

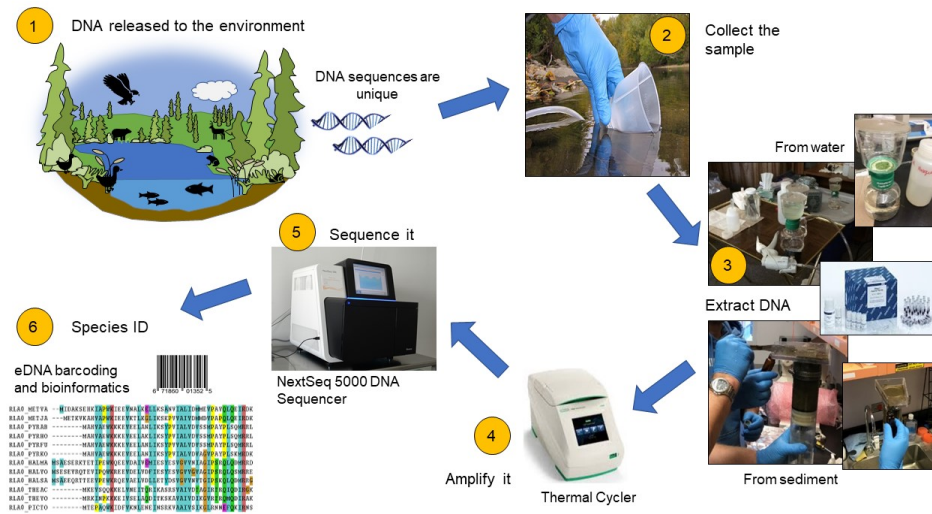
BIOLOGY AND MOLECULAR BIOLOGY

Biodiversity Surveys, DNA Extraction, Sequencing, and Bioinformatics

Environmental Analysis Program
Teaching and Research Laboratories, Seeley G Mudd
Pomona College

Marc Los Huertos

Environmental Analysis Program Coordinator



Pomona College
Claremont, California

BIOLOGY AND MOLECULAR BIOLOGY

Biology, Ecology, and Molecular Biology

Biodiversity surveys, organism sampling, DNA extraction, sequencing, and bioinformatics

EA Program – Biogeochemistry Lab, Pomona College

Compiled: June 26, 2026

SOP Number	Title
SOP-F602	iNaturalist for Biodiversity Documentation
SOP-F603	Vegetation Transect Surveys
SOP-F604	Tree DBH and Forest Inventory
SOP-F605	Leaf Area Index Measurement
SOP-F606	Canopy Cover (Densiometer and Hemispherical Photography)
SOP-F607	Tree Tagging for Restoration Monitoring
SOP-F608	Plant Tissue Sampling
SOP-F610	Benthic Macroinvertebrate Sampling
SOP-F611	Pollinator Observation Surveys
SOP-F620	Camera Trap Deployment and AI-Assisted Image Analysis
SOP-F630	Drone Operations (DJI Mavic and Matrice UAVs)
SOP-F700	eDNA Field Sampling and Preservation
SOP-F701	Soil Microbial Field Sampling for Molecular Analysis
SOP-L601	Preparing a Wet Mount for Microscopy
SOP-L602	Macroinvertebrate Sorting, Subsampling, and Identification
SOP-L603	Plant Tissue Drying, Grinding, and Preparation for Analysis
SOP-L604	Algae / Periphyton Identification and Enumeration
SOP-L605	Soil Enzyme Assays (Fluorometric Microplate Method)
SOP-L700	DNA Extraction, Quantification, and Purity Assessment
SOP-L701	Primer Selection and Design for Nitrogen Cycle Genes – ddPCR
SOP-L702	ddPCR Applications Guide – Absolute Quantification of Soil Nitrogen-Cycle Functional Genes
SOP-L702	ddPCR Data Export and Analysis – Nitrogen Cycle Gene Abundance
SOP-L703	PCR Amplification and Gel Electrophoresis
SOP-L704	Amplicon Library Preparation for Sequencing
SOP-L705	Bioinformatics Pipeline (QIIME 2 / DADA2)
SOP-L706	eDNA Metabarcoding Lab Workflow



Environmental Analysis Program

Pomona College
Biogeochemistry Laboratory

SOP-F602

iNaturalist for Biodiversity Documentation

Standard Operating Procedure

SOP Number	SOP-F602
Revision	0.3
Author(s)	Marc Los Huertos
Date	2026-06-26
Organization	EA Program, Pomona College

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1. Purpose

This SOP describes how to use iNaturalist (<https://www.inaturalist.org>) as a standardized tool for documenting biodiversity at EA Program field sites. iNaturalist is a community science platform that combines field observation, AI-assisted species identification, and expert community verification into a single workflow (ueda2024?). Observations that receive community agreement on identification reach “Research Grade” status and are automatically shared with the Global Biodiversity Information Facility (GBIF), making them part of the global scientific record (Global Biodiversity Information Facility, 2024).

Community science platforms like iNaturalist generate large volumes of occurrence data, but the scientific value of those data depends critically on the quality of individual observations, including photographic evidence, spatial accuracy, and taxonomic verification (Dickinson et al., 2010). When data quality is maintained, iNaturalist records can support large-scale biodiversity monitoring and contribute meaningfully to national and international biodiversity assessments (Callaghan et al., 2021; Chandler et al., 2017). This SOP covers making high-quality observations, creating and managing collaborative projects for EA Program research, and exporting data for analysis in R.

2. Scope

- **Applies to:** All EA Program biodiversity surveys, species inventories, and community science activities where iNaturalist is used to document plant, animal, fungal, or other organism occurrences
- **Does not apply to:** Quantitative vegetation transect surveys (SOP-F603); camera trap image processing (SOP-F620); formal specimen collection requiring voucher preparation; eDNA sampling
- **Location:** All EA Program field sites and campus

3. Responsibilities

Role	Responsibility
PI / Field Lead	Create and administer iNaturalist projects; review data quality
Project Admin	Manage project settings, curate observations, invite members
Trained Personnel	Make observations per this SOP; contribute identifications
New Students	Create an account and complete training before using iNaturalist for research data

4. Definitions

- **Observation:** A record of an encounter with an individual organism at a specific place and time, documented with at least one photo or sound recording.

- **Research Grade:** An observation that has a date, location, photo or sound, and community agreement on the identification (at least two-thirds of identifiers agree at the species level). Research Grade observations are shared with GBIF (Global Biodiversity Information Facility, 2024).
- **Needs ID:** An observation that has not yet received enough community identifications to reach Research Grade. These records are scientifically useful but carry higher taxonomic uncertainty.
- **Casual:** An observation that is missing date, location, or evidence (photo/sound), or that the community has voted as not meeting quality standards. Casual observations are excluded from most research exports.
- **Collection Project:** A project that automatically includes all observations matching defined criteria (taxa, place, users, dates, quality grade). No manual curation needed.
- **Traditional Project:** A project where observations must be added manually by members. Can require custom observation fields. Requires the creator to have at least 50 verifiable observations.
- **Umbrella Project:** A project that aggregates observations from multiple other projects. Useful for coordinating across sites or taxa.
- **Observation Field:** A custom data field that can be attached to observations (e.g., “habitat type,” “number of individuals,” “flowering status”). Only Traditional Projects can require observation fields.
- **Computer Vision (CV) Model:** The machine learning model trained on iNaturalist’s community-verified image database that suggests identifications for new observations. The model is retrained periodically and performs best for taxa and regions with large training datasets; accuracy declines for rare, under-photographed, or morphologically cryptic taxa (Ioalari2023?).
- **Taxon Geoprivacy:** An automatic system that obscures the coordinates of observations for threatened, endangered, or sensitive species to protect them from collection or disturbance.
- **Coordinate Uncertainty:** The estimated spatial accuracy of the GPS location, expressed as a radius in meters. High uncertainty (greater than 1000 m) reduces the value of occurrence records for fine-scale spatial analyses.

5. Safety and Hazards

Standard field safety applies when making observations in the field. Refer to [SOP-F001 Field Safety](#). Additional considerations specific to iNaturalist use:

- **Location privacy:** iNaturalist observations include GPS coordinates by default. Be aware that your location is visible to other users unless you obscure or hide it. Do not post observations from private residences without obscuring the location.
- **Sensitive species:** iNaturalist automatically obscures the locations of threatened and endangered species (“taxon geoprivacy”). Do not override this obscuring for sensitive taxa.
- **Disturbance:** Do not disturb, handle, or collect organisms solely for an iNaturalist photo. Photograph organisms in situ where possible.

6. Required Materials

Item	Notes
Smartphone with iNaturalist app	iOS or Android; free download
iNaturalist account	Free at https://www.inaturalist.org
Camera (optional)	For higher-quality photos; phone camera is sufficient for most uses
Field notebook	For recording additional data not captured in iNaturalist
GPS unit (optional)	Phone GPS is usually adequate

7. Procedure

7.1. Account Setup

1. Go to <https://www.inaturalist.org> or download the iNaturalist app
2. Create an account using your Claremont email address
3. In your profile settings:
 - Set your default photo license to **CC-BY** or **CC-BY-NC** (this allows your photos to be used in research with attribution)
 - Set your default observation license to **CC0** or **CC-BY** (this allows your observation data to be shared with GBIF)
 - Set your default location to “open” (you can obscure individual observations as needed)
4. Send your iNaturalist username to the project administrator so you can be added to the EA Program project

! License Settings Matter for Research

Observations with “All Rights Reserved” licenses are excluded from GBIF exports and many research datasets. For EA Program work, always use a Creative Commons license. CC-BY is recommended.

7.2. Making High-Quality Observations

A useful iNaturalist observation needs four things: a photo (or sound), a date, a location, and enough visual detail for identification. Follow these guidelines to maximize the chances of reaching Research Grade.

7.2.1. Photography Tips

1. Take multiple photos of each organism from different angles:
 - **Plants:** Whole plant habit, close-up of leaves (both sides), flowers or fruit, stem, bark (for trees)

- **Insects:** Dorsal view (top), lateral view (side), close-up of head and wing venation if possible
 - **Birds:** Best angle available; include habitat context if possible
 - **Fungi:** Top of cap, underside (gills/pores), stem, habitat context
2. Include something for scale (a finger, a coin, a ruler) when practical
 3. Photograph the organism in its natural position; do not relocate it
 4. For plants, photograph vegetative and reproductive structures together if both are present
 5. Ensure the photo is in focus and adequately lit

7.2.2. Recording the Observation

6. Open the iNaturalist app and tap the camera icon to start a new observation
7. Take photos directly in the app, or select photos from your camera roll
8. The app will suggest an identification using its computer vision

(CV) model (**loarie2023?**). Accept the suggestion if it looks correct, or refine the identification manually. When in doubt, identify to the broadest level you are confident in (e.g., “Plants” or “Oaks” rather than guessing a specific species). The CV model performs best for common, well-photographed taxa in North America and Europe; for under-represented groups (e.g., lichens, mosses, many invertebrate families), manual identification by the observer or community identifiers is essential.

9. Verify that the date and location are correct. The app captures these automatically from the photo metadata, but check that the GPS pin is accurate on the map. If coordinate uncertainty exceeds 500 m, manually reposition the pin.
10. Add any relevant notes in the “Notes” field (habitat, behavior, abundance, associated species)
11. If the observation is part of a Traditional Project, add it to the project before submitting (see Section 7.4)
12. Tap **Submit**

Sound Observations

For birds, frogs, and insects, a sound recording can be as useful as (or more useful than) a photo. The iNaturalist app can record audio directly. Hold the phone steady and minimize background noise.

7.3. Bulk Uploading Observations via CSV

For observations recorded outside the app (for example, transcribed from a field notebook, exported from another platform, or compiled in a spreadsheet during data entry), iNaturalist accepts a bulk CSV import. This is useful when you have many records to create at once and do not want to enter them one at a time. The importer creates the observation records only; it does not upload photos or sound files, so media must be attached afterward (see below).

