Heberle Laboratory

Department of Chemistry, University of Tennessee, Knoxville

STANDARD OPERATING PROCEDURE: PHOSPHATE ASSAY FOR PHOSPHOLIPID QUANTITATION

Title	Determination of Phospholipid Concentration by Inorganic Phosphate Assay
Procedure Reference	SOP-PIASSAY-001
Principal Investigator	Bhavya Sharma
Prepared by	Frederick Heberle
Revision Date	July 2026
Supersedes	None (new SOP)

1. Purpose and Scope

This SOP describes the determination of phospholipid stock concentration by wet-ashing followed by colorimetric quantitation of released inorganic phosphate. The method (Bartlett, 1959, with subsequent refinements) allows accurate determination of phospholipid concentration independent of the specific headgroup, provided the phospholipid contains a known number of phosphate groups per molecule. For essentially all phospholipids routinely used in this lab (PC, PE, PS, PG, PI, PA, and sphingomyelin), the ratio is one phosphate per lipid; cardiolipin is an exception at two phosphates per lipid.
Scope: quantitation of lipid stocks in chloroform, dispensed in per-tube volumes of approximately 0.5–5 µL. The working range of the standard curve (0–60 nmol phosphate) comfortably brackets these samples. Samples in aqueous buffer are within scope, provided the buffer contains no phosphate. Non-lipid samples and lipid samples in phosphate-containing buffer are outside the scope of this SOP.

2. Summary of Hazards and Controls

Hazard	Source	Controls
Corrosive; eye and skin burn	10% sulfuric acid (H_2SO_4) added to each ashing tube	Handle in the fume hood. Safety glasses, lab coat, nitrile gloves. Dispense with a pipette or repeat dispenser directly into tubes; do not pour.
Strong oxidizer; chemical burn; sputtering	30% hydrogen peroxide (H_2O_2) added to hot ashing tubes	Handle in the fume hood. Face shield or splash-rated safety glasses when dispensing into hot tubes. Nitrile gloves. Do not allow contact with metals, organic combustibles, or reducing agents. Store per SDS.
Fire / ignition	30% H_2O_2 in contact with organic combustibles (paper towels, sawdust, kimwipes)	Never absorb H_2O_2 spills with paper towels or kimwipes. Use vermiculite or an aqueous-compatible spill pillow.
Toxic if ingested; irritant	5% ammonium molybdate stock (component of the color reagent)	Standard chemical hygiene: gloves, no eating/drinking, wash hands after handling. Do not aerosolize.
Thermal burn	200 °C heat block; hot glass test tubes	Use a wire (metal) test tube rack — not plastic — to transfer hot tubes. Wait for tubes to cool before further handling. Do not touch tube bottoms when holding the rack.
Sputtering /	Peroxide addition to hot	Face shield or splash-rated safety glasses. Do not lean

Hazard	Source	Controls
splash	ashing tubes	over tubes when dispensing. Sputtering is expected and is not a fault, but does mean droplets may fly.
Sharps	Broken glass from failed test tubes	Inspect tubes before use; discard any with chipped rims or cracks. Broken glass goes to the sharps container.
Chloroform exposure	Chloroform used to fill and clean the dispensing syringe when transferring lipid	Governed by SOP-CHCl3-001. Handle in the fume hood, double nitrile gloves, halogenated waste stream.

3. Equipment and Reagents

3.1 Equipment

- Chemical fume hood (Buehler 302 or 303)
- Techne Dri-Block DB-3D heat block (range: ambient to 200 °C)
- Lauda-Brinkmann ECO RE 2025 G circulating bath, operated at 45 °C for this assay
- Vortex mixer
- Mettler Toledo UV5 UV/Vis spectrophotometer, with an aspirator system on the bench next to the instrument for cuvette handling
- 25 µL Hamilton syringe with PB600 repeating dispenser (“clicker”)
- 13 × 100 mm disposable borosilicate glass test tubes
- Wire (metal) test tube rack for cooling hot tubes
- Standard plastic test tube rack for room-temperature work
- Aluminum foil (for optional overnight covering of ashed tubes)
- Vacuum line at the spectrophotometer bench (for cuvette rinsing)
- UV/Vis-transparent sample cuvette (quartz or equivalent)
- Kimwipes

