# Quantification of Total and Soluble Inorganic Phosphate

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**[Abstract]** A simple, rapid, and sensitive colorimetric microassay for inorganic phosphate (Pi) relies upon the absorption at 660 nm of a molybdenum blue complex that forms upon reduction of an ammonium molybdate-Pi complex in acid. The method for determination of total Pi uses plant tissues that have been ashed at 500 °C, whereas quantification of soluble Pi is performed with tissues extracted under mild acid conditions (which preserves acid-labile phosphate ester bonds).

## Materials and Reagents

1. Plant tissues
2. 17.5 M Glacial acetic acid
3. 12 M Concentrated HCl
4. 16 M Concentrated $HNO_3$
5. Sodium phosphate, monobasic ($NaH_2PO_4$) (Bioshop, catalog number: SPM400)
6. Ascorbic acid (Bioshop, catalog number: AS0704)
7. Ammonium molybdate (Bioshop, catalog number: AMN333)
8. Zinc acetate (Sigma-Aldrich, catalog number: Z0625)
9. Quartz crucibles (Thermo Fisher Scientific, catalog number: 08-072 series)
10. Acid extraction solution (see Recipes)
11. Pi stock (for standard curve) (see Recipes)
12. Pi assay reagent (see Recipes)

## Equipment

1. Crucible
2. Drying oven
3. Repeat pipetor
4. Isotemp Muffle Furnace (Thermo Fisher Scientific, model: 10-650-14)
5. Microcentrifuge
6. A computer supported microplate spectrophotometer (*e.g.*, Spectromax Plus, Molecular Devices, Sunnyvale, CA, U.S.A.)

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## Procedure

A. Total Pi (Hurley *et al*., 2010)

1. Acid wash crucibles by incubating for at least 1 h in 0.1 N HCl at room temperature, then rinse with $dH_2O$ and dry.
2. Pre-weigh crucibles and place at least 60 mg (fresh weight) of tissue in each.
3. Dry in oven at 50-80 °C for at least 16 h (*e.g.,* overnight), and then record tissue's dry weight (mg) in each crucible.
4. Ash the tissue in the furnace using a temperature ramp program (20 min at 150 °C, 1 h at 250 °C, and 3 h at 500 °C).
5. Weigh crucible and ash. Add 25 µl of acid extraction solution per mg of ash, mix well, and centrifuge at 11,000 *x g* for 10 min.
6. Dilute the supernatant 50-fold in $dH_2O$.
7. Assay Pi using the Drueckes *et al*. (1995) protocol as modified for plant tissues (Bozzo *et al*., 2006) by preparing a standard curve over the range 1-133 nmol of Pi using the following template.

| Well # | Vol of Pi stock (3.3 mM) (µl) | Volume of $dH_2O$ (µl) | Amount of Pi added (nmol) |
|---|---|---|---|
| 1A | 0 | 40 | 0 |
| 1B | 2 | 38 | 6.6 |
| 1C | 4 | 36 | 13.2 |
| 1D | 8 | 32 | 26.4 |
| 1E | 12 | 28 | 39.6 |
| 1F | 16 | 24 | 52.8 |
| 1G | 20 | 20 | 66.0 |
| 1H | 24 | 16 | 79.2 |
| 2A | 30 | 10 | 99.9 |
| 2B | 35 | 5 | 116.6 |
| 2C | 40 | 0 | 133.2 |

a. Pipette 1-40 µl of unknown(s) into adjacent wells(s). Add $dH_2O$ to bring each well to 40 µl final volume.
b. Add 200 µl of Pi assay reagent to each well using a repeat pipetor.
c. Incubate at 37 °C for 30 min.
d. Measure $A_{660}$ values and use the Pi calibration (standard) curve to determine Pi content of unknowns.
e. Express the data as: nmol Pi $mg^{-1}$ dry weight.

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B. Soluble Pi (Bozzo *et al*., 2006)
   1. Extract snap-frozen tissues (1:5, w/v) with 1% (v/v) glacial acetic acid.
   2. Centrifuge samples at 11,000 *x g* for 10 min.
   3. Assay the supernatant for Pi as described above.
   4. Esterified-Pi is calculated from the difference between total and free Pi concentrations.

## Recipes

1. Acid extraction solution
   30 ml 12 M concentrated HCl
   10 ml 16 M concentrated $HNO_3$
   60 ml $dH_2O$
2. Pi stock (for standard curve)
   3.3 mM $NaH_2PO_4$
3. Pi assay reagent
   a. Ammonium molybdate reagent
   b. Ammonium molybdate is added to an aqueous solution of 15 mM zinc acetate to give a 10 mM solution of molybdate. The solution is then adjusted to pH 5.0 with HCl. This solution is stored at 4 °C in the dark and is stable for several months.
   c. Reducing reagent
      A 10% (w/v) solution of ascorbic acid is adjusted to pH 5.0 with NaOH.
      *Note: This solution must be prepared fresh daily.*
   d. The Pi assay reagent is prepared by mixing one part of the ammonium molybdate reagent with four parts of the reducing reagent (prepare fresh daily).

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