Dec 05, 2025

Quantitative Root Ferric Reductase Activity Assay with *Arabidopsis thaliana* Seedlings

DOI
https://dx.doi.org/10.17504/protocols.io.5qpvod5y9g4o/v1

Monique Eutebach[1], Enya Becker[1], Petra Bauer[1]

[1]Institute of Botany, Heinrich Heine University, Universitätsstraße. 1, 40225 Düsseldorf, Germany

Petra Bauer

DOI: https://dx.doi.org/10.17504/protocols.io.5qpvod5y9g4o/v1

Protocol Citation: Monique Eutebach, Enya Becker, Petra Bauer 2025. Quantitative Root Ferric Reductase Activity Assay with Arabidopsis thaliana Seedlings. **protocols.io** https://dx.doi.org/10.17504/protocols.io.5qpvod5y9g4o/v1

Protocol status: Working

Created: July 14, 2025

Last Modified: December 05, 2025

Protocol Integer ID: 222416

Keywords: Quantitative Root Ferric Reductase activity, Arabidopsis thaliana, Arabidopsis thaliana, root ferric reductase activity, iron uptake, iron deficiency, ferrozine, FeNaEDTA, root length normalization, ImageJ, enzymatic activity measurement, Fe(III) reduction, seedling root system., quantitative root ferric reductase activity assay, quantitative differences in ferric reductase activity, ferric reductase activities among genotype, ferric reductase activity, ferric reductase oxidase, ferric reductase oxidases such as fro2, fe reductase solution, ferric reductase solution, iron reduction strategy at the root surface, molecular mechanisms of iron reduction, physiological responses to iron limitation, observations of iron reduction, bioavailable ferrous form, optical density of fe reductase solution, iron reduction, iron reduction strategy, ferric iron, efficient iron acquisition, arabidopsis thaliana, iron limitation, iron, arabidopsis thaliana seedlings background, fe, essential for plant development, specific root

Abstract

Background:
Efficient iron acquisition is essential for plant development and metabolism. However, in most soils, ferric iron (Fe^{3+}) is poorly soluble and therefore scarcely available to plants. To overcome this limitation, *Arabidopsis thaliana* and other non-grass species employ an iron reduction strategy at the root surface, mediated by ferric reductase oxidases such as FRO2. This enzymatic system converts Fe^{3+} to its bioavailable ferrous form (Fe^{2+}), enabling subsequent uptake through specific root transporters.

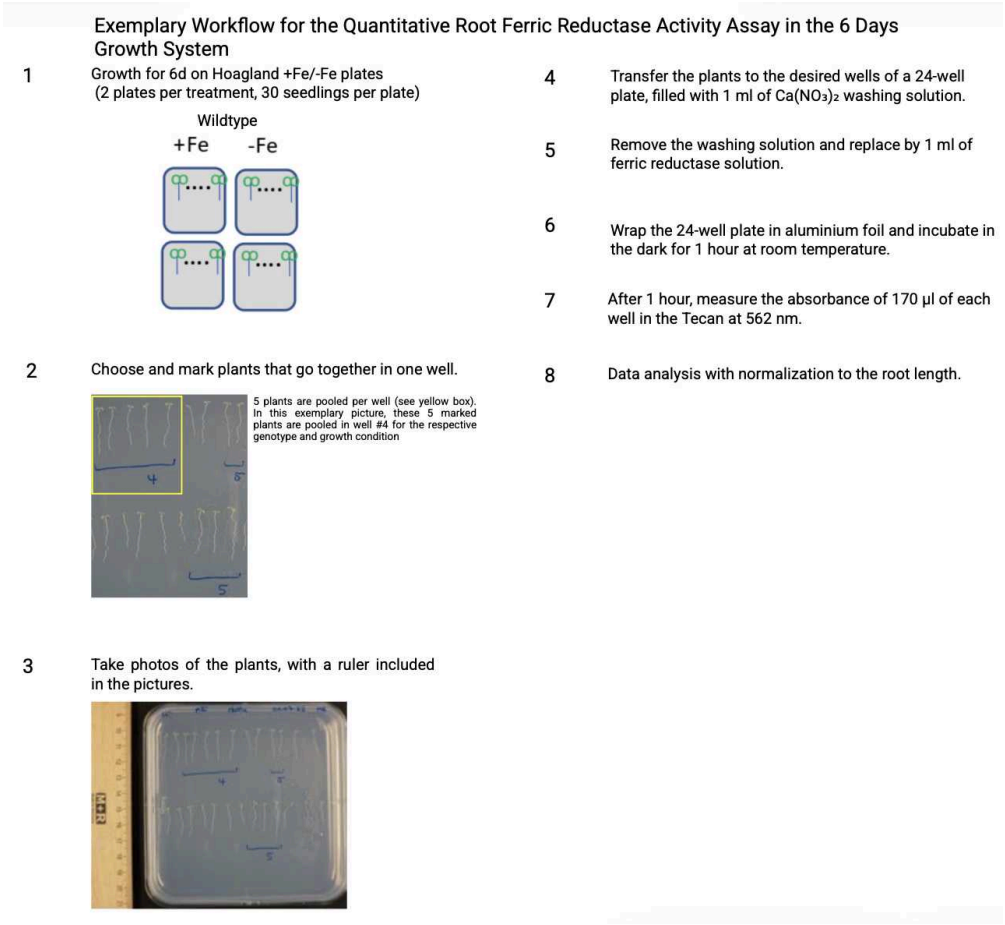
Open question:
While the molecular mechanisms of iron reduction are well described, quantitative differences in ferric reductase activity between genotypes and under different nutritional conditions are less systematically characterized. Moreover, methodological consistency is crucial to ensure the reproducibility and comparability of enzymatic activity data across experiments.