3.2 Reagents

- 10% (v/v) sulfuric acid (H_2SO_4) in water
- 30% hydrogen peroxide (H_2O_2), highest available purity — the assay’s blank absorbance is dominated by trace phosphate in the peroxide, so reagent quality matters
- 5% (w/v) ammonium molybdate in water — will develop a white precipitate on storage; the supernatant remains usable if the precipitate is not disturbed
- Ascorbic acid, solid
- Standard inorganic phosphate solution: 5.805 mM KH_2PO_4 in water
- Ultrapure (Milli-Q) water
- Ethanol
- Chloroform (for syringe cleaning; handled per SOP-CHCl3-001)

4. PPE

- Lab coat
- Safety glasses (splash-rated) or face shield when dispensing peroxide into hot tubes
- Chemical-resistant nitrile gloves. Double-glove for chloroform handling per SOP-CHCl3-001, and when handling concentrated peroxide or acid.

5. Procedure

The full assay takes approximately three hours of active work spread over about four hours total. A safe stop point exists after the ashing/peroxide-decomposition step (§5.4).

5.1 Reagent and Bench Preparation

- Confirm availability of all reagents. Inspect the ammonium molybdate stock — the supernatant should be clear; if the solution has darkened or contains dispersed fine particulate, prepare fresh (see §6).
- Set out the 10% H_2SO_4, 30% H_2O_2, and phosphate standard in the fume hood.
- Verify the fume hood sash is at working height and airflow is normal.

5.2 Sample Tube Setup

Prepare the standard curve and sample tubes in disposable 13 × 100 mm glass test tubes. Inspect each tube for cracks or chipped glass and discard any damaged tubes.

Standard curve. Prepare a series covering approximately 0–60 nmol phosphate. Include at least two blanks (0 nmol) for the zero point. A representative setup using the 5.805 mM phosphate standard:

Volume of 5.805 mM standard (µL)	nmol phosphate
0	0.0
0	0.0
2	10.2
4	20.4
5	25.5
6	30.6
8	40.7
10	50.9
12	61.1

Note on units. The 25 µL Hamilton syringe delivers 50 clicks per full stroke, so 1 click = 0.5 µL. With a nominally 5 mM phosphate standard, that corresponds to approximately 2.5 nmol phosphate per click (10 clicks = 5 µL ≈ 25 nmol Pi). Lab members typically move fluently between all three units — clicks, µL, and nmol phosphate — when setting up standard curves and dispensing samples.

Samples. For each lipid stock to be assayed, prepare 5–10 replicate tubes (5 minimum; ~10 needed for the 1% SD typical of a well-run assay). Sample volume per tube depends on the expected concentration:

- ~25 mg/mL stock: dispense 1.5–2.0 µL per tube
- ~10 mg/mL stock: dispense 3–4 µL per tube
- Unknown concentration: dispense a range (e.g., 0.5, 1, 2, 3, 5 µL) to ensure at least one point falls within the calibrated range

Chloroform aliquots ≤20 µL evaporate within a few minutes at room temperature. Larger organic-solvent volumes should be dried under a gentle N_2 stream before ashing.

5.3 Ashing (in the fume hood)

All steps in this section are performed in a chemical fume hood.

1. Add 200 µL of 10% H_2SO_4 to each tube, dispensing straight down to the bottom of the tube (not down the sides).
2. Transfer the tube rack into the 200 °C heat block positioned in the fume hood. Distribute tubes randomly across the block so that any heat gradient across the block averages across the sample set rather than concentrating in one region.
3. Heat for **1 hour**.
4. Without removing the tubes from the block, add 20 µL of 30% H_2O_2 to each tube, dispensing straight down. **Sputtering is expected** and is not a cause for concern; keep the face shield in place and do not lean over the tubes while dispensing.
5. Heat for **40 minutes** after the peroxide addition.
6. Inspect each tube. Any tube still showing brown, black, or grey color indicates incomplete oxidation of organic material. If any tube shows residual color, add another 20 µL of 30% H_2O_2 to **every** tube in the run (to keep treatment uniform across the sample set) and heat for another 40 minutes. Repeat as needed.

Notes on peroxide usage:

- For typical phospholipid samples, a single 20 µL H_2O_2 addition is sufficient. Multiple additions are more often needed when the sample tube contains a large volume of aqueous buffer alongside the lipid.
- Total peroxide additions above ~150 µL noticeably raise the blank absorbance (roughly 0.1 absorbance units per additional 150 µL). This threshold is rarely reached in routine use.

5.4 Cooling and Optional Stop Point

- After the final 40-minute post-peroxide heating step, transfer the tubes from the heat block to a **wire (metal) test tube rack**. Do not use a plastic rack immediately — the tube bottoms are hot enough to soften plastic on contact.
- Cool the tubes to room temperature (~15 minutes).
- **Safe stop point:** once cooled, tubes may be covered with aluminum foil and stored on the bench for up to 1–2 days without loss of assay quality. This is a convenient stopping point if starting color development the following day.

5.5 Color Development

- Turn on the 45 °C water bath.
- Turn on the UV/Vis spectrophotometer. The lamp requires approximately 15–30 minutes to warm up before it is stable enough for use.
- Prepare fresh color reagent immediately before use. **Do not prepare in advance** — color reagent decomposes over hours once ammonium molybdate has been added.

Color reagent recipe (per 10 assay tubes; scale linearly):

Component	Volume / Amount
Milli-Q water	4.5 mL
Ascorbic acid (solid)	0.1 g
5% ammonium molybdate solution	0.5 mL (added last)
Total	**5.0 mL**

Preparation:

1. Combine the Milli-Q water and ascorbic acid in a screw-cap tube (~15 mL). Vortex to dissolve. Verify complete dissolution by holding the tube up to the light and watching for crystals settling at the bottom.
2. Add the ammonium molybdate solution last, immediately before adding the color reagent to the assay tubes.

Practical note: prepare enough color reagent for the actual number of tubes plus a small excess (~5 tubes' worth). Volumetric transfer losses mean a nominal 10 mL preparation typically yields ~9.8 mL, so slight over-preparation avoids running short.

To each cooled ashed tube:

1. Add 500 µL of Milli-Q water down the walls of the tube (to wash any material off the walls into the bottom). The pipette tip must **not** touch the tube walls. Vortex briefly.
2. Add 500 µL of color reagent, dispensed **directly down the center of the tube** — do not rinse the walls with the color reagent. Vortex immediately.

Incubate the tubes in the 45 °C water bath for **20 minutes**. **Do not cover the water bath** — condensation on the cover will drip into the samples.

Remove the tubes from the water bath, vortex, and cool to room temperature (~10–15 min) before reading absorbance. Read absorbance the same day; do not leave color-developed samples overnight.

5.6 Absorbance Measurement

Measurements are performed on the Mettler Toledo UV5, using the bench-side aspirator system for sample handling between tubes.

Workflow:

- Measure the blank (Milli-Q water) first, then the standard curve tubes in order, then samples in order.
- For each measurement: pipette the sample into the cuvette and read absorbance at **820 nm**, or take the average of A_{818} and A_{820} for improved noise performance (the standard practice inherited from the Feigenson lab). The signal is largest at the red end of the molybdate-phosphate absorption band, but slightly noisier there — hence the two-wavelength average.
- After each measurement, aspirate the sample out of the cuvette, rinse the cuvette once with Milli-Q water from a squirt bottle, aspirate the rinse, and pipette in the next sample. The aspirator feeds directly into the phosphate-assay waste container (see §7).
- **Record each absorbance value directly in the lab notebook as it is measured**, in addition to whatever the instrument stores or exports electronically. Instruments and files can fail; the notebook is the primary record.

5.7 Data Analysis

- Fit a linear standard curve of A_{820} (or the averaged $A_{818-820}$ signal) versus nmol phosphate.
- Convert each sample's absorbance to nmol phosphate using the fitted curve.
- Calculate lipid concentration:
  [lipid] (mM) = (nmol phosphate per sample) × (moles lipid per mole phosphate) / (sample volume in µL)
  For most phospholipids (PC, PE, PS, PG, PI, PA, SM), the ratio is 1 lipid : 1 phosphate. For cardiolipin, apply a factor of ½.
- Quality metrics:
  - Standard curve relative standard error (RSE): < 0.01 for good data
  - Sample replicate error: < 1% at typical concentrations; up to ~2% is acceptable for very dilute stocks (~1 mM), where the phosphate signal is inherently small

6. Special Considerations

- **Oxidiser–organic interaction at the ashing step.** The combination of 30% H_2O_2 with residual organic material (chloroform, lipid, partially-ashed carbon) at 200 °C is an oxidiser–organic interaction with potential for a vigorous exothermic reaction. This is inherent to the assay chemistry — the peroxide is doing exactly what it is meant to, oxidising residual carbon — and is controlled by the small, defined per-tube peroxide volume (20 µL) and mandatory fume hood working. The rapid decomposition also evolves oxygen while the tubes are open, which is the origin of the expected sputtering. This is a controlled pressure release, and is the reason for the face-shield requirement in §4 and the "do not lean over the tubes" instruction in §5.3.
- **Reagent storage separation.** The three reactive reagents used in this assay must be stored separately from each other and from incompatible materials:
  - 30% H_2O_2 stored away from organics, flammables, alcohols, ketones, nitric acid, and other acids or oxidisers.
  - 10% H_2SO_4 stored in an acid-proof cabinet, away from strong bases and organic materials.
  - Ammonium molybdate stock stored away from strong acids and strong oxidisers.
  - H_2SO_4 and H_2O_2 are not stored in the same cabinet.
- **Second-person requirement for the ashing step.** Per CHP §3 rule #6, work with concentrated acids or bases (≥1 M) requires a second person present in the lab or within earshot. The ashing step of this assay uses ~1.8 M H_2SO_4 and 30% H_2O_2 added to hot tubes with expected sputtering — do not perform this step alone.
- **Reagent quality is the largest determinant of assay quality.** The 30% H_2O_2 is the dominant source of the blank signal — purchase the lowest-Pi ("ultrapure" or "trace-metals grade") product available.
- **Ammonium molybdate storage.** A white precipitate forms with age. The clear supernatant remains usable indefinitely, but must be handled carefully — knocking or shaking the bottle

disperses the precipitate as a fine powder that is much harder to avoid drawing up. If the excess color reagent (after use) fails to develop the characteristic blue color within ~30 minutes, prepare fresh ammonium molybdate solution.

- **Color reagent lifetime.** Once ammonium molybdate is added, the color reagent decomposes over hours. It must be used within an hour of preparation and cannot be stored.
- **Particulate contamination during ashing is a known failure mode.** Historically, ashing runs conducted in the presence of nearby wood-cutting or other particulate-generating activity have produced entirely black tubes (assay failure). Do not conduct particulate-generating work in the same room as an active ashing run.
- **Chloroform handling.** Syringe operation for lipid dispensing is governed by SOP-CHCl3-001. At the end of use, rinse the syringe and plunger with water, then ethanol, then chloroform (in that order). Do not store the plunger inside the syringe — the plunger's Teflon tip is required to seal properly and is damaged by prolonged compression.
- **Phosphate-containing buffers cannot be assayed by this method.** If lipid stocks are in phosphate-containing buffer, the phosphate must first be removed (e.g., by extensive dialysis into a phosphate-free buffer) or an alternative assay must be used.
- **Standard curve preparation.** Standards should be taken through the entire assay procedure — including ashing and peroxide addition — even though the standards contain only inorganic phosphate. Heating and H_2O_2 affect the final absorbance, and treating standards identically to samples eliminates this as a source of systematic error.

7. Waste Disposal

- **All phosphate-assay waste** — the acidified color-developed solutions in the assay tubes after measurement, together with all aspirated cuvette rinsates — is collected in the dedicated **"phosphate assay waste"** container maintained by the lab. The aspirator system on the bench next to the UV5 feeds directly into this container.
- **Concentrated H_2O_2 and H_2SO_4 waste** (unused reagent, spills): collect in the appropriate labeled containers and dispose via EHS pickup.
- **Chloroform waste** from syringe cleaning: halogenated solvent stream, per SOP-CHCl3-001.
- **Broken glass** from failed test tubes: sharps container.

8. Equipment-Failure Response

- **Heat block failure mid-ashing.** Turn off the block. Allow tubes to cool in place before removing. Samples interrupted mid-ashing are typically not salvageable and should be discarded; document the incident and repeat the assay.
- **H_2O_2 spill.** Do not use paper towels or kimwipes for undiluted 30% H_2O_2; saturated cellulose is a fire hazard. Absorb with vermiculite or an aqueous-compatible spill pillow per Appendix F of the CHP. Notify Zidan Khan.
- **Sulfuric acid spill (10%).** Neutralize with sodium bicarbonate; absorb with vermiculite. Dispose per EHS guidance.
- **Spectrophotometer failure or software crash.** Data may be lost if not exported. As a matter of routine, export standards and samples after each measurement set rather than at the end of the session. If the software becomes unresponsive, save the current state before restarting; the reference spectrum will need to be re-measured after restart.

9. Training

Prerequisites:

- Current UTK General Lab Safety and PPE training
- Familiarity with SOP-CHCl3-001 (chloroform handling)
- Familiarity with SOP-LIPID-001 (lipid stock solutions)

In-lab training is delivered by Zidan Khan (QLD) and includes:

- Reagent preparation, with particular attention to the color reagent (most common source of assay-day errors)
- Use of the Hamilton syringe with PB600 repeating dispenser
- Ashing hood procedure with supervised H_2O_2 addition to a hot tube (technique observation before independent work)
- Spectrophotometer operation and data export
- Supervised first assay against a lipid stock of known concentration to verify technique
- Verification that the trainee can identify common failure modes: residual color after peroxide, poor curve linearity, unusually high blank absorbance

Annual review of this SOP is required; signatures collected below.

10. References

- Bartlett, G.R. (1959). Phosphorus assay in column chromatography. *J. Biol. Chem.* **234**, 466–468.
- Chen, P.S. Jr., Toribara, T.Y., & Warner, H. (1956). Microdetermination of phosphorus. *Anal. Chem.* **28**, 1756–1758.
- Morrison, W.R. (1964). A fast, simple and reliable method for the microdetermination of phosphorus in biological materials. *Anal. Biochem.* **7**, 218–224.
- Kingsley, P.B., & Feigenson, G.W. (1979). The synthesis of a perdeuterated phospholipid: 1,2-dimyristoyl-sn-glycero-3-phosphocholine-d72. *Chem. Phys. Lipids* **24**, 135–147.
- Feigenson Lab (Cornell), “Assay for Organic Phosphate” reference protocol, updated Jan 2013.
- Petruzielo, R. (Feigenson Lab), procedural notes for phosphate assay, Jul 2014.

11. Approval and Acknowledgment

Principal Investigator approval:

Name: Bhavya Sharma Signature: _____ Date: _____

Personnel acknowledgment: I have read and understand the content of this SOP and agree to adhere to its requirements.

Name (printed) / Signature	Date