Each dilution has enough amount of standard to set up duplicate reading (2 x 100 µL).
11. 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.

11.1. Tissue samples:

11.1.1. Harvest the amount of tissue necessary for each assay (initial recommendation = 10 mg tissue).

11.1.2. Wash tissue in cold PBS.

11.1.3. Homogenize tissue in 4-10 volumes of Iron Assay Buffer using a Dounce homogenizer sitting on ice, with 10 – 15 passes.

11.1.4. Centrifuge at 16,000 x g for 10 minutes to remove insoluble materials.

11.1.5. Collect supernatant and transfer to a clean tube. Keep on ice.

11.2. Serum, Urine and other biological fluids:

Urine and cell culture media can be tested directly.

Serum:

11.2.1. For serum preparation, collect whole blood in a covered test tube on ice.

11.2.2. After collection of the whole blood, allow the blood to clot by leaving it undisturbed at room temperature (typically 15 – 30 minutes).
11.2.3. Remove the clot by centrifuging samples at 1000 – 2000 x g for 10 minutes in a cold microcentrifuge.

11.2.4. Following centrifugation, it is important to immediately transfer the serum into a clean polypropylene tube using a Pasteur pipette.

11.2.5. If samples are not analyzed properly, serum should be aliquoted into 0.5 mL aliquots. Store at -80°C.

11.2.6. Normal serum iron concentration ~ 10 – 40 µM.

*Plasma*: Due to the presence of iron binding transferrin in plasma, the measurement of free iron is not accurate and therefore plasma is not a suitable sample for this product.

*NOTE*: We suggest using different volumes of sample to ensure readings are within the Standard Curve range.
12. 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).

12.1. Set up Reaction wells (see table below):

<table>
<thead>
<tr>
<th># order in reaction</th>
<th>Component</th>
<th>Standard (µL)</th>
<th>Iron (II) (µL)</th>
<th>Total Iron (II + III) (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Standard</td>
<td>100</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Sample</td>
<td>-</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>Iron Reducer</td>
<td>5</td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>Iron Buffer</td>
<td>-</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>Iron Probe</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

- 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.

12.2. Add 5 µL Iron Reducer to each Standard well.
12.3. For iron (II) assay: add 5 µL of Assay Buffer to each sample.
12.4. For total iron (II+III) assay: add 5 µL of Iron Reducer to each sample.
12.5. Mix and incubate standards and samples at 37°C for 30 minutes.
12.6. Add 100 µL Iron Probe to each well containing the Iron Standard and test samples.
12.7. Mix and incubate at 37°C for 60 minutes protected from light.
12.8. Measure output immediately on a colorimetric microplate reader. (OD 593 nm).
**13. 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.

13.1. Average the duplicate reading for each standard and sample.

13.2. Subtract the mean absorbance value of the blank (Standard #1) from all standard and sample readings. This is the corrected absorbance.

13.3. Plot the corrected absorbance values for each standard as a function of the final concentration of Iron.

13.4. 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).

13.5. Iron (II) and Total Iron (II+III) contents of the test samples can be acquired directly from the standard curve following equation on Step 13.5.

13.6. Iron (III) content of the test samples can be calculated as:

\[
\text{Iron (III)} = \text{Total Iron (II+III)} - \text{Iron (II)}
\]

13.7. Concentration of Iron (Iron(II), Iron(III) and Total Iron) in the test samples is calculated as:

\[
\text{Iron Concentration} = \left(\frac{S_a}{S_v}\right) * D
\]

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

Iron ion molecular weight: is 55.845 g/mol
14. TYPICAL DATA

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:** Total iron, ferrous iron (Fe$^{2+}$) and ferric iron (Fe$^{3+}$) measured in human urine.
Figure 3: Assay of soluble free iron from a soil sample (5 μL of 100 μL buffer into which 100 mg of soil had been stirred), 5 μL of FBS and 5 μL of a 100 μM sample of iron standard.
15. QUICK ASSAY PROCEDURE

NOTE: This procedure is provided as a quick reference for experienced users. Follow the detailed procedure when initially performing the assay.

- Prepare Iron standard dilution [2 – 10 nmol/well].
- Prepare samples in optimal dilutions to fit standard curve readings.
- Set up plate in duplicate for standard (100 µL) and samples (100 µL).
- Add 5 µL Iron Reducer to each Standard well.
- For iron (II) assay: add 5 µL of Assay Buffer to each sample.
- For iron (II+III) assay: add 5 µL of Iron Reducer to each sample.
- Mix and incubate at 37°C for 30 minutes.
- Add 100 µL Iron Probe to each well containing the Iron Standard and test samples.