Aim and workplan:
This assay aims to quantitatively assess root ferric reductase activity in *Arabidopsis thaliana* under iron-sufficient (+Fe) and iron-deficient (–Fe) conditions. Two agar growth systems are applied: the 6-day system, where seedlings are grown directly on +Fe or –Fe Hoagland medium agar plates, and the 14 + 3-day system, where plants are grown for 14 days on +Fe Hoagland medium agar plates before being transferred to +Fe or –Fe Hoagland medium agar plates for three additional days.

Work steps:
Seedlings are transferred into a ferric reductase solution containing FeNaEDTA and ferrozine. After incubation in the dark for one hour, the amount of Fe^{2+}–ferrozine complex formed is quantified. For that, the optical density of Fe reductase solution is spectrophotometrically (at 562 nm) analyzed using a microplate reader. Enzymatic activity is calculated and normalized to root length (6 days) or root weight (14 + 3 days) giving numbers on the amount of Fe^{2+} production per root unit and time. Data are analyzed to compare ferric reductase activities among genotypes and treatment conditions.

Perspectives and conclusion:
This quantitative assay provides a reproducible and scalable method to measure root ferric reductase activity and complements qualitative observations of iron reduction. The combined application of the 6-day and 14 + 3-day systems enables the comparison of developmental and physiological responses to iron limitation. Ultimately, this approach supports the standardized documentation of experimental protocols on platforms such as *protocols.io*, contributing to reproducible plant science and improved data transparency.

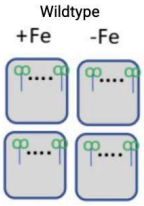
Guidelines



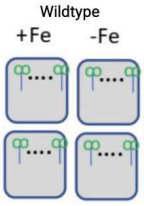
Exemplary workflow for the quantitative root ferric reductase activity assay in the 6 days growth system (illustration created with BioRender.com).

Exemplary Workflow for the Ferric Reductase Activity Assay in the 6 Days Growth System

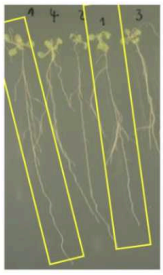
1 Growth for 14d on Hoagland +Fe plates (2 plates per treatment, 15 seedlings per plate)



2 Transfer to fresh Hoagland +Fe/ -Fe plates. (2 plates per treatment, 10-12 seedlings per plate). Growth for another 3 days.



