5.2. Expert Experimentalist Rating: *"A Heart as Strong as Iron"*

Techniques Checklist:

- Use of a centrifuge ❑

Equipment:

- Disposable UV-Vis cuvettes (1-mL capacity)
- Pipetmen: 20 P, 100 P, 1000 P
- Pipet tips
- Eppendorf tubes (safe-lock)
- Hot plate
- Centrifuge
- Boiling plate or rack to hold Eppendorf tubes
- Large crystallizing dish

Goals:

• From the CC-level experiment, you know the concentration of protein in your sample. Now you will determine the concentration of iron in bovine heart cytochrome c.

Experiment Outline:

The Ferrozine Assay

Ferrozine is an iron-chelating agent. When it forms a complex with ferrous iron (Fe^{II}), it shows a characteristic UV-Vis absorption at 562 nm. By comparing the A_{562} of your sample to a calibration curve of iron standards, you will determine the concentration of iron in your protein sample.

Solutions provided by your TA:

-Fe AA standard (AA = atomic absorption)

-Buffer - 25 mM MOPS, pH 7

-Ultrapure HNO_3 (5 M)

-75 mM Ascorbic acid solution

-10 mM Ferrozine solution

-Saturated ammonium acetate solution

1. Preparation of Standards:

• Prepare a fresh set of iron standards in 2 mL Eppendorf tubes, as illustrated below. Carefully label each tube. Also fill 2 tubes with 300 μL of your protein sample.

μL of Fe AA standard (99 μg/mL)	μL of Buffer to add
0	300
6	294
12	288
18	282
24	276

• Add 30 μL of ultrapure HNO_3 (5 M) to each standard and sample tube.

• Place the closed Eppendorf tubes in a rack, and boil them for 30 minutes in a hot water bath (a large Pyrex dish over a heating plate).

• Centrifuge for 1-2 minutes, making sure the centrifuge is properly balanced.

• Remove 300 μL of the supernatant liquid from each tube, and transfer to fresh tubes (labeled!).

• Add 1020 μL of distilled water.

• Add 60 μL of 75 mM ascorbic acid.

• Add 60 μL of 10 mM ferrozine.

• Add 60 μL of saturated ammonium acetate.

• Shake each tube and wait 10-15 minutes, (the solutions should become purplish in color).

• Transfer to a 1.5 mL cuvette, and determine the A_{562} for each standard and your two samples.

• Generate a calibration curve of A_{562} *vs.* [Fe] from your standards.

• Determine the [Fe] in your unknown.

Results

• To obtain your "EE Rating" in Protein Assays and Error Analysis, the line fit for your standard curve must have a 0.995 correlation coefficient or higher. Additionally, the absorbance values for your unknown samples must have a standard deviation of 0.035 or less. Finally, you must determine the number of molecules of iron per molecule of protein.