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# Dissolved Fe(II/III) colorimetric assay using a plate reader (96-well plate)

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## ABSTRACT

This protocol describes the adaption of an Iron assay originally described in [Viollier 1999](#) and [Stookey 1970](#), modified for use on a multi-mode plate reader spectrophotometer (BioTek, Synergy 2, Winooski, VT, USA), using standard [96-well plates](#) for rapid measurements of 1 mL water samples. This assay measures the concentration of both Fe(II) and Fe(III) ions in solution.

Samples for this assay should be filtered ([0.2 µm syringe filter](#)), measured or diluted to 1.20 mL volume and placed in [1.5 mL microcentrifuge tubes](#). Care should be taken to avoid oxidation of Fe(II) ions by oxygen in the atmosphere, either by performing the steps quickly under a stream of N<sub>2</sub> gas right after sampling or by performing the first step of this assay inside an anaerobic chamber.

## GUIDELINES

### Working principle

Ferrozine binds to both Fe(II) and Fe(III) ions in solution and turns into a magenta-colored complex (note: Fe(II)-induced complex has ~60x stronger absorbance than Fe(III), thus constituting a larger part of the signal). Maximum absorbance is at  $\lambda=562\text{ nm}$  at pH 4-9. To determine the concentrations of Fe(II) and Fe(III) ions, two spectrophotometric absorbance measurements are made, termed  $A_1$  and  $A_2$ , where  $A_1$  is an initial measurement and  $A_2$  after a reduction step to reduce all Fe(III) to Fe(II) ions.

After measuring  $A_1$  and  $A_2$ , the following system of two equations is solved to find  $C_{\text{FeII}}$  and  $C_{\text{FeIII}}$ , the molar concentrations of Fe(II) and Fe(III) ions, respectively:

$$A_1 = \beta_{\text{FeII}} C_{\text{FeII}} + \beta_{\text{FeIII}} C_{\text{FeIII}}$$

$$A_2 = \beta_{\text{FeII}} (C_{\text{FeII}} + C_{\text{FeIII}}) \alpha$$

where  $\beta_x = \epsilon_x l$ ,  $x$  is the species to be measured,  $\epsilon_x$  is the molar absorbance coefficient of the specific complex,  $l$  the path length.  $\beta$  can be interpreted as the instrument-specific absorbance coefficient and  $\alpha$  is the dilution factor ( $0 < \alpha < 1$ ) due to the addition of reductant reagent to obtain  $A_2$ .

In order to solve for  $C_{\text{FeII}}$  and  $C_{\text{FeIII}}$ , all we need to determine are  $\beta_{\text{FeII}}$ ,  $\beta_{\text{FeIII}}$ , and  $\alpha$  (and estimate their uncertainties). We can obtain these values by analyzing a dilution series of standard solutions where the concentrations of Fe(II) and Fe(III) ions are known. A realistic  $\alpha$  value (along with its uncertainty estimates) is obtained by doing the reduction step a second time to the standards only, obtaining  $A_3$  (even though all Fe(III) has already been reduced). Hence  $\alpha = A_3/A_2$ . All analytical uncertainty estimates can be obtained by regression analysis after this step (usually linear regression, estimating error of the slope), using the same dataset already obtained.

After obtaining  $\beta_{\text{FeII}}$ ,  $\beta_{\text{FeIII}}$ , and  $\alpha$ , we may derive  $C_{\text{FeII}}$  and  $C_{\text{FeIII}}$  as the following:

$$C_{\text{FeII}} = \frac{A_2 - \alpha A_1}{\alpha(\beta_{\text{FeII}} - \beta_{\text{FeIII}})}$$

$$C_{FeIII} = \frac{\alpha\beta_{FeII} A_1 - \beta_{FeIII} A_2}{\alpha(\beta_{FeII} - \beta_{FeIII})\beta_{FeII}}$$

## Measurement range

The range of measurement of this assay is between **0 - 80 µM Fe(II/III)**, thus for samples containing a higher amount of dissolved Fe, especially anoxic water samples, dilution is required. For unknown samples, it is useful to add a 10x dilution sample. For samples that may contain elevated Fe, an additional 100x dilution is recommended. An accurate way to perform dilution is by measuring mass instead of volume. A pipette (which measures volume) has about 1-3% accuracy, whereas an analytical balance (0.1-1 mg accuracy) is vastly better. Do not switch pipettes during colorimetric assay procedures and pay careful attention.

## MATERIALS TEXT

### Materials

- [1.5 mL Eppendorf® Microcentrifuge Tubes](#)
- [Corning® 96-Well EIA/RIA Assay Microplate](#)

### Reagents

All reagents are prepared with nanopure water and stored in 4 °C fridge in the dark.

(A) **Ferrozine: [M]0.01 Molarity (M)** *Ferrozine* (FW 492.47, 97%, [Aldrich #160601](#)) is prepared in [M]0.1 Molarity (M) *ammonium acetate* (FW 77.08, 99.999%, [Aldrich #372331](#)) solution.

Preparation steps: add  nanopure water in a clean bottle, add  *ammonium acetate* powder, mix well, then add  *Ferrozine*, mix well and store at 4 °C in the dark.

(B) **Reducing Agent: [M]1.4 Molarity (M)** of *hydroxylamine hydrochloride* (FW 69.49, 99.99%, [Aldrich #379921](#)) is prepared in a solution of analytical grade *hydrochloric acid* at a final concentration of [M]2.0 Molarity (M) (diluted from stock *hydrochloric acid*: HCl suprapur, 30%, [M]9.46 Molarity (M) , [Aldrich #1.00318](#)).

Preparation steps: add  nanopure water in a clean bottle, add  (or by mass  ) 30% HCl. Add  *hydroxylamine hydrochloride*. Mix well.

(C) **Buffer: [M]10 Molarity (M)** *ammonium acetate* (FW 77.08, 99.999%, [Aldrich #372331](#)) is prepared and adjusted pH to  with a solution of *ammonium hydroxide* (28-30%, [Aldrich #221228](#)).

Preparation steps: dissolve  *ammonium acetate* in  of nanopure water. Adjust pH to  with a pH meter by adding concentrated *ammonium hydroxide* dropwise while keeping the solution mixed.

### Standards

Standards are diluted from [M]1000 Parts per Million (PPM) Fe(III) stock solution (prepared from [M]0.0179 Molarity (M) of FeCl<sub>3</sub> (FW 162.20 [Aldrich #8.03945](#)) in 0.01 M *hydrochloric acid* (diluted from stock *hydrochloric acid*: HCl suprapur, 1 M (1 N), [Aldrich #1.09137](#))). Series of Fe(III) standards are prepared and diluted in NaCl solution matching sample salinity. Salinity should be matched within 20%.

Preparation steps: add **200 ml** nanopure water in a clean bottle, add **2.02 ml** 1N HCl, mix well, add NaCl (amount to match expected/measured salinity of sample), then add **0.5807 g** FeCl<sub>3</sub> powder. Mix well.

#### SAFETY WARNINGS

Use safety goggles and nitrile gloves when performing the steps outlined in this assay. Dispose of left-over chemicals by evaporating off all liquids inside a chemical hood (96-well plate), or by collecting these liquids in designated chem-waste jars.

#### BEFORE STARTING

Samples for this assay should be filtered (**0.2 µm syringe filter**), measured or diluted to 1.20 mL volume and placed in **1.5 mL microcentrifuge tubes**. Care should be taken to avoid oxidation of Fe(II) ions by oxygen in the atmosphere, either by performing the steps quickly under a stream of N<sub>2</sub> gas right after sampling or by performing the first step of this assay inside an anaerobic chamber.

This assay has a limited detection range and therefore dilutions of the sample may be required. Refer to the Guideline section to understand the requirements.

- 1 Measure absorbance  $A_1$ :  $A_1$  is measured *immediately* after adding **120 µl** of *reagent A* to **1.2 ml** of filtered/filtered-diluted samples or undiluted standard solutions. Mix the mixture well with the pipette by gently pipetting up and down a few times. Load three **200 µl** mixtures onto a 96-well plate to make triplicate measurements on the spectrophotometer.
- 2 Reduction and measure absorbance  $A_2$ : collect **640 µl** of the remaining mixture after step 1, then add **120 µl** of *reagent B*. Mix with the pipette and begin the timer. The solution is allowed to react for **00:10:00** to complete the reduction from Fe(III) to Fe(II). At the 10 min mark, add **40 µl** *reagent C*, mix with the pipette and *immediately* measure  $A_2$  by loading triplicate **200 µl** mixtures on the well plate.
- 3 Steps 2 is repeated once only for the standards, from which  $A_3$  is measured and  $\alpha$  calculated. In the procedure above  $\alpha$  should be close to 0.8. The goal of executing this step is to obtain a realistic  $\alpha$  together with estimates of its uncertainty during the assay procedure.



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