3 Choose and mark plants that go together in one well.



2 plants are pooled per well (see yellow boxes). In this exemplary picture, these 2 marked plants are pooled in well #1 for the respective genotype and growth condition

4 Take photos of the plants, with a ruler included in the pictures.

5 Transfer the plants to the desired wells of a 24-well plate, filled with 2 ml of $Ca(NO_3)_2$ washing solution.

6 Remove the washing solution and replace by 1.5 ml of ferric reductase solution.

7 Wrap the 24-well plate in aluminium foil and incubate in the dark for 1 hour at room temperature.

8 After 1 hour, measure the absorbance of 170 µl of each well in the Tecan at 562 nm.

9 Measure the root weight of each pooled plants per well.

Materials

• Intact plants,e.g. *Arabidopsis thaliana* plants exposed to + and -Fe (Please note that the assay is performed with living and intact plants), e.g. plants raised as described in Elke Wieneke, Enya Becker, Petra Bauer 2025. Plant Growth on plant medium agar plates for iron deficiency response assays. **protocols.io** https://dx.doi.org/10.17504/protocols.io.6qpvrw84blmk/v1

• 100 ml 0.1 M $Ca(NO_3)_2$ washing solution to remove apoplectic Fe, exact quantity depending on the experiment size

• 100 ml Ferric reductase solution, containing 100 µM FeNaEDTA and 300 µM ferrozine (final concentrations). Stock solutions: 50 mM ferrozine (Keep in a falcon tube that is wrapped in aluminium foil (light-sensitive solution) in a cold room) and 10 mM FeNaEDTA. Exact quantity depending on the experiment size.

• 24-well plate(s), exact quantity of wells depending on the experiment size. Plates can be rinsed thoroughly and be re-used after the experiment.
• plastic forceps (iron-free, not metal/steel forceps)
• A 96-well plate for photometric assessment. More specific: "Microplate 96-well, PS, Half area, clear" (Greiner Bio-One GmbH, Frickenhausen, Germany). Plates can be rinsed thoroughly and be re-used after the experiment.
• Microplate Reader (e.g. Tecan, Microplate Reader, Tecan Group Ltd., Männedorf, Switzerland)
• A ruler, which is included in the photos to determine the root lengths of analyzed plants (needed for normalization to root length in case of 6-day seedlings)
• A camera/ photo station
• In case of performing the assay with 14+3 day old plants: Scalpel to separate the roots from shoots. Fine scale to determine the root weights of analyzed plants (needed for normalization to root weight in case of 14+3 day or older plants)

Troubleshooting

Problem
No difference in Fe reductase activity between +and -Fe plants
Solution
Check that the ambient temperature is below 24°C. Check that plants are exposed in light prior to the assay.

Safety warnings

- **Protect all solutions and samples from light.** Ferrozine and Fe^{2+}–ferrozine complexes are light-sensitive. Cover tubes or plates with aluminum foil during incubation.
- **Avoid metal contamination** by using only plastic or acid-washed glassware and freshly prepared solutions.
- **Keep incubation time constant** (e.g., 1 h) for all samples to ensure comparability.
- **Perform the assay at room temperature** (approx. 21 °C), as enzymatic activity is temperature-dependent.
- **Always include and subtract a reagent blank** (without roots) to correct for background Fe^{3+} reduction.
- **Ferrozine is harmful. Avoid contact with skin and eyes**, and dispose of all waste according to institutional safety regulations.

Before start

Preparations in advance:
Grow your plants in the desired system:

- 6 days vertical growth on Hoagland +Fe plates (50 µM final concentration of 10 mM FeNaEDTA stock solution) or Hoagland -Fe medium agar plates (no FeNaEDTA is added)
- 14+3 days system: Plants are grown for 14 days vertically on Hoagland +Fe plates, then all plants are transferred to fresh +Fe / -Fe Hoagland medium agar plates and grown vertically for three additional days.

Think of proper controls!
Tips:
For the 6 day system, it is recommended to use two 12 cm square agar plates per genotype and growth condition, each containing 30 seedlings. This ensures you have sufficient backup in case of loss or variability.
For the 14+3 day system, aim to end up with one to two 12 cm square plates per genotype and condition, each with 10 to 12 healthy seedlings.
Please note: to achieve this, you will need to start with more seedlings on the +Fe plates, as not all may germinate or grow properly. For example, some might fail to emerge or grow into the agar.

Note that for the assays, plants have to be pooled, e.g. 5 plants per sample for the 6-day seedlings or 2 plants per sample for the 14+3 day seedlings.