<table>
<thead>
<tr>
<th># order in reaction</th>
<th>Component</th>
<th>Standard (µL)</th>
<th>Iron (II) (µL)</th>
<th>Total Iron (II + III) (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Standard</td>
<td>100</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Sample</td>
<td>-</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>Iron Reducer</td>
<td>5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>Iron Buffer</td>
<td>-</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>Iron Probe</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

- Mix and incubate at 37°C for 60 minutes protected from light.
- Measure plate immediately at OD 593 nm.
### 16. TROUBLESHOOTING

<table>
<thead>
<tr>
<th>Problem</th>
<th>Cause</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay not working</td>
<td>Use of ice-cold buffer</td>
<td>Buffers must be at room temperature</td>
</tr>
<tr>
<td></td>
<td>Plate read at incorrect wavelength</td>
<td>Check the wavelength and filter settings of instrument</td>
</tr>
<tr>
<td></td>
<td>Use of a different 96-well plate</td>
<td>Colorimetric: Clear platesFluorometric: black wells/clear bottom plate</td>
</tr>
<tr>
<td>Sample with erratic readings</td>
<td>Samples not deproteinized (if indicated on protocol)</td>
<td>Use provided protocol for deproteinization</td>
</tr>
<tr>
<td></td>
<td>Cells/tissue samples not homogenized completely</td>
<td>Use Dounce homogenizer, increase number of strokes</td>
</tr>
<tr>
<td></td>
<td>Samples used after multiple free/thaw cycles</td>
<td>Aliquot and freeze samples if needed to use multiple times</td>
</tr>
<tr>
<td></td>
<td>Use of old or inappropriately stored samples</td>
<td>Use fresh samples or store at -80°C (after snap freeze in liquid nitrogen) till use</td>
</tr>
<tr>
<td></td>
<td>Presence of interfering substance in the sample</td>
<td>Check protocol for interfering substances; deproteinize samples</td>
</tr>
<tr>
<td>Lower/ Higher readings in samples and standards</td>
<td>Improperly thawed components</td>
<td>Thaw all components completely and mix gently before use</td>
</tr>
<tr>
<td></td>
<td>Allowing reagents to sit for extended times on ice</td>
<td>Always thaw and prepare fresh reaction mix before use</td>
</tr>
<tr>
<td></td>
<td>Incorrect incubation times or temperatures</td>
<td>Verify correct incubation times and temperatures in protocol</td>
</tr>
<tr>
<td></td>
<td>Standard stock is at incorrect concentration</td>
<td>Always refer to dilutions described in the protocol</td>
</tr>
<tr>
<td>Problem</td>
<td>Cause</td>
<td>Solution</td>
</tr>
<tr>
<td>----------------------------------------------</td>
<td>--------------------------------------------</td>
<td>--------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Standard readings do not follow a linear</td>
<td>Pipetting errors in standard or reaction</td>
<td>Avoid pipetting small volumes (&lt; 5 µL) and prepare a master mix whenever</td>
</tr>
<tr>
<td>pattern</td>
<td>mix</td>
<td>possible</td>
</tr>
<tr>
<td></td>
<td>Air bubbles formed in well</td>
<td>Pipette gently against the wall of the tubes</td>
</tr>
<tr>
<td></td>
<td>Standard stock is at incorrect</td>
<td>Always refer to dilutions described in the protocol</td>
</tr>
<tr>
<td></td>
<td>concentration</td>
<td></td>
</tr>
<tr>
<td>Unanticipated results</td>
<td>Measured at incorrect wavelength</td>
<td>Check equipment and filter setting</td>
</tr>
<tr>
<td></td>
<td>Samples contain interfering substances</td>
<td>Troubleshoot if it interferes with the kit</td>
</tr>
<tr>
<td></td>
<td>Sample readings above/ below the linear</td>
<td>Concentrate/ Dilute sample so it is within the linear range</td>
</tr>
<tr>
<td></td>
<td>range</td>
<td></td>
</tr>
</tbody>
</table>
17. INTERFERENCES

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

- Chromium (III).
- Copper (II): the interfering effect of copper can be blocked by thiourea.
- Transferrin – iron binding protein present in plasma

18. 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 x 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 12.4. Incubate for 30 min at 37°C as stated in step 12.5 and then follow the protocol.
This SDS will clear up the turbidity (by dissolving any lipoproteins in the samples).

**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.

**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.

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

This kit is not compatible with plasma samples.
19. NOTES