7.3.1. Preparing the CSV

1. Build the spreadsheet with up to eight columns, in this order. Only the taxon column is mandatory.

Column	Format / example	Notes
Taxon name	text (e.g., <i>Quercus agrifolia</i>)	Mandatory. Use the scientific name for reliable matching.
Date observed	YYYY-MM-DD HH:MM	Use day-level precision or finer for research use.
Description	text	Free-text notes (habitat, behavior, abundance).
Place name	text	Optional named locality.
Latitude	decimal degrees (34.1064)	Use a decimal point, not a comma.
Longitude	decimal degrees (-117.7120)	Use a decimal point, not a comma.
Tags	tag,tag	Comma-separated tags.
Geoprivacy	open, obscured, or private	Set to obscured for sensitive taxa.

2. Keep the file under 10,000 rows. Split larger datasets into multiple files.
3. Wrap any field that contains a comma in double quotes so it is not misread as a column break.
4. Confirm that coordinates use decimal points. Spreadsheets configured for some locales export decimal commas, which the importer rejects.

7.3.2. Importing

1. Go to the import page at https://www.inaturalist.org/observations/import#csv_import (or from the upload page, click **More import options** and choose **CSV**).
2. Choose your file and upload it.
3. Open **Edit Observations** to review the created records. Use **Batch Edit** to select multiple observations, attach photos or sound files, and apply a shared location from the map.
4. Verify dates, coordinates, and identifications, then save.

CSV Imports Do Not Include Media

The CSV importer creates observation records without photos or sounds. An observation with no media cannot reach Research Grade and is treated as Casual, so it will be excluded from most research exports and from GBIF. Attach media through Batch Edit after import if the records are intended for analysis.

Large or Photo-Linked Datasets: Use the API

For large batches, or when you need to match many photos to the correct records automatically, the spreadsheet importer becomes impractical. In those cases, script the upload against the iNaturalist API (<https://api.inaturalist.org>). The **rinat** package is read-only (it retrieves data but does not create observations), so creating observations programmatically requires

direct authenticated calls, for example with `httr2` in R:

```
library(httr2)

# Requires an OAuth API token from your iNaturalist account.
# Create the observation, then POST the photo to /observation_photos
# using the returned observation id. See the API reference for the
# full request schema before running against your account.
```

Test any upload script on a handful of records first, and never use it to create observations for organisms you did not personally encounter (see Responsible Use).

7.4. Contributing Identifications

Identifying other people’s observations is as valuable as making your own. It builds community expertise, helps observations reach Research Grade, and improves the CV model.

1. Go to the **Identify** tab on the website (or use the Explore page and filter by “Needs ID”)
2. Filter by your area of expertise (taxa, location)
3. Add your identification. Agree with the existing suggestion if correct, or provide a different identification with a brief explanation.
4. Use the “Compare” tool to check similar species
5. Mark observations as “No, it’s as good as it can be” only if the evidence is genuinely insufficient for identification

7.5. Creating and Managing Projects

iNaturalist offers three project types. Choose based on your research needs:

Feature	Collection Project	Traditional Project	Umbrella Project
Observations added	Automatically (by filter criteria)	Manually by members	From member projects
Custom observation fields	No	Yes (can be required)	No
Creator requirements	Any user	50+ verifiable observations	Any user
Best for	Site-based inventories, class bioblitzes	Projects needing custom data fields	Multi-site or multi-taxon coordination
Obscured coordinate access	Yes (with Trust enabled)	Yes (with member permission)	No (via member projects)

7.5.1. Creating a Collection Project

This is the recommended type for most EA Program site inventories and class projects. Observations matching your criteria are included automatically.

1. Go to <https://www.inaturalist.org/projects/new>
2. Click **Get Started** under Collection Projects
3. Fill in the project details:
 - **Title:** Use a descriptive name (e.g., “EA Program – Bernard Field Station Biodiversity 2025”)
 - **Icon:** Upload the EA Program logo or a representative photo
 - **Banner:** Upload a site photo
 - **Description:** Explain the project’s purpose, study site, and time frame
4. Set the project requirements (filters):
 - **Place:** Select the iNaturalist Place for your study area. If one does not exist, create a custom Place by drawing a boundary on the map (Community > Places > Add a New Place).
 - **Taxa:** Leave blank to include all organisms, or restrict to specific groups (e.g., Plantae, Aves)
 - **Users:** Leave blank to include anyone, or list specific usernames for a closed team
 - **Date range:** Set start and end dates if the project covers a specific period (e.g., a class semester or field season)
 - **Quality grade:** Select “Research Grade” and “Needs ID” for most research projects
5. Under **Trust** (optional but recommended for research): enable Trust to allow project admins to access the true coordinates of observations with obscured or private locations. Members must individually opt in to share their coordinates.
6. Click **Save**

Creating a Custom Place

If your study area (e.g., the Bernard Field Station, a specific watershed) does not exist as an iNaturalist Place, create one:

1. Go to <https://www.inaturalist.org/places/new>
2. Name the place (e.g., “Bernard Field Station, Claremont, CA”)
3. Draw the boundary on the map using the polygon tool
4. Save

The place will be available as a filter in your Collection Project. Note that very small places (e.g., a single backyard) work better with Traditional Projects because GPS accuracy and obscured coordinates can cause observations to fall outside tiny boundaries.

7.5.2. Creating a Traditional Project

Use when you need custom observation fields or when the study area is too small for reliable GPS-based filtering.

1. You must have at least 50 verifiable observations on your account
2. Go to <https://www.inaturalist.org/projects/new>
3. Look for the small text link at the bottom of the page: “click here to create a traditional project”
4. Fill in the project details (title, icon, banner, description)
5. Set the membership model:
 - **Open:** Anyone can join
 - **Invite-only:** Only users you invite can join (recommended for research teams)
6. Set the submission model:
 - **Any member:** All members can add observations
 - **Project curators:** Only curators can add observations (recommended when the PI needs to control data quality)
7. Add observation fields if needed (e.g., “Habitat type,” “Transect number,” “Number of individuals”).

8. Data Quality Considerations

iNaturalist data are heterogeneous in quality because they are generated by observers with widely varying skill levels, equipment, and motivations (Dickinson et al., 2010). For EA Program research, apply the following data quality filters when exporting records for analysis:

- **Quality grade:** Use Research Grade observations only for quantitative analyses. Needs ID observations can supplement species inventories but carry higher misidentification risk.
- **Coordinate uncertainty:** Exclude records with positional accuracy greater than 1000 m unless the analysis does not require fine spatial resolution.
- **Date precision:** Exclude records without day-level date precision for phenological analyses.
- **Taxonomic verification:** For critical analyses, manually verify identifications against the photographic evidence, especially for taxa where the CV model is known to struggle (e.g., grasses, juvenile insects, sterile plants).
- **Spatial bias:** iNaturalist data are spatially biased toward accessible, human-frequented areas (trails, roads, urban parks). This bias must be acknowledged and, where possible, corrected for in species distribution models (Callaghan et al., 2021).

These filters should be documented in the methods section of any publication or report using iNaturalist-derived data.

9. Data Export and Analysis

9.1. Exporting from iNaturalist

1. Go to the **Explore** page and filter to your project, place, or taxa of interest
2. Click **Filters** to narrow results
3. Click **Download** (bottom of the page) to download a CSV file

9.2. Exporting from GBIF

For Research Grade observations that have been shared with GBIF:

1. Go to <https://www.gbif.org/occurrence/search>
2. Filter by dataset (search for “iNaturalist”), location, and taxa
3. Click **Download** and select the desired format
4. GBIF assigns a DOI to every download; cite the DOI in your publications

9.3. Using `rinat` in R

The `rinat` package provides programmatic access to iNaturalist data ([barve2025?](#)):

```
library(rinat)

# Get observations from a specific project
obs <- get_inat_obs_project("ea-program-bfs-biodiversity-2025",
                           type = "observations")

# Get observations by taxon and place
birds <- get_inat_obs(taxon_name = "Aves",
                     place_id = 12345,
                     quality = "research",
                     maxresults = 500)
```

10. Citing iNaturalist Data

- **Platform citation:** iNaturalist. Available at: <https://www.inaturalist.org>. Accessed [date].
- **GBIF download citation:** Use the DOI provided with the GBIF download.
- **Individual observations:** Cite by observation URL (e.g., <https://www.inaturalist.org/observations/123456>) when referencing specific records.

When using iNaturalist data in publications, describe the data quality filters applied (e.g., Research Grade only, coordinate uncertainty threshold) and acknowledge the community identifiers whose expertise validated the records (Chandler et al., 2017; Dickinson et al., 2010).

11. Responsible Use

- Respect copyright: each user retains ownership of their observations and photos. Your rights to use them are governed by the license the user selected. Most iNaturalist photos use CC-BY-NC.
- Do not create observations for specimens you did not personally encounter (no uploading photos from field guides, the internet, or other people’s cameras)
- Do not harass, collect, or relocate organisms for the purpose of getting a better photo

- For sensitive species (rare plants, nesting sites), manually obscure the location even if iNaturalist does not do so automatically
- Follow iNaturalist’s Community Guidelines: <https://www.inaturalist.org/pages/community+guidelines>

Student Use

Before assigning students to use iNaturalist, read the iNaturalist Teacher’s Guide (<https://www.inaturalist.org/pages/teacher's+guide>). The majority of low-quality observations and inappropriate content on iNaturalist comes from poorly managed class assignments. Set clear expectations, demonstrate proper technique, and monitor student contributions. Structured class projects with specific data quality goals produce better scientific outcomes and learning (Aristeidou & Herodotou, 2020).

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- Global Biodiversity Information Facility. (2024). *What is GBIF?* <https://www.gbif.org/what-is-gbif>

13. Training Record

Before using iNaturalist for EA Program research data, personnel must:

1. Read this SOP in full
2. Create an iNaturalist account with appropriate license settings (Section 7.1)
3. Make at least 10 observations that reach Research Grade (or “Needs ID” with correct identifications added by the community)
4. Contribute at least 10 identifications to other users’ observations
5. Demonstrate familiarity with the project type relevant to their research (Collection or Traditional)
6. Be signed off by the PI or project lead



Environmental Analysis Program

Pomona College
Biogeochemistry Laboratory

SOP-F603

Vegetation Transect Surveys

Standard Operating Procedure

SOP Number	SOP-F603
Revision	0.3
Author(s)	Marc Los Huertos
Date	2026-06-26
Organization	EA Program, Pomona College

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1. Purpose

This SOP describes field procedures for conducting vegetation transect surveys using three common methods: line-point intercept (LPI), line intercept (LI), and quadrat (cover class) sampling. These methods quantify plant species composition, percent cover, and community structure across field sites and form the basis for vegetation monitoring, ecological assessment, and restoration evaluation (Bonham, 2013; Kent, 2012; **mueller-dombois1974?**).

2. Scope

- **Applies to:** Terrestrial vegetation surveys at all EA Program field sites, including the Bernard Field Station, Pomona College Farm, the Pit, restoration sites, and photovoltaic panel study areas
- **Does not apply to:** Aquatic or submerged vegetation surveys; tree-only forest inventory (DBH-based methods; see [SOP-F604](#)); remote sensing vegetation analysis
- **Location:** All EA Program field sites

3. Definitions

- **Percent cover:** The proportion of the ground surface covered by the vertical projection of the aerial parts of a plant species, expressed as a percentage. Cover is the standard metric for characterizing non-woody vegetation communities because it integrates plant size and density into a single measure (Bonham, 2013).
- **Line-point intercept (LPI):** A method that estimates cover by recording what a vertically lowered pin contacts at fixed intervals along a transect. Originally described by Levy & Madden (1933), LPI produces objective, repeatable cover estimates with minimal observer bias (Herrick et al., 2005).
- **Line intercept (LI):** A method that measures the length of transect tape intercepted by the canopy of each species. Best suited for shrubs and other plants with clearly defined canopy boundaries.
- **Quadrat:** A fixed-area frame (typically 1 m x 1 m) within which percent cover is visually estimated for each species. Visual estimation introduces more observer subjectivity than point-based methods but permits rapid assessment and better detection of rare species (Daubenmire, 1959; God'inez-'Alvarez et al., 2009).
- **Daubenmire cover class:** A categorical scale for estimating cover within quadrats: 1 (0–5%), 2 (5–25%), 3 (25–50%), 4 (50–75%), 5 (75–95%), 6 (95–100%). The midpoint of each class is used in calculations (Daubenmire, 1959).