Ferric Reductase Activity Assay

1 **Prepare the 0.1 M $Ca(NO_3)_2$ washing solution**
Calculate beforehand how much 0.1 M $Ca(NO_3)_2$ washing solution you'll need:
For 6d old seedlings: 5 wells per genotype per growth condition, with 1 ml of 0.1 M $Ca(NO_3)_2$ washing solution per well (e.g., 10 wells = 10 ml in total for one genotype under +Fe and –Fe conditions)

For 14+3 d old seedlings: 5 wells per genotype per growth condition, with 2 ml of 0.1 M $Ca(NO_3)_2$ washing solution per well. (e.g., 10 wells = 20 ml in total for one genotype under +Fe and –Fe conditions)

Dissolve the calculated amount of $Ca(NO_3)_2$ in millipore H_2O and keep in a bottle.
Always prepare the 0.1 M $Ca(NO_3)_2$ washing solution freshly on the day of the assay.

2 **Prepare the ferric reductase solution**
Calculate beforehand how much solution you'll need:
For 6d old seedlings: 5 wells per genotype per growth condition, with 1 ml of ferric reductase solution per well (e.g., 10 wells = 10 ml in total for one genotype under +Fe and –Fe conditions)

For 14+3 d old seedlings: 5 wells per genotype per growth condition, with 1.5 ml of ferric reductase solution per well. (e.g., 10 wells = 15 ml in total for one genotype under +Fe and –Fe conditions)

3 **Prepare the 24-well plate(s)**
label the setup of plate(s) and pipet the 0.1 M $Ca(NO_3)_2$ washing solution into the wells. Keep a lid on the plates.

4 **Select and label plants to be used in the assay**
Several plants are to be incubated in the same well. Check your plates and select and mark equally grown plants that are to be analyzed together in one well: Don't select plants that have grown into the agar or have growth deficits.

For 6d old plants: select 5 plants for one well X 5 wells per genotype/growth condition (5 biological replicates).
For 14+3d old plants: select 2 plants for one well X 5 wells per genotype/growth condition (5 biological replicates).

5 **Take photos of the plates**
Use a photo station and place a ruler next to the plate. Make sure to have the whole plate in the focus. Take photo in manual mode.

6 **Transfer plants to washing solution**
After taking photos, transfer the selected and marked plants to the 0.1 M $Ca(NO_3)_2$ washing solution inside the desired wells. Use plastic forceps for the transfer. Make sure that all plants are covered with 0.1 M $Ca(NO_3)_2$ washing solution and that no root is sticking to the dry wall of the 24-well plate. You can gently shake the plate and/or use the plastic forceps and gently put the roots into the solution. Be careful to not squeeze the plants too much to avoid stress.

7 **Remove 0.1 M $Ca(NO_3)_2$ washing solution**
When all plants are transferred, remove the 0.1 M $Ca(NO_3)_2$ washing solution thoroughly with a 1 ml pipet. Clean the pipet tips with water in between.

8 **Fill the wells with ferric reductase solution**
Replace with ferric reductase solution

For 6d old seedlings: 1 ml per well of ferric reductase solution
For 14+3d old plants: 1.5 ml per well of ferric reductase solution

Make sure that all plants are covered with ferric reductase solution and that no root is sticking to the dry wall of the 24-well plate.
Include one well for the blank, which is ferric reductase solution only.

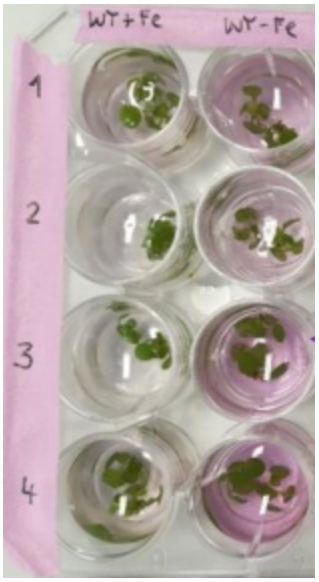
9 **Incubate in the dark for 1 hour at room temperature**
Wrap the 24-well plate(s) if needed or incubate in the dark (e.g. in a drawer) for 1 hour at room temperature (20°C). It is best to start the incubation in the ferric reductase solution around 11 AM.

Note that darkness is needed to avoid light-triggered Fe reduction in the assay solution. The prepared Fe reductase assay solution is to be kept in the dark until usage. For else, the plants themselves mobilize and reduce more Fe when they are exposed in the light!

Note that plants display high Fe reductase activity during the day, while it is low at night (Trofimov et al. 2024). High temperature strongly affects the outcome. Make sure the environmental temperature is not above 24°C.

10 **After 1 hour incubation, unwrap your plate**

Gently shake the plate to mix the ferric reductase solution in the wells.
In -Fe samples, you should observe pink/magenta staining of the solution.



Exemplary picture: *Arabidopsis thaliana* wild-type seedlings after 1 hour of incubation in ferric reductase solution in the 14 + 3 days growth system. Each well contains 1.5 ml of ferric reductase solution and 2 plants. A clear colour shift of the solution to pink/magenta is visible for seedlings grown under –Fe conditions, indicating increased ferric reductase activity.

11 **Transfer solution and measure absorbance**

Transfer 170 µl ferric reductase solution of each well to a half-area 96-well plate. Clean pipet tips with water for every well.
To guarantee good mixing you should additionally pipet the solution in the 24-well plate up and down several times before transferring to the 96-well plate.
Also pipet 170 µl of the Blank to the 96-well plate.

11.1 Measure the OD of the solution at 562 nm in the microplate reader .

11.2 After the measurement, rinse the 96-well plate thoroughly with distilled water, it can be re-used.
6d old seedlings can be discarded.

14+3d old seedlings have to be kept to determine root weights. Only afterwards they can be discarded.
24-well plates can be rinsed thoroughly with distilled water and be re-used.

Determining Root Lengths of 6-d Seedlings

12 Determine the root lengths of the plants from the photos using image analysis software (e.g., ImageJ)

Determining Root Weights of 14+3 d Seedlings

13 Separate the roots from shoots with a scalpel, e.g., on an agar plate, drain off excess ferric reductase solution on filter paper and weigh the roots per well with a fine scale. Note the values.

After the Assay: Data Analysis and calculation of Root ferric Reductase activity

14 The ferric reductase activity is calculated using the Lambert-Beer law.
The calculation happens in consecutive steps:

14.1 a) Determine the concentration of Fe^{2+} in the ferric reductase solution using the OD values and the molar extinction coefficient of ferrozine-Fe^{2+} (ε = 28.6 mmol L^{-1} cm^{-1})
b) Calculate the moles of Fe^{2+} in the 1 ml / 1.5 ml reaction volume
c) Normalize the amount of Fe^{2+} in the 1 ml / 1.5 ml reaction volume to the root lengths/weights of all plants in the sample (per one hour)
This will result in the moles of Fe^{2+} generated in the reaction volume per root unit per time unit, e.g. 1 hour → you will finally have determined the enzymatic activity in number of Fe2+ produced per root unit (weight or length) and per time unit.
Typically, the root Fe reductase activity should be in the nanomolar range for seedlings.

Exemplary calculations for the 6d and 14+3d growth system

Quantitative Ferric Reductase Activ... 30.1KB

Protocol references

Hornbergs J, Montag K, Loschwitz J, Mohr I, Poschmann G, Schnake A, Gratz R, Brumbarova T, Eutebach M, Angrand K, Fink-Straube C, Stühler K, Zeier J, Hartmann L, Strodel B, Ivanov R, Bauer P. SEC14-GOLD protein PATELLIN2 binds IRON-REGULATED TRANSPORTER1 linking root iron uptake to vitamin E. Plant Physiol. 2023 May 2;192(1):504-526. doi: 10.1093/plphys/kiac563. PMID: 36493393; PMCID: PMC10152663.

Trofimov K, Gratz R, Ivanov R, Stahl Y, Bauer P, Brumbarova T. FER-like iron deficiency-induced transcription factor (FIT) accumulates in nuclear condensates. J Cell Biol. 2024 Apr 1;223(4):e202311048. doi: 10.1083/jcb.202311048. Epub 2024 Feb 23. PMID: 38393070; PMCID: PMC10890924.

Original description of ferrozine-based plant Fe reduction assay:
Yi Y, Guerinot ML. Genetic evidence that induction of root Fe(III) chelate reductase activity is necessary for iron uptake under iron deficiency. Plant J. 1996 Nov;10(5):835-44. doi: 10.1046/j.1365-313x.1996.10050835.x. PMID: 8953245.

Original description of a plant Fe reduction assay:
Chaney RL, Brown JC, Tiffin LO. Obligatory reduction of ferric chelates in iron uptake by soybeans. Plant Physiol. 1972 Aug;50(2):208-13. doi: 10.1104/pp.50.2.208. PMID: 16658143; PMCID: PMC366111.

Acknowledgements

We thank all lab members who tested the protocol and contributed to its improvement over the years.