- **Top hit / lower hit:** In LPI, the “top hit” is the first species the pin contacts as it descends through the canopy; subsequent contacts with other species or canopy layers are “lower hits.” Recording both allows multi-layer canopy analysis.
- **Species richness:** The total number of species recorded in a defined area. Richness is strongly influenced by plot size and sampling effort (**wilson2012?**).
- **Voucher specimen:** A pressed, dried, and identified plant specimen archived for verification of field identifications. See Section 9.
- **Basal cover:** The proportion of ground surface occupied by the base (stem or root crown) of a plant, as distinct from foliar cover. Basal cover is more stable than foliar cover across seasons and is sometimes used for perennial grass monitoring (Herrick et al., 2005).

4. Responsibilities

Role	Responsibility
Lab PI	Approve survey design, ensure training compliance
Field Lead	Coordinate logistics, assign transect locations
Trained Personnel	Conduct surveys, record data, collect voucher specimens
New Students	Complete training before independent surveying

5. Safety and Hazards

Hazards

- **Biological:** Poison oak, stinging nettle, ticks, snakes, bees (all common at local field sites)
- **Physical:** Uneven terrain, heat exposure, sharp stakes and rebar
- **Allergenic:** Pollen exposure during peak growing season

5.1. Required PPE

- ☐ Closed-toed shoes or field boots
- ☐ Long pants (required in areas with poison oak or ticks)
- ☐ Sun protection (hat, sunscreen)
- ☐ Nitrile gloves (when handling unknown plants)
- ☐ Tick repellent (DEET or permethrin-treated clothing)

5.2. Required Training

- [SOP-F001 Field Safety](#)

6. Summary of Methods

Three survey methods are covered in this SOP. Select the method that best fits your vegetation type and research objectives (Elzinga et al., 1998; Herrick et al., 2005).

Method	Best For	Accuracy	Speed	Observer Bias
Line-point intercept	Grasslands, herbaceous communities	High	Moderate	Low
Line intercept	Shrublands, plants with dense canopies >1 m	High	Slow	Low
Quadrat (cover class)	Mixed communities, rapid assessment, rare species detection	Moderate	Fast	Moderate

Choosing a Method

For most EA Program projects in southern California grasslands and shrublands, **line-point intercept** is the default method because it minimizes observer bias in cover estimation (Godínez-Alvarez et al., 2009; Herrick et al., 2005). Use **quadrats** when rare species detection is important or when rapid assessment is needed, recognizing that visual cover estimation varies significantly among observers even after training (Bonham, 2013). Use **line intercept** for shrub-dominated communities where canopy boundaries are distinct. When a study spans multiple years, method consistency across years is more important than selecting the “best” method in any given year (symstad2008?).

7. Required Materials

7.1. Field Equipment

Item	Notes
50 m measuring tape	Fiberglass preferred (doesn't stretch)
Chaining pins or rebar stakes	For anchoring transect endpoints
Pin flags	For marking transect start/end and quadrat locations
Sampling pin or rod	Thin metal rod (~3 mm diameter) for point intercept
1 m x 1 m quadrat frame	PVC or metal; for cover class method
Compass	For establishing transect bearings
GPS unit or smartphone	For recording transect coordinates
Field data sheets	Printed from Appendix A
Clipboard and pencils	Mechanical pencils work well in the field

Item	Notes
Plant identification guides	Regional flora; Jepson Manual for California (Baldwin et al., 2012)
Camera	For photo documentation at each transect
Collection bags and plant press	For voucher specimens of unknown species
Flagging tape	For temporary marking

8. Estimated Time

Task	Time
Site preparation and transect setup	15–30 minutes per transect
Line-point intercept survey	30–60 minutes per 50 m transect
Line intercept survey	45–90 minutes per 50 m transect
Quadrat survey	10–15 minutes per quadrat
Data entry and QC	30 minutes per transect (post-field)

9. Procedure

9.1. Survey Timing

Conduct vegetation surveys during the peak growing season for the target community. In southern California:

- **Grasslands and annual forbs:** March through May
- **Coastal sage scrub:** April through June
- **Riparian:** June through August

Consistency in timing across years is essential for detecting temporal changes (**symstad2008?**). Surveys conducted outside the peak window will underestimate cover for deciduous and annual species.

9.2. Transect Layout

1. Determine transect locations using the project sampling design (random, stratified random, or systematic placement within habitat zones). The choice of sampling design affects the inferences that can be drawn from the data; random or stratified random designs support unbiased estimation of population-level cover, while systematic designs maximize spatial coverage but can coincide with periodic landscape features (Kent, 2012).
2. Navigate to the starting point and record GPS coordinates
3. Establish the transect bearing using a compass (typically perpendicular to environmental gradients, e.g., slope, moisture, or distance from structures)
4. Anchor the 0 m end of the measuring tape with a chaining pin

5. Walk out the tape to the desired length (typically 50 m), keeping it taut and as straight as possible
6. Anchor the far end with a second chaining pin
7. Photograph the transect from both ends

Permanent Transects

For long-term monitoring, mark transect endpoints with permanent markers (capped rebar driven flush with the ground, GPS coordinates recorded to sub-meter accuracy). Take a compass bearing and distance to at least two nearby permanent features for relocation.

9.3. Method A: Line-Point Intercept

This method estimates percent cover by recording what a vertical sampling pin contacts at regular intervals along the transect. The method was originally developed by Levy & Madden (1933) for pasture analysis and has since become the standard for rangeland and grassland monitoring (Herrick et al., 2005).

1. Begin at the 0 m end of the transect; always work from the same side of the tape
2. At each sampling point (every 0.5 m or 1 m, depending on project design), lower the sampling pin vertically through the vegetation canopy directly over the tape
3. Record each plant species the pin contacts as it descends from the canopy to the ground (**top hit** is the first species contacted; record all species for multi-layer canopies)
4. If the pin does not contact any vegetation, record the ground surface type: bare soil, litter, rock, moss/lichen, or other
5. Record the height (cm) of the tallest species contacted at each point
6. Advance to the next sampling point and repeat

Pin Angle Matters

The pin must be lowered vertically (plumb). Tilting the pin even slightly increases the probability of contacting vegetation and inflates cover estimates. Some protocols use a pin guide or frame to enforce verticality.

Calculations:

$$\text{Percent cover for species } i = \frac{\text{Number of hits for species } i}{\text{Total number of sampling points}} \times 100$$

Total cover may exceed 100% when canopy layers overlap. The number of sampling points required depends on the diversity and patchiness of the vegetation; 100 points per 50 m transect (0.5 m interval) is standard for diverse grasslands, while 50 points (1 m interval) may suffice in low-diversity or homogeneous stands (Elzinga et al., 1998).

9.4. Method B: Line Intercept

This method measures the length of transect tape intercepted by each plant species. Best suited for shrubs and plants with distinct canopy boundaries (Bonham, 2013).

1. Begin at the 0 m end of the transect
2. Walk slowly along the tape, noting where each plant canopy begins and ends (intercepts the tape)
3. Record the species, the start point (cm), and the end point (cm) on the data sheet
4. If a gap occurs within a single plant's canopy that is longer than the canopy width on either side, treat it as two separate intercepts
5. Record gaps between plants as the appropriate ground cover type

Calculations:

$$\text{Percent cover for species } i = \frac{\sum \text{intercept lengths for species } i}{\text{Total transect length}} \times 100$$

9.5. Method C: Quadrat (Cover Class)

This method uses visual estimates of cover within fixed-area plots placed at intervals along the transect (Daubenmire, 1959).

1. Place quadrats at predetermined intervals along the transect (e.g., every 5 m or 10 m), or at random distances
2. Ensure enough distance between quadrats for them to be independent sampling units (typically at least 5 m apart)
3. For each quadrat, identify every plant species present
4. Estimate percent cover for each species using the modified Daubenmire cover classes:

Class	Cover Range	Midpoint
1	0–5%	2.5%
2	5–25%	15%
3	25–50%	37.5%
4	50–75%	62.5%
5	75–95%	85%
6	95–100%	97.5%

5. Also record percent cover of bare ground, litter, and rock
6. Note the maximum vegetation height (cm) in each quadrat
7. Photograph each quadrat from directly above

Visual estimation of cover is inherently subjective and sensitive to observer experience, viewing angle, and vegetation height; training with known-cover reference images or calibration frames improves consistency but does not eliminate inter-observer variability (Bonham, 2013; God'inez-Alvarez et al., 2009).

10. Voucher Specimens

When a plant cannot be identified in the field:

1. Collect a representative sample including leaves, stems, and flowers or fruit if available
2. Place in a labeled collection bag (site, transect, date, collector)
3. Press specimens the same day
4. Identify in the lab using the Jepson Manual (Baldwin et al., 2012) or other regional flora
5. Update the data sheet with the confirmed identification

11. Quality Control

- Two observers should independently estimate cover for at least 10% of quadrats to assess observer agreement. Inter-observer variability in cover estimation is the largest source of error in quadrat-based surveys; training reduces but does not eliminate this bias (Bonham, 2013; Elzinga et al., 1998).
- For LPI surveys, duplicate readings at 10% of points should agree on species identity for at least 90% of hits. Disagreements on ground cover type (e.g., litter vs. bare soil) are most common and should be resolved by applying consistent definitions.
- Photograph each transect and quadrat for archival verification
- Review data sheets in the field before leaving the site (check for missing entries, illegible writing, and species code consistency)
- Enter data within 48 hours of collection
- Maintain a species list with standardized codes used across all surveys

12. Data Management

12.1. Field Data Sheets

Use the data sheets in Appendix A. Record in pencil (ink smears when wet). Use Rite in the Rain paper if available.

12.2. Data Entry

Enter data into the project spreadsheet or database within 48 hours. Each row should represent one observation (one point, one intercept, or one quadrat-species combination). Required fields:

- Date, site, transect ID, observer
- Method (LPI, LI, or Quadrat)
- Point/interval/quadrat number
- Species (use standardized 6-letter codes: first 3 letters of genus + first 3 of species, e.g., BRODIA for *Bromus diandrus*)
- Cover value or hit/no-hit
- Height (cm)
- Ground cover type (if applicable)

13. Waste and Site Impact

- Remove all flagging tape, pin flags, and temporary markers after the survey
- Do not trample vegetation outside the immediate transect corridor
- Leave permanent markers flush with the ground to avoid hazards to people and wildlife
- Pack out all trash and supplies

14. References

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15. Training Record

Before conducting vegetation surveys independently, personnel must:

1. Read this SOP and [SOP-F001 Field Safety](#) in full
2. Demonstrate familiarity with at least 20 common species at the study site
3. Participate in a supervised survey using each method to be employed
4. Demonstrate acceptable inter-observer agreement on cover estimates (within one cover class for quadrats, or within 5% for line-point intercept)
5. Be signed off by the field lead or PI

16. Appendix A: Field Data Sheets

16.1. Line-Point Intercept Data Sheet

Site: _____ Transect ID: _____ Date: _____

Observer: _____ **Bearing:** _____ **Length:** _____ m

Sampling interval: every _____ m **Start GPS:** _____

Point (m)	Top Hit Species	Lower Hit(s)	Ground Cover	Height (cm)
0.0				
0.5				
1.0				
1.5				
2.0				

(Continue on additional sheets as needed)

16.2. Quadrat Cover Class Data Sheet

Site: _____ **Transect ID:** _____ **Date:** _____

Observer: _____ **Quadrat size:** _____ m x _____ m

Cover classes: 1 = 0–5%, 2 = 5–25%, 3 = 25–50%, 4 = 50–75%, 5 = 75–95%, 6 = 95–100%

Quadrat #	Position (m)	Species	Cover Class	Max Height (cm)

Ground cover for each quadrat:

Quadrat #	Bare Soil (%)	Litter (%)	Rock (%)	Other (%)



Environmental Analysis Program

Pomona College
Biogeochemistry Laboratory

SOP-F604

Tree DBH and Forest Inventory

Standard Operating Procedure

SOP Number	SOP-F604
Revision	0.3
Author(s)	Marc Los Huertos
Date	2026-06-26
Organization	EA Program, Pomona College

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1. Purpose

This SOP describes standardized procedures for measuring tree diameter at breast height (DBH), conducting fixed-area plot-based forest inventories, and estimating basal area using prism or angle-gauge sampling. These measurements are fundamental to characterizing forest structure, estimating stand density and biomass, monitoring tree growth, and providing inputs to allometric equations for carbon accounting (Avery & Burkhardt, 2002; Westfall & Westfall, 2020). The measurement conventions follow the USDA Forest Service Forest Inventory and Analysis (FIA) national standards (USDA Forest Service, 2023).

Accurate DBH measurement is the single most important field variable in forest ecology because nearly all derived quantities (basal area, volume, biomass, and carbon) depend on it through nonlinear allometric relationships. Small systematic errors in DBH propagate disproportionately into biomass estimates, especially for large-diameter trees, which store a disproportionate fraction of stand-level carbon (Lutz et al., 2018; **west2009?**).

2. Scope

- **Applies to:** All EA Program field sites with tree cover, including riparian forests, oak woodlands, mixed conifer stands, and planted trees at restoration and campus sites
- **Does not apply to:** Shrubs with DBH below 2.5 cm (use [SOP-F603](#) for shrub cover); timber cruising for commercial harvest; urban tree risk assessment
- **Location:** All EA Program terrestrial field sites with woody vegetation

3. Definitions

- **Diameter at breast height (DBH):** The diameter of a tree trunk measured at 1.37 m (4.5 ft) above ground level on the uphill side of the tree. DBH is the standard dimension for characterizing tree size (Avery & Burkhardt, 2002; USDA Forest Service, 2023).
- **Basal area (BA):** The cross-sectional area of a tree trunk at breast height, calculated as $BA = \frac{\pi}{4} \times DBH^2$. Often expressed per hectare (m^2/ha) as a measure of stand density.
- **Basal area factor (BAF):** The conversion factor for a wedge prism or angle gauge that translates the number of “in” trees to stand basal area (m^2/ha).
- **Variable-radius plot (point sampling):** A sampling method where the probability of a tree being included is proportional to its basal area, achieved using a BAF prism. Originally described by
 - 1.
- **Fixed-area plot:** A plot of known area (e.g., 0.04 ha circular plot, radius 11.28 m) within which all trees above a minimum DBH threshold are measured (Laar & Ak cca, 2007).
- **Allometric equation:** A mathematical model relating an easily measured tree dimension (usually DBH) to a harder-to-measure quantity (usually biomass or volume) through a power function (Chojnacky et al., 2014; Jenkins et al., 2003).
- **Crown class:** A classification of a tree’s position in the canopy: dominant, co-dominant, intermediate, or suppressed. Crown class reflects competitive status and light availability.

- **Snag:** A standing dead tree. Snags provide habitat for cavity nesters and are recorded separately from live trees in inventories.
- **Stand density index (SDI):** A relative measure of stand density that accounts for tree size, allowing comparison across species and sites (**pretzsch2009?**).
- **Size distribution:** The frequency distribution of tree diameters in a stand. Reverse-J distributions characterize uneven-aged stands with continuous recruitment; normal or bimodal distributions suggest even-aged cohorts or past disturbance (**muller-landau2006?**).

4. Responsibilities

Role	Responsibility
PI / Field Lead	Approve plot design and minimum DBH threshold
Trained Personnel	Measure trees and record data per this SOP
New Students	Complete training before independent measurement

5. Safety and Hazards

Hazards

- **Physical:** Dead or leaning trees (snags, widow-makers) may shed branches. Look up before working under any tree. Do not measure trees during high winds.
- **Biological:** Poison oak, ticks, stinging insects, and snakes in wooded habitats. Wasp nests may be present in tree cavities.
- **Terrain:** Steep slopes in forested areas increase fall risk. Use caution when navigating uneven terrain with equipment.

5.1. Required PPE

- ☐ Closed-toed shoes or field boots
- ☐ Long pants
- ☐ Hard hat (in areas with overhead hazard trees)
- ☐ Sun protection (hat, sunscreen)
- ☐ Tick repellent

5.2. Emergency Procedures

Refer to [SOP-F001 Field Safety](#). Work in pairs in remote forested areas.

6. Equipment and Materials

Item	Notes
Diameter tape (DBH tape, metric)	Graduated in diameter units (pi tape); reads directly in cm
Standard measuring tape (50 m)	For establishing plot boundaries
Clinometer (Suunto or equivalent)	For measuring tree height (optional)
Basal area factor (BAF) prism or angle gauge	For variable-radius plot sampling
GPS unit or smartphone	Per SOP-F006
Field notebook or data sheet	Pre-printed inventory form (Appendix A)
Compass	For plot layout and stem mapping
Flagging tape	For marking plot center and measured trees
Tree tags and nails (aluminum)	For permanent plot re-measurement
Paint marker or timber crayon	For marking the DBH measurement point
Laser rangefinder (optional)	For measuring tree height and distances
Calculator	For basal area and density calculations

7. Procedure

7.1. Measuring DBH

DBH is measured at 1.37 m (4.5 ft) above ground level on the uphill side of the tree (Avery & Burkhardt, 2002; USDA Forest Service, 2023).

1. Identify the measurement height: 1.37 m above the ground surface on the uphill side of the tree. If the ground is level, measure from any side.
2. Wrap the diameter tape around the trunk at 1.37 m, perpendicular to the axis of the trunk (not perpendicular to the slope).
3. Ensure the tape is snug against the bark, not twisted, and level. Read the diameter directly from the DBH tape to the nearest 0.1 cm.
4. Record the measurement in the field notebook or data sheet.

Special cases:

Situation	Measurement Rule
Tree on a slope	Measure at 1.37 m from the uphill side of the base
Forked below 1.37 m	Measure each fork as a separate tree
Forked at or above 1.37 m	Measure as one tree below the fork
Swelling or abnormality at 1.37 m	Measure above and below the abnormality and average
Leaning tree	Measure 1.37 m along the underside of the trunk
Buttressed tree	Measure above the buttress where the trunk becomes cylindrical

 Tip

For permanent monitoring plots, mark the DBH measurement point on the bark with a paint marker or timber crayon so that subsequent measurements are taken at the exact same height. For long-term studies, tag each tree with a numbered aluminum tag nailed above breast height.

7.2. Fixed-Area Plot Inventory

Fixed-area plots are the standard method for forest inventory (Laar & Ak cca, 2007; **west2009?**). Plot size depends on tree density.

Tree Density	Recommended Plot Size	Radius (circular)
Dense forest	0.01 ha (100 m ²)	5.64 m
Moderate forest	0.04 ha (400 m ²)	11.28 m
Open woodland	0.10 ha (1000 m ²)	17.84 m

Procedure:

1. Navigate to the plot center and record GPS coordinates per [SOP-F006](#).
2. Mark the plot center with a stake or flagging tape.
3. For circular plots, use the measuring tape as a radius to determine which trees are inside the plot boundary. A tree is “in” if more than half of its trunk at DBH lies within the plot radius.
4. Measure and record the following for every tree with DBH at or above the minimum threshold (typically 2.5 cm or 10 cm, as specified by the project):
 - Species (common name and/or scientific name)
 - DBH (cm)
 - Condition class: live, standing dead (snag), leaning
 - Crown class (optional): dominant, co-dominant, intermediate, suppressed
 - Height (optional; measured with a clinometer or rangefinder)
5. Record the total number of stems and species per plot.

7.3. Variable-Radius Plot Sampling (Basal Area Prism)

The BAF prism provides a rapid estimate of stand basal area without establishing fixed plot boundaries (Avery & Burkhart, 2002; Grosenbaugh, 1952).

1. Stand at the sample point and hold the BAF prism at arm’s length, looking through it at each surrounding tree at breast height.
2. For each tree, the prism refracts the image of the trunk. Compare the offset image to the original:
 - **Tree is “in”:** The offset image overlaps the unrefracted trunk. Count the tree.
 - **Tree is “out”:** The offset image is completely separated from the unrefracted trunk. Do not count.

- **Borderline:** The offset image just touches the trunk edge. Count every other borderline tree.
3. Rotate 360 degrees, counting all “in” trees.
 4. Calculate basal area:

$$\text{Basal area (m}^2/\text{ha)} = n \times \text{BAF}$$

where n is the number of “in” trees and BAF is the basal area factor of the prism (typically 1, 2, 4, or 5 m²/ha per tree counted; check the prism label).

7.4. Tree Height Measurement (Optional)

Use a clinometer or laser rangefinder to measure total tree height (Laar & Ak cca, 2007; west2009?):

1. Stand at a known horizontal distance from the tree base (e.g., 15–30 m; measure with a tape or rangefinder).
2. Sight the tree top through the clinometer and record the angle to the top (α).
3. Sight the tree base and record the angle to the base (β). If the base is below eye level, β is negative.
4. Calculate height:

$$H = D \times (\tan \alpha - \tan \beta)$$

where D is the horizontal distance (m) and H is the total height (m). This formula assumes the observer’s line of sight to the top of the tree is unobstructed and that the tree is vertical; for leaning trees, measure height along the leaning axis and record the lean angle.

8. Derived Calculations

8.1. Basal Area per Tree

$$\text{BA (m}^2) = \frac{\pi}{4} \times \left(\frac{\text{DBH}}{100} \right)^2$$

where DBH is in cm.

8.2. Stand Density and Basal Area per Hectare

$$\text{Trees/ha} = \frac{n}{\text{plot area (ha)}}$$

$$\text{BA/ha (m}^2/\text{ha)} = \frac{\sum \text{BA}_i}{\text{plot area (ha)}}$$

8.3. Biomass Estimation

Above-ground biomass can be estimated using published allometric equations of the form:

$$\text{Biomass (kg)} = a \times \text{DBH}^b$$

where a and b are species-specific coefficients from published sources (Chojnacky et al., 2014; Jenkins et al., 2003). Document the allometric equation, source, and species used in the project README. Because the exponent b typically ranges from 2 to 3, measurement errors in DBH are amplified in biomass estimates: a 1 cm error at DBH = 50 cm produces a roughly 4–6% error in biomass (west2009?). Large-diameter trees store a disproportionately large fraction of aboveground carbon in most forest types; accurate measurement of the largest individuals in a stand is therefore critical for unbiased biomass and carbon stock estimates (Lutz et al., 2018).

8.4. Interpreting Size Distributions

Plotting the diameter distribution (number of trees per DBH class) provides insight into stand history and dynamics. A reverse-J (negative exponential) distribution suggests continuous recruitment across size classes, typical of uneven-aged or mixed-species stands. A bell-shaped or bimodal distribution suggests one or more distinct cohorts established after disturbance events (muller-landau2006?; pretzsch2009?).

9. Quality Control

- Measure DBH twice (wrap the tape, read, remove, re-wrap, re-read). The two readings should agree within 0.5 cm. Record the mean.
- On 10% of plots, have a second observer independently measure all trees. DBH measurements should agree within 1 cm; tree counts should match exactly.
- Verify the diameter tape calibration at the start of each field season by measuring a cylinder of known diameter.
- For permanent plots, verify that tags are intact and measurement points are correctly located at each re-measurement.
- When comparing DBH across re-measurement intervals, confirm that the measurement point has not shifted due to bark ingrowth over tags or paint marks (Westfall & Westfall, 2020).

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11. Related SOPs

SOP	Title	Relationship
F001	Field Safety	Required prerequisite
F006	GPS and Site Navigation	Plot center coordinates
F603	Vegetation Transects	Understory and shrub cover
F605	Leaf Area Index	Canopy measurement companion
F606	Canopy Cover	Densiometer and photo methods

12. Training Record

Before conducting tree measurements independently, personnel must:

1. Read this SOP in full
2. Demonstrate correct DBH measurement technique, including special cases (forks, slopes, buttresses)
3. Participate in a supervised plot inventory
4. Demonstrate BAF prism technique with results within 10% of the trainer's count
5. Be signed off by the PI or field lead

13. Appendix A: Forest Inventory Data Sheet

Site: _____ Plot ID: _____ Date: _____

Observer: _____ Plot size: _____ m² Min DBH: _____ cm

GPS: _____ Elevation: _____ m Slope: _____ %

Tree #	Species	DBH (cm)	Height (m)	Condition	Crown Class	Notes
--------	---------	----------	------------	-----------	-------------	-------

Basal area prism count (BAF = _____): _____ trees in = _____ m²/ha



Environmental Analysis Program

Pomona College
Biogeochemistry Laboratory

SOP-F605

Leaf Area Index Measurement

Standard Operating Procedure

SOP Number	SOP-F605
Revision	0.3
Author(s)	Marc Los Huertos
Date	2026-06-26
Organization	EA Program, Pomona College

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1. Purpose

This SOP describes field methods for estimating Leaf Area Index (LAI), the total one-sided area of leaf tissue per unit ground surface area (m^2 leaf / m^2 ground). LAI is a key structural parameter governing light interception, transpiration, and primary productivity (Jonckheere et al., 2004). It is used in the EA Program for characterizing canopy structure at photovoltaic panel sites, restoration areas, and agricultural plots.

Both ceptometer and hemispherical photography methods estimate an “effective” LAI that assumes a random spatial distribution of foliage elements within the canopy. In most real canopies, foliage is clumped rather than random, and the effective LAI underestimates the true LAI by 20–50% depending on species and canopy architecture (Br’eda, 2003; Chen et al., 1997). When reporting LAI values, specify whether a clumping correction has been applied and which correction method was used. Ground-based LAI measurements also serve as critical reference data for validating satellite-derived LAI products (garrigues2008?; zheng2009?).

2. Scope

- **Applies to:** Indirect optical measurement of LAI using hemispherical photography and/or a ceptometer (AccuPAR or equivalent linear PAR sensor) at all EA Program field sites
- **Does not apply to:** Direct destructive leaf area measurement (harvest methods); satellite-derived LAI products (though ground measurements may be used to validate them); root area measurements
- **Location:** All EA Program field sites

3. Definitions

- **Leaf Area Index (LAI):** The total one-sided area of leaf tissue per unit ground area. Dimensionless ($\text{m}^2 \text{ m}^{-2}$). Typical values range from 0 (bare ground) to 1–3 (grasslands and crops) to 5–10 (closed-canopy forests) (Jonckheere et al., 2004).
- **Effective LAI (L_e):** The LAI value obtained from optical methods that assume random foliage distribution. Effective LAI underestimates true LAI in clumped canopies because light passes through gaps between clumps more easily than a random model predicts (Chen et al., 1997).
- **True LAI:** The actual one-sided leaf area per unit ground area, obtained either by destructive harvest or by applying a clumping correction (Ω) to the effective LAI: $\text{LAI}_{\text{true}} = L_e / \Omega$, where $\Omega < 1$ for clumped canopies (Chen et al., 1997).
- **Clumping index (Ω):** A dimensionless parameter (0 to 1) describing the degree to which foliage deviates from a random distribution. Values near 1 indicate nearly random distribution; values of 0.5–0.7 are typical for conifer canopies.
- **PAR:** Photosynthetically Active Radiation (400–700 nm).
- **Ceptometer:** A linear array of PAR sensors used to measure light transmission through a canopy. LAI is estimated from the ratio of below-canopy to above-canopy PAR using the Beer-Lambert extinction model (Norman & Campbell, 1989).
- **Gap fraction:** The proportion of the sky hemisphere visible through the canopy at a given zenith angle. Gap fraction is the primary measurement from hemispherical photography and is inverted to estimate LAI.
- **Extinction coefficient (K):** A parameter in the Beer-Lambert model relating LAI to light transmission; depends on solar zenith angle and leaf angle distribution.
- **Hemispherical photography:** A technique using a fisheye lens pointed upward to photograph the canopy. Gap fraction analysis of the image yields LAI estimates (Frazer et al., 2001).

4. Responsibilities

Role	Responsibility
PI / Field Lead	Approve measurement design, review data
Trained Personnel	Collect field measurements per this SOP
New Students	Complete training before independent use

5. Safety and Hazards

Hazards

- **Physical:** Uneven terrain, tripping hazards from dense vegetation
- **Biological:** Tick and insect exposure in dense canopy understories
- **Heat:** Measurements are often taken in open or partially shaded areas during peak growing season

5.1. Required PPE

- ☐ Closed-toe shoes or field boots
- ☐ Sun protection (hat, sunscreen)
- ☐ Long pants and long sleeves (tick protection in dense vegetation)

5.2. Emergency Procedures

Refer to [SOP-F001 Field Safety](#).

6. Required Materials

Item	Notes
Ceptometer (AccuPAR LP-80 or equivalent)	Linear PAR sensor with datalogger
External PAR sensor (if not built in)	For above-canopy reference
Camera with fisheye lens adapter	For hemispherical photography
Tripod with self-leveling head	For consistent camera positioning
GPS unit or smartphone	For recording measurement locations
Field data sheet	Pre-printed with site/date fields
Compass	For orienting measurements
Measuring tape (50 m)	For locating measurement points

7. Estimated Time

Task	Time
Site setup and orientation	10–15 minutes
Ceptometer measurements (10 points)	20–30 minutes
Hemispherical photographs (10 points)	30–45 minutes
Data download and QC	15 minutes

8. Procedure

8.1. Measurement Timing

LAI measurements should be taken under uniform overcast skies or within one hour of solar noon under clear skies (Weiss et al., 2004). Avoid measurement during patchy cloud conditions, as rapidly changing light causes inconsistent above-canopy reference readings. For hemispherical photography, early morning, late afternoon, or overcast conditions produce the best contrast between sky and foliage (Br'eda, 2003).

8.2. Sampling Design

1. Determine measurement locations using the project sampling design (random, stratified random, or collocated with vegetation transects from [SOP-F603](#))
2. At each location, take a minimum of 5 below-canopy readings (ceptometer) or photographs (hemispherical) to capture spatial variability (Weiss et al., 2004)
3. Record GPS coordinates at each measurement location

8.3. Method A: Ceptometer (AccuPAR)

1. Power on the ceptometer and set the date, time, and site ID
2. Enter the leaf angle distribution parameter (χ). Use 1.0 for a spherical distribution (default for most broadleaf canopies) unless species-specific values are available (Norman & Campbell, 1989).
3. Take an above-canopy PAR reading by holding the sensor bar horizontally in full sunlight (or use the external above-canopy sensor if available)
4. Move to the first below-canopy measurement point. Hold the ceptometer horizontally at approximately 1 m height (or just above the understory layer), perpendicular to the dominant row or canopy orientation.
5. Record the below-canopy PAR reading
6. Rotate the ceptometer 90 degrees and take a second reading at the same point to account for canopy anisotropy
7. Average the two readings for that point
8. Repeat at each of the remaining measurement points (minimum 5 per location)
9. Take another above-canopy reading at the end of the measurement sequence to verify that light conditions have not changed substantially (within 10% of the initial reading)

10. The instrument calculates LAI internally using the Beer-Lambert model:

$$\text{LAI} = \frac{-\ln(\tau)}{K}$$

where τ is the ratio of below-canopy to above-canopy PAR and K is the extinction coefficient (a function of solar zenith angle and leaf angle distribution). This value is the effective LAI (L_e) and does not include a clumping correction.

8.4. Method B: Hemispherical Photography

1. Mount the camera with fisheye lens adapter on the tripod at 1 m height (or just above the understory)
2. Level the camera using the tripod's bubble level
3. Orient the top of the image toward magnetic north using a compass
4. Set the camera to manual exposure: use the sky brightness (not the canopy) to set exposure. Bracket exposures if uncertain (one stop above and below). Exposure settings strongly influence the sky/foilage classification threshold and can introduce systematic bias in LAI estimates (Br'eda, 2003).
5. Take the photograph pointing directly upward (zenith)
6. Record the image number, GPS coordinates, and any notes (e.g., understory density, canopy species) on the field data sheet
7. Repeat at each measurement point

8.4.1. Image Analysis

8. Transfer images to the lab computer
9. Analyze using Gap Light Analyzer (GLA) or the **hemiphot** R package (Steege, 2018):
 - a. Threshold the image to classify pixels as sky (gap) or foliage
 - b. Calculate gap fraction at multiple zenith angle rings
 - c. Invert gap fractions to estimate LAI using a canopy model (e.g., Miller's formula or the ellipsoidal leaf angle model)
10. Report the mean LAI and standard error for each site. Specify whether the reported value is effective LAI or clumping-corrected true LAI.

i Ceptometer vs. Hemispherical Photography

The two methods measure slightly different quantities and may not agree at the same site. Ceptometers integrate PAR transmission along a linear transect and are sensitive to solar angle and sky conditions; hemispherical photographs sample the full sky hemisphere and are analyzed offline, allowing more flexible post-processing. Agreement between methods is typically within 0.5–1.0 LAI units, but systematic offsets can occur, especially in heterogeneous canopies (Jonckheere et al., 2004; **thimonier1996?**). Using both methods at a subset of sites allows cross-validation and helps quantify method-specific bias.

9. Quality Control

- Take at least 5 below-canopy readings per measurement location to quantify within-site variability
- Report the coefficient of variation (CV) across readings; if CV exceeds 30%, increase the number of readings
- For ceptometer measurements, verify that above-canopy PAR did not change by more than 10% between the start and end of the measurement sequence
- For hemispherical photography, visually inspect thresholded images to ensure correct sky/foilage classification; manually adjust the threshold if needed
- Cross-validate: where possible, compare ceptometer and hemispherical photography estimates at the same points (Br'eda, 2003)
- When LAI data will be used to validate remote sensing products, document the spatial footprint (measurement spacing and extent) and the timing relative to the satellite overpass (garrigues2008?)

10. Data Management

1. Download ceptometer data to the lab computer within 48 hours
2. Organize hemispherical images by site and date: LAI_SiteID_YYYYMMDD/
3. Archive raw data and images on the lab shared drive
4. Version-control analysis scripts per SOP-L801

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12. Training Record

Before measuring LAI independently, personnel must:

1. Read this SOP and [SOP-F001 Field Safety](#)
2. Demonstrate correct ceptometer operation (power on, set parameters, above/below readings, data download)
3. Demonstrate correct hemispherical photography technique (leveling, orientation, exposure settings)
4. Process one set of images through gap fraction analysis under supervision
5. Be signed off by the PI or field lead



Environmental Analysis Program

Pomona College
Biogeochemistry Laboratory

SOP-F606

Canopy Cover (Densiometer and Hemispherical Photography)

Standard Operating Procedure

SOP Number	SOP-F606
Revision	0.3
Author(s)	Marc Los Huertos
Date	2026-06-26
Organization	EA Program, Pomona College

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1. Purpose

This SOP describes two methods for estimating canopy cover: the convex spherical densiometer and hemispherical (fisheye) photography. Canopy cover is a key variable in stream habitat assessments (SWAMP protocol), forest ecology, microclimate studies, and solar radiation modeling. Separating this SOP from [SOP-F304](#) allows the methods to be applied in any habitat, not just stream reaches (Jennings et al., 1999; Lemmon, 1956).

The distinction between canopy cover and canopy closure is important for data interpretation. Canopy cover is the proportion of the forest floor covered by the vertical projection of tree crowns; canopy closure is the proportion of the sky hemisphere obscured by vegetation as seen from a single point below the canopy. The two metrics are related but not equivalent, and values can differ by 10–20% at the same location depending on canopy heterogeneity and the method used (Jennings et al., 1999; Paletto & Tosi, 2009).

2. Scope

- **Applies to:** Measurement of overhead canopy cover in forests, riparian corridors, oak woodlands, restoration sites, and any habitat where tree or tall shrub canopy is present; includes both the spherical densiometer method and hemispherical photography
- **Does not apply to:** Canopy closure measured by line-intercept or vertical projection methods (see [SOP-F603](#)); leaf area index (see [SOP-F605](#)); remote sensing canopy cover from aerial or satellite imagery
- **Location:** All EA Program field sites with tree or tall shrub cover

3. Definitions

- **Canopy cover:** The proportion of the forest floor covered by the vertical projection of tree crowns, expressed as a percentage. Canopy cover is an areal measurement that integrates across an entire stand or plot (Jennings et al., 1999).
- **Canopy closure:** The proportion of the sky hemisphere obscured by vegetation when viewed from a single point below the canopy. Canopy closure is a point measurement and is what both the densiometer and hemispherical photography directly estimate (Jennings et al., 1999).
- **Canopy openness:** The complement of canopy closure: openness = 100% - closure. Some software and instruments report openness rather than closure.
- **Convex spherical densiometer:** A hand-held instrument with a convex mirror etched with a grid. The observer counts sky-visible grid intersections to estimate canopy openness (Lemmon, 1956).
- **Gap fraction:** The fraction of the sky visible through the canopy at a given zenith angle, measured from hemispherical photographs. Gap fraction is the basis for estimating both canopy closure and effective LAI.
- **Binary threshold:** The image-processing step in hemispherical photography analysis where each pixel is classified as either sky (white) or canopy (black). The threshold value strongly influences the estimated canopy closure.
- **SWAMP:** Surface Water Ambient Monitoring Program (California). The SWAMP protocol uses densiometer readings to characterize stream canopy cover as part of physical habitat assessment.

4. Responsibilities

Role	Responsibility
PI / Field Lead	Specify method and number of measurement points
Trained Personnel	Perform measurements per this SOP
New Students	Complete training before independent measurement

5. Safety and Hazards

Hazards

- **Physical:** Looking upward while walking increases trip and fall risk. Stop walking before looking up.
- **Biological:** Ticks, poison oak, and snakes in forested and riparian habitats.
- **Environmental:** Bright sunlight reflecting off the densiometer can cause glare. Wear sunglasses when not actively reading the instrument.

5.1. Required PPE

- ☐ Closed-toed shoes or field boots
- ☐ Long pants
- ☐ Sun protection (hat, sunscreen)
- ☐ Tick repellent (riparian and woodland sites)

5.2. Emergency Procedures

Refer to [SOP-F001 Field Safety](#).

6. Equipment and Materials

6.1. Method A: Spherical Densiometer

Item	Notes
Convex spherical densiometer (Forestry Suppliers Model A or equivalent)	Concave models also exist; specify which model is used
Bubble level (built into the densiometer)	Essential for consistent readings
Field notebook or data sheet	For recording dot counts
Compass	For orienting the four cardinal readings

6.2. Method B: Hemispherical Photography

Item	Notes
Camera with fisheye lens	Full-frame fisheye (180 degrees circular) preferred; or smartphone with clip-on fisheye lens
Self-leveling tripod or monopod	Camera must be level and facing directly upward
Bubble level (hot-shoe mounted or tripod-integrated)	For leveling the camera
Gray card or exposure reference	For consistent exposure settings
Laptop with canopy analysis software	Gap Light Analyzer (GLA), hemIMAGE, or ImageJ with the Hemispherical plugin
GPS unit	Per SOP-F006

7. Procedure

7.1. Measurement Point Selection

Canopy cover measurements are typically taken at systematic intervals along a transect or at the center and edges of fixed-area plots.

- **Stream habitat surveys (SWAMP):** Measure at 11 equally spaced points along each cross-section transect (left bank, center, right bank at each of the main transect positions). See [SOP-F304](#).
- **Forest inventory plots:** Measure at the plot center and at 4 points on the plot boundary (N, E, S, W).
- **Transect-based surveys:** Measure at regular intervals (e.g., every 10 m or 25 m along a 50 m transect).

Record the GPS coordinates of each measurement point or its position along the transect.

7.2. Method A: Convex Spherical Densimeter

The densimeter has a convex mirror with an etched grid. The observer counts the number of grid intersections that are open to the sky (not obstructed by vegetation) in each of four cardinal directions (Lemmon, 1956; Strickler, 1959).

1. Hold the densimeter at elbow height (approximately 30–40 cm in front of your body), level it using the bubble level.
2. Position your head so that its reflection is just outside the grid area. This ensures a consistent viewing angle.
3. Face **north**. Imagine the grid divided into 24 squares (4 rows x 6 columns). At each of the 24 grid intersections, determine whether the sky or vegetation is reflected. Count the number of open (sky) intersections. Record this count.
4. Rotate 90 degrees and face **east**. Repeat the count.
5. Repeat for **south** and **west**.
6. Calculate canopy cover:

$$\text{Canopy openness (\%)} = \frac{\text{sum of 4 counts}}{96} \times 100$$

$$\text{Canopy cover (\%)} = 100 - \text{canopy openness (\%)}$$

where 96 is the total number of possible open points across 4 directions (24 per direction x 4 directions).

! Known Densiometer Bias

The convex densiometer systematically overestimates canopy openness (and therefore underestimates canopy cover) compared to hemispherical photography because of the wide-angle optics and the observer's head position (Cook et al., 1995; Fiala et al., 2006). The magnitude of the bias varies with stand density, observer experience, and lighting conditions but is typically 5–15 percentage points (Korhonen et al., 2006; Paletto & Tosi, 2009). Report the method used alongside all canopy cover values, and do not mix methods within a dataset or monitoring program.

7.3. Method B: Hemispherical Photography

Hemispherical photographs capture a 180-degree view of the canopy and are analyzed digitally to classify sky vs. canopy pixels (Frazer et al., 2001).

In the field:

1. Mount the camera on the tripod at breast height (1.37 m) or at a height specified by the project protocol.
2. Level the camera using the bubble level. The lens must point directly upward (zenith).
3. Orient the top of the camera toward magnetic north. Record the declination if relevant.
4. Set the camera to manual exposure. Bracket the exposure (take 3 photos: one at the metered exposure, one stop under, one stop over) to ensure at least one well-exposed image.
5. Shoot in overcast or uniformly cloudy conditions whenever possible. Direct sunlight produces bright spots that confound automatic thresholding.
6. Include a gray card in one reference photo per site for exposure calibration.
7. Record the photo number, GPS coordinates, date, time, and sky conditions in the field notebook.

In the lab:

1. Import images into canopy analysis software (Gap Light Analyzer, hemIMAGE, or ImageJ with the Hemispherical plugin).
2. Register the image (set lens projection, define the circular boundary, orient to north).
3. Apply a binary threshold to classify each pixel as sky (white) or canopy (black). Adjust the threshold manually if automated thresholding produces obvious errors.
4. The software calculates canopy openness, canopy cover, leaf area index (effective), and gap fraction by zenith angle.
5. Export results as a .csv file. Save to the project repository under `data/canopy/`.

7.4. Reporting

Report canopy cover values with the following metadata:

- Method (densiometer or hemispherical photography)
- Instrument model (densiometer type; camera and lens model)
- Measurement height above ground
- Date, time, and sky conditions
- Software and thresholding method (for photography)
- Number of measurement points and their spatial arrangement
- Whether the reported metric is canopy cover (vertical projection) or canopy closure (hemispherical view); see Section 3 definitions

8. Quality Control

- **Densiometer:** Have two observers independently read the same point. Readings should agree within 5 percentage points. If they disagree by more than 10 points, re-read together to identify the source of discrepancy. Observer bias is the largest error source for densiometer measurements (Korhonen et al., 2006).
- **Hemispherical photography:** Process the same image twice using different threshold values (+/- 10 units). If the resulting canopy cover differs by more than 5 percentage points, the image may be poorly exposed; re-shoot if possible.
- At a subset of sites, measure canopy cover with both methods and report the comparison. This allows future users to relate datasets collected with different methods and to quantify the systematic offset between them (Fiala et al., 2006; Paletto & Tosi, 2009).
- Record the observer's name with every densiometer measurement.

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10. Related SOPs

SOP	Title	Relationship
F001	Field Safety	Required prerequisite
F304	Stream Physical Habitat	Canopy cover in stream reaches
F603	Vegetation Transects	Understory cover companion
F604	Tree DBH and Forest Inventory	Stand structure companion
F605	Leaf Area Index	Related canopy metric

11. Training Record

Before measuring canopy cover independently, personnel must:

1. Read this SOP in full
2. Demonstrate correct densiometer technique (leveling, head position, cardinal-direction counts) with results within 5 percentage points of the trainer’s reading on at least 5 test points
3. If using hemispherical photography, demonstrate camera setup, exposure bracketing, and image analysis in the lab
4. Be signed off by the PI or field lead



Environmental Analysis Program

Pomona College
Biogeochemistry Laboratory

SOP-F607

Tree Tagging for Restoration Monitoring

Standard Operating Procedure

SOP Number	SOP-F607
Revision	0.1
Author(s)	Ellena Reposa
Date	2026-06-26
Organization	EA Program, Pomona College

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1. Purpose

This SOP describes standardized procedures for tagging trees during restoration field work at EA Program field sites. Tree tagging creates a permanent, individual record for each tree so that

management decisions are consistent, repeatable, and transparent across field crews and over time. A future student or technician should be able to revisit a tagged tree and understand exactly why it was flagged and how its condition has changed.

Tree tagging is used to:

- Identify which trees will be retained, removed, or monitored
- Track individual tree condition over time
- Create a permanent record for future groups and project documentation
- Support consistent decision-making across crew members

Recording a consistent set of fields for each individual tree allows data to be combined, compared, and reused across projects and seasons (Handley et al., 2021).

2. Scope

- **Applies to:** All EA Program field sites with tree cover, including riparian forest, oak woodland, mixed conifer stands, and planted trees at restoration and campus sites
- **Does not apply to:** Shrubs with DBH below 2.5 cm (use [SOP-F603](#) for shrub cover); diameter, height, and plot-based inventory measurement (use [SOP-F604](#)); timber cruising for commercial harvest; formal urban tree risk assessment, which requires a qualified arborist (International Society of Arboriculture, 2017)
- **Location:** All EA Program terrestrial field sites with woody vegetation

3. Definitions

- **DBH (diameter at breast height):** The diameter of a tree trunk measured at 1.37 m (4.5 ft) above ground on the uphill side of the tree. The standard dimension for comparing tree sizes. Measurement procedure is defined in [SOP-F604](#) (Avery & Burkhart, 2002; USDA Forest Service, 2023).
- **Tag:** A numbered aluminum disk fastened to the tree. Each tag number is unique within the project.
- **Flagging:** Colored plastic tape tied loosely around a tree at eye level. Color communicates the management decision for that tree.
- **Condition rating:** A five-level score (Excellent, Good, Fair, Poor, Dead) describing tree health and structure.
- **Crown:** The upper part of a tree, including all branches and foliage.
- **Crown spread:** The average horizontal width of the crown, taken as the mean of the widest spread and a second spread measured at right angles to it (American Forests, 2014).
- **Retain:** Leave the tree in place. Do not remove or damage.
- **Remove:** Tree is scheduled for cutting or restoration thinning.

4. Responsibilities

Role	Responsibility
PI / Field Lead	Approve flag color codes before the season starts; review completed field forms and photo logs weekly; ensure the project meets restoration goals
Trained Personnel	Tag trees per this SOP; complete all fields in the tree assessment record; take required photos; flag trees correctly the first time
New Students	Complete training before tagging independently

Minimum crew size is two persons at all times (safety requirement; see [SOP-F001 Field Safety](#)).

5. Safety and Hazards

Hazards

- **Overhead:** Dead branches and leaning or unstable trees (widow-makers) can fall without warning. Look up before approaching any tree. Do not work under or near hazard trees in high wind.
- **Sharp tools:** Hand saws, loppers, and pruners. Keep blades sheathed while walking, pass tools handle-first, and never carry open blades through brush.
- **Terrain, biota, and weather:** Uneven terrain, poison oak, ticks, snakes, stinging insects, and heat are common at EA field sites and are covered in [SOP-F001 Field Safety](#).

5.1. Required PPE

- ☐ Hi-vis vest
- ☐ Closed-toed shoes or field boots
- ☐ Long sleeves and long pants (poison oak protection)
- ☐ Safety glasses or goggles
- ☐ Cut-resistant gloves (when using cutting tools)
- ☐ Hard hat (in areas with overhead hazard trees)
- ☐ Sun protection (hat, sunscreen)

5.2. Emergency Procedures

Refer to [SOP-F001 Field Safety](#) for stop-work conditions, biotic and weather hazards, emergency contacts, and incident response. Work in pairs at all times in forested areas.

6. Equipment and Materials

Item	Notes
Numbered aluminum tags	Pre-numbered; one per tree
Aluminum or stainless nails	For attaching tags; see Section 8.2 on growth allowance
Flagging tape (color set)	Per the color codes in Section 7
Diameter (DBH) tape	Reads diameter directly in cm (pi tape)
GPS unit or smartphone	Record coordinates per SOP-F006
Camera or smartphone	Minimum three photos per tree
Field data sheets	Printed from Appendix A
Clipboard and pencils	Pencils work better than pens in wet conditions
Long measuring tape	For crown spread
Clinometer or laser rangefinder	For tree height (optional); see SOP-F604
Lumber crayon or paint marker	For marking the DBH measurement point
Hand saw or loppers (optional)	For small branch clearance to access the trunk

7. Flagging Color Codes

! Important

Confirm color codes with the crew and PI before working. Project-specific changes must be documented in the field log.

Flag Color	Designation
ORANGE	Confirmed removal: invasive species (primary target)
YELLOW	Confirmed removal: dead, dying, or hazard tree
PINK	Confirmed removal: restoration thinning
GREEN	Retain: do not remove
WHITE	Under review: secondary evaluation required (do not remove yet)
RED	Monitoring tree: reassess next cycle
BLUE	Approved native for transplant or seed collection

Rule: Tie flagging loosely at eye level. Do not wrap tightly; flagging can girdle the tree over time.

8. Procedure

8.1. Before Entering the Field (Daily Pre-Work)

Complete this checklist before leaving the vehicles each day:

- ☐ All PPE inspected and worn
- ☐ GPS units charged; project boundaries pre-loaded
- ☐ Tag number range assigned to each crew member (no duplicates)

- ☐ Communication check complete; phones functional
- ☐ Weather reviewed against the stop-work conditions in [SOP-F001](#)
- ☐ Crew briefed: target species, flag color codes, today's objectives
- ☐ Cutting tools sharpened, sheathed, and inspected

8.2. Tree Tagging Step-by-Step

Step	Action	Why it matters
1. Approach and evaluate	Assess tree form, lean, root zone, crown condition, and nearby hazards from more than one side before tagging	A tree that looks sound from one side may have rot, lean, or deadwood visible from another angle (International Society of Arboriculture, 2017)
2. Identify species	Confirm common and scientific name; cross-check against the project Invasive Priority List and Retain Species List. Use SOP-F602 iNaturalist for ID support where needed	Tagging the wrong species leads to the wrong management decision
3. Measure DBH	Measure at 1.37 m on the uphill side per SOP-F604	DBH drives removal priority, allometric calculations, and monitoring frequency
4. Assign flag color	Select the color per Section 7 and tie loosely at eye level	Color communicates the decision to every future crew member
5. Install tag	Fasten the numbered tag on the uphill side at DBH height with a single aluminum or stainless nail. Leave the nail head protruding about 2 to 3 cm so the tree can grow without engulfing the tag	A consistent, accessible tag is easy to relocate and read in later seasons; leaving the nail proud accommodates radial growth and bark ingrowth (USDA Forest Service, 2023)
6. Photograph	Take at least three photos: (a) full tree form, (b) tag number close-up, (c) notable defects or hazards	Photos resolve disputes and provide evidence if a tag is lost
7. Record data	Complete all fields in the tree assessment record (Appendix A) before moving to the next tree	If it is not written down, it did not happen

i Crown spread (optional field)

If crown spread is recorded, measure the widest spread across the crown, then measure a second spread at right angles to the first, and average the two: $(A + B) / 2$ (American Forests, 2014). Tree height, if recorded, is measured with a clinometer or laser rangefinder per SOP-F604.

8.3. Tree Condition Ratings

Use a consistent five-level scale. Note structural defects (lean, cracks, cavities, deadwood, included bark) that influence the rating and any hazard flag (International Society of Arboriculture, 2017).

Condition	Field indicators
EXCELLENT	No significant defects; vigorous, full crown; normal growth
GOOD	Minor defects; healthy overall; normal growth
FAIR	Moderate defects; reduced vigor; some dieback
POOR	Major defects; significant dieback; structural risk
DEAD	No live tissue; standing snag or fallen log

9. Quality Control

Perform these checks every tagging day:

1. **Tag uniqueness:** Confirm no tag number was used twice. Reconcile the day's tags against the assigned number ranges.
2. **Species cross-check:** Verify each species identification against the Invasive Priority and Retain Species lists. Flag uncertain IDs for review rather than guessing.
3. **Data completeness:** Confirm every tagged tree has a fully completed assessment record (Appendix A) with no blank required fields.
4. **Photo completeness (end of day):** Before leaving the site, scroll through the day's photos and confirm that every tag number has the three required photos (full form, tag close-up, defects). Re-photograph any tree that is missing photos before leaving.
5. **Second-observer check:** On a subset of trees (for example, 10%), have a second crew member independently confirm species, condition rating, and flag color. Resolve any disagreements with the field lead.

10. References

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11. Related SOPs

SOP	Title	Relationship
F001	Field Safety	Required prerequisite; hazards, stop-work, emergencies
F006	GPS and Site Navigation	Recording tree coordinates
F602	iNaturalist	Species identification support
F603	Vegetation Transects	Understory and shrub cover
F604	Tree DBH and Forest Inventory	DBH, height, and crown measurement
F606	Canopy Cover	Stand structure companion

12. Training Record

Before tagging trees independently, each student or new technician must:

1. Read this SOP in full
2. Read [SOP-F001 Field Safety](#) and pass a field safety check (PPE, stop-work conditions, emergency contacts)
3. Know the correct flag color codes
4. Demonstrate correct DBH measurement technique (per [SOP-F604](#))
5. Correctly tag and flag three practice trees with the crew lead observing
6. Complete a tree assessment record without errors
7. Take an acceptable three-photo set (full tree, tag close-up, defects)
8. Be signed off by the PI or field lead

Trainee	Date read SOP	Date practice trees	Signed off by	Date

13. Appendix A: Individual Tree Assessment Record

Print one copy per tree (or one per plot) before going to the field. Circle the appropriate option where choices are given.

Tag number: _____ GPS coordinates: _____

Species (common): _____

Species (scientific): _____

DBH: _____ (circle: in / cm) **Est. height (ft):** _____ (clinometer or rangefinder; see SOP-F604)

Crown spread (ft): _____ (average of two perpendicular axes: $(A + B) / 2$)

Condition rating: EXCELLENT / GOOD / FAIR / POOR / DEAD (circle one)

Flag color: ORANGE / YELLOW / PINK / GREEN / WHITE / RED / BLUE (circle one)

Proximity hazards (e.g., lean toward trail, overhanging deadwood, near utility): _____

Adjacent utilities? YES / NO (if yes, note type and distance): _____

Removal rationale (e.g., invasive species, hazard, restoration thinning, dead): _____

Photo file references (e.g., TAG042_full, TAG042_tag, TAG042_defect): _____

Assessor initials: _____ **Date and time:** _____

Additional notes / sketch (trunk abnormalities, lean direction, nearby landmarks):

14. Appendix B: Daily Photo Log

Minimum three photos per tree. Store photos with the tag number in the filename (for example, TAG042_full_form.jpg).

Photo #	Tag #	Subject	Direction facing	Notes
1		Full tree form		
2		Tag number close-up		
3		Defects / hazards		
4				
5				

15. Appendix C: Field Note

This SOP is a field guide. It does not replace project permits or crew lead direction. Review this SOP with your crew lead at the start of each project season.



Environmental Analysis Program

Pomona College
Biogeochemistry Laboratory

SOP-F608

Plant Tissue Sampling

Standard Operating Procedure

SOP Number	SOP-F608
Revision	0.3
Author(s)	Marc Los Huertos
Date	2026-06-26
Organization	EA Program, Pomona College

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1. Purpose

This SOP describes procedures for collecting, handling, and preserving plant tissue samples in the field for subsequent laboratory analysis. Plant tissue analysis provides data on nutrient concentrations (C, N, P, K, metals), stable isotope ratios ($\delta^{13}\text{C}$, $\delta^{15}\text{N}$), and structural traits such as leaf mass per area (LMA) (Kalra, 1998; Mills & Jones, 2004). Proper sampling technique is essential to ensure that results represent the target tissue and are not contaminated or degraded during collection and transport.

Foliar nutrient concentrations and LMA form part of the “leaf economics spectrum” (LES), a global axis of plant strategy that links leaf structure to function across biomes: species with low LMA and high leaf nitrogen tend toward fast resource acquisition and short leaf lifespans, while species with high LMA and low nitrogen invest in durable, long-lived leaves (Reich, 2014; Wright et al., 2004). Extending this framework, the “fast-slow” plant economics spectrum integrates leaf, stem, and root traits into a whole-plant strategy axis (Reich, 2014; Westoby et al., 2002). Standardized sampling protocols allow EA Program data to be compared with global trait databases (e.g., TRY, BIEN) and regional reference values (Cornelissen et al., 2003; P’erez-Harguindeguy et al., 2013).

2. Scope

- **Applies to:** Collection of leaf, stem, root, and reproductive tissue from vascular plants for nutrient and isotope analysis at all EA Program field sites
- **Does not apply to:** Soil organic matter sampling (see [SOP-F400](#)); algal tissue sampling; plant tissue culture; DNA extraction from plant tissue (see [SOP-L700](#))
- **Location:** All EA Program field sites; initial processing in SGM 133/134

3. Definitions

- **Tissue type:** The specific plant organ sampled (e.g., fully expanded mature leaves, current-year stem growth, fine roots less than 2 mm diameter, seeds).
- **Composite sample:** A sample formed by combining tissue from multiple individuals of the same species within a plot to represent the population average. Compositing reduces analytical cost but eliminates information about individual-level variation.
- **Voucher specimen:** A pressed and identified plant specimen collected alongside tissue samples to verify species identification.
- **Leaf mass per area (LMA):** The ratio of leaf dry mass to one-sided leaf area (g m^{-2}). LMA is a key functional trait that reflects the trade-off between resource capture (photosynthesis per unit area) and leaf durability. High-LMA leaves are thicker or denser, with lower nitrogen per unit mass but greater physical toughness (Poorter et al., 2009; Wright et al., 2004).
- **Specific leaf area (SLA):** The inverse of LMA: $\text{SLA} = 1/\text{LMA}$, expressed as $\text{m}^2 \text{kg}^{-1}$. SLA and LMA contain the same information but are used in different contexts; report whichever is standard for the analysis.
- **Leaf economics spectrum (LES):** A global pattern relating leaf traits (LMA, nitrogen, phosphorus, photosynthetic capacity, and leaf lifespan) along a single axis of variation. High-nitrogen, low-LMA species occupy the “fast return” end; low-nitrogen, high-LMA species occupy the “slow return” end (Wright et al., 2004).

- **Sun leaf / shade leaf:** Leaves developed in high-light (sun) or low-light (shade) conditions. Sun and shade leaves from the same tree can differ by 20–40% in nitrogen concentration and by a factor of two or more in LMA (Cornelissen et al., 2003; P’erez-Harguindeguy et al., 2013). Sampling must standardize canopy position to avoid confounding light environment with species-level traits.
- **Field blank:** An empty labeled sample container opened and exposed at the field site, then sealed and processed identically to real samples. Blanks detect contamination introduced during collection and transport.

4. Responsibilities

Role	Responsibility
PI / Field Lead	Approve sampling design and target species
Trained Personnel	Collect and process tissue per this SOP
New Students	Complete training before independent sampling

5. Safety and Hazards

Hazards

- **Biological:** Contact with allergenic plants (poison oak, stinging nettles). Some target species may have thorns or irritating sap.
- **Physical:** Use of pruning shears and scissors poses a laceration risk.
- **Chemical:** If using liquid nitrogen for snap-freezing, follow cryogenic safety procedures (gloves, face shield, ventilated area).

5.1. Required PPE

- ☐ Nitrile gloves
- ☐ Closed-toe shoes or field boots
- ☐ Sun protection
- ☐ Leather gloves (for thorny species)
- ☐ Cryogenic gloves and face shield (if using liquid nitrogen)

5.2. Emergency Procedures

For cuts from pruning shears, apply direct pressure and clean the wound. For poison oak exposure, wash with cold water and soap as soon as possible. Refer to [SOP-F001 Field Safety](#).

6. Required Materials

Item	Notes
Stainless steel pruning shears or scissors	Clean between species
Paper coin envelopes or labeled paper bags	For drying samples; avoid plastic for fresh tissue intended for drying
Ziplock bags (gallon size)	For fresh tissue requiring freezing
Cooler with ice packs	For transport of fresh tissue
Permanent markers (Sharpie)	For labeling
Pre-printed labels	Site, species, tissue type, date, collector
DI water squeeze bottle	For rinsing contamination
Ethanol (70%) in a spray bottle	For decontaminating tools between species
Paper towels	For drying tools after decontamination
GPS unit	For recording sampling locations
Plant identification guides	For species verification
Plant press (if collecting vouchers)	See SOP-F603 Section 10
Field data sheet	Pre-printed
Liquid nitrogen dewar and cryo-vials	Only if snap-freezing is required
Analytical balance (field-portable, optional)	For recording fresh mass
Leaf area meter or scanner (optional)	For measuring fresh leaf area in the lab before drying (needed for LMA calculation)

7. Estimated Time

Task	Time
Site setup and species identification	15–30 minutes
Tissue collection (10 samples)	30–60 minutes
Labeling and packing for transport	15 minutes
Lab processing (cleaning, drying)	Variable (see Section 9)

8. Procedure

8.1. Sampling Design

1. Define the target species, tissue type, and number of replicates in the sampling plan before going to the field
2. Determine whether individual or composite samples are needed. For nutrient analysis where individual variation is important, collect tissue from individual plants. For site-level averages, composite tissue from 5–10 individuals per species per plot.
3. Select individuals randomly within the sampling area. Avoid plants at plot edges, obviously damaged or diseased individuals, and plants with heavy herbivory or epiphyte loads (unless these are the target condition).

8.2. Leaf Tissue Collection

1. Put on clean nitrile gloves. Change gloves between species to avoid cross-contamination.
2. Select fully expanded, mature leaves from the outer canopy (sun leaves) unless the project specifically targets shade leaves or another position. Standardizing canopy position is critical for comparability: sun and shade leaves from the same individual can differ by 20–40% in nitrogen concentration and by a factor of two in LMA (Cornelissen et al., 2003; P'erez-Harguindeguy et al., 2013). For trees, sample from the sunlit portion of the mid- to upper crown; for herbs and grasses, sample the most recently fully expanded leaf.
3. Using clean pruning shears, clip the petiole at its base. For grasses, clip the most recent fully expanded blade at the ligule.
4. Collect enough tissue mass for the planned analyses. As a guideline:

Analysis	Minimum dry mass
C, N, S by EA-IRMS	2–5 mg
$\delta^{13}\text{C}$, $\delta^{15}\text{N}$	2–5 mg
Total P, metals (acid digestion)	100–500 mg
LMA (requires leaf area scan)	3–5 leaves per individual
Multiple analyses from one sample	2–5 g fresh weight recommended

5. For composite samples, collect an approximately equal mass of tissue from each individual and combine in a single bag.
6. Place tissue immediately into a labeled paper envelope (if air-drying) or a labeled Ziplock bag (if freezing or transporting fresh). For frozen samples, place bags into the cooler with ice packs.

8.3. Measuring Leaf Area (for LMA/SLA)

If LMA or SLA is needed, leaf area must be measured on fresh tissue before drying (P'erez-Harguindeguy et al., 2013):

1. Transport fresh leaves to the lab within 6 hours of collection, keeping them in sealed bags in the cooler to minimize wilting
2. Scan leaves on a flatbed scanner (600 dpi) or measure with a leaf area meter. Record total one-sided area (cm^2) for each sample.
3. Dry and weigh as described in Section 9.1
4. Calculate $\text{LMA} = \text{dry mass (g)} / \text{area (m}^2\text{)}$, or $\text{SLA} = \text{area (m}^2\text{)} / \text{dry mass (kg)}$

8.4. Stem, Root, and Reproductive Tissue

- **Stems:** Clip current-year growth. Record the diameter and length of the stem segment.
- **Fine roots (less than 2 mm diameter):** Excavate carefully around the base of the target plant using a trowel. Separate live roots from dead roots and soil. Rinse briefly with DI water in the field or at the first opportunity in the lab.
- **Seeds/fruits:** Collect from a minimum of 5 individuals. Record the phenological stage (e.g., immature, mature, dehiscent).

8.5. Decontamination Between Species

1. Spray pruning shears with 70% ethanol
2. Wipe dry with a clean paper towel
3. Allow to air-dry for 30 seconds before cutting the next species
4. For metal analysis, use stainless steel tools only (no galvanized or painted tools) and rinse with DI water after ethanol to remove residual salts

8.6. Labeling

Every sample must be labeled with:

- Site name and plot/transect ID
- Species (use the 6-letter code from [SOP-F603](#) Section 12.2)
- Tissue type (leaf, stem, root, seed)
- Individual or composite (if composite, record the number of individuals)
- Date (YYYY-MM-DD)
- Collector initials

Write on both the bag/envelope and a separate tag inside (in case the exterior label smudges).

8.7. Field Data Sheet

Record the following for each sample:

Field	Entry
Sample ID	Unique sequential number
Site / Plot	
Species	
Tissue type	
Number of individuals	(if composite)
Canopy position	Sun / shade / mixed
GPS coordinates	
Fresh mass (g)	(if field balance available)
Notes	Condition, herbivory, phenology

9. Sample Preservation and Lab Processing

9.1. Drying

The default preservation method for nutrient and isotope analysis (Jones, 2001):

1. Transport paper envelopes to the lab within 24 hours

2. Dry at 60 C in the drying oven ([SOP-L105](#)) for at least 48 hours or until a constant mass is reached
3. Record the dry mass
4. Store dried tissue in labeled coin envelopes in a desiccator or sealed container until grinding

! Temperature Matters

Do not exceed 60 C for tissue intended for nitrogen or carbon isotope analysis. Higher temperatures can volatilize nitrogen compounds and alter $\delta^{15}\text{N}$ values (Kalra, 1998). For samples intended only for total C and N by combustion (not isotope analysis), drying at 70 C is acceptable and reduces drying time.

9.2. Freezing

For analyses requiring fresh tissue (enzyme assays, gene expression, volatile compounds):

1. Transport in cooler with ice packs (or snap-freeze with liquid nitrogen in the field)
2. Transfer to the -80 C freezer ([SOP-L106](#)) within 6 hours of collection
3. Record in the freezer inventory

9.3. Grinding

1. Grind dried tissue to a fine powder using a ball mill or mortar and pestle
2. Clean the grinding equipment between samples (brush, then wipe with ethanol)
3. Store ground tissue in labeled microcentrifuge tubes or coin envelopes

10. Quality Control

- Collect at least one field blank per sampling event: open an empty labeled bag at the site, expose it to the same conditions as the samples, then seal and process identically
- For metal analysis, verify that tools are stainless steel and DI-rinsed
- Record the time between collection and drying/freezing on the data sheet; flag any samples exceeding the 24-hour (drying) or 6-hour (freezing) window
- For composite samples, verify that approximately equal mass was contributed by each individual
- When interpreting foliar nutrient results, compare against published reference ranges for the target species or functional group (Mills & Jones, 2004; Reuter & Robinson, 1997). Values outside the expected range may indicate contamination, analytical error, or genuine ecological signal (e.g., nutrient deficiency or toxicity).
- For LMA, verify that leaf area was measured on fresh, fully turgid tissue; wilted leaves underestimate area and inflate LMA (P'erez-Harguindeguy et al., 2013)

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12. Training Record

Before collecting plant tissue independently, personnel must:

1. Read this SOP and [SOP-F001 Field Safety](#)
2. Demonstrate species identification for the target community
3. Demonstrate proper collection technique (glove changes, tool decontamination, labeling, canopy position standardization)
4. Process one set of samples through drying and grinding under supervision
5. Be signed off by the PI or field lead



Environmental Analysis Program

Pomona College
Biogeochemistry Laboratory

SOP-F610

Benthic Macroinvertebrate Sampling

Standard Operating Procedure

SOP Number	SOP-F610
Revision	0.1
Author(s)	Marc Los Huertos
Date	2026-06-26
Organization	EA Program, Pomona College

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1. Purpose

This SOP describes procedures for collecting benthic macroinvertebrate (BMI) samples from wadeable streams. BMI communities are widely used indicators of water quality and biological integrity because different taxa have varying tolerances to pollution, habitat degradation, and hydrologic alteration. This SOP follows the California Surface Water Ambient Monitoring Program (SWAMP) protocols to enable comparison with statewide bioassessment datasets (Ode et al., 2016).

2. Scope

- **Applies to:** BMI sampling in wadeable streams (depth less than 1 m, velocity allowing safe wading) at all EA Program study sites
- **Does not apply to:** Non-wadeable rivers or lakes (require different sampling gear); estuarine or marine macroinvertebrates; terrestrial invertebrates; meiofauna (organisms passing through a 500 um sieve)
- **Location:** All EA Program stream sites

3. Responsibilities

Role	Responsibility
PI / Field Lead	Approve sampling plan; ensure permit compliance
Trained Personnel	Collect and preserve samples per this SOP
New Students	Complete training before independent sampling

4. Safety and Hazards

Hazards

- **Drowning and swift water:** Do not wade in flows deeper than waist height or where footing is unstable. Always face upstream. Use a wading rod for support.
- **Slippery substrates:** Algae-covered rocks are extremely slippery. Wear felt-soled or studded wading boots.
- **Biological:** Waterborne pathogens, ticks in riparian vegetation, stinging insects
- **Chemical:** Ethanol (95%) preservative is flammable. Keep away from ignition sources.

4.1. Required PPE

- ☐ Chest or hip waders with felt or studded soles

- ☐ Nitrile gloves
- ☐ Safety glasses (when handling preservatives)
- ☐ Sun protection

4.2. Permits

BMI sampling may require a Scientific Collecting Permit (California Department of Fish and Wildlife). The PI must confirm permit status before fieldwork at any new site. Some sites may also fall under federal Section 7 or Section 10 requirements if listed species are present.

4.3. Invasive Species Prevention

Decontaminate all equipment (nets, waders, buckets) between sites to prevent transfer of invasive species and pathogens. Inspect, scrub, and dry all gear, or treat with dilute bleach or hot water (60 C). See [SOP-F304 Section 4.3](#).

4.4. Emergency Procedures

A minimum of two people must be present for in-stream work. Refer to [SOP-F001 Field Safety](#).

5. Definitions

- **Benthic macroinvertebrate (BMI):** An invertebrate organism living on or in the stream bottom substrate that is retained by a 500 um mesh sieve. Includes aquatic insects, crustaceans, mollusks, worms, and other taxa.
- **Riffle:** A shallow stream section with fast, turbulent flow over coarse substrate. The primary habitat sampled for BMI in wadeable streams.
- **Reach:** A 150 m to 250 m stream segment (typically 150 m in California SWAMP protocols) within which all sampling occurs.
- **Targeted riffle composite (TRC):** The SWAMP standard method in which BMI samples are collected from multiple riffles and composited into a single reach-level sample.
- **D-frame kick net:** A D-shaped net with 500 um mesh mounted on a frame, used for dislodging and capturing BMI from the streambed.

6. Required Materials

Item	Notes
D-frame kick net (500 um mesh)	Standard SWAMP gear
500 um sieve (brass or stainless steel)	For field processing
White sorting trays	For inspecting samples
Sample jars (1 L, wide-mouth, HDPE)	Pre-labeled

Item	Notes
95% ethanol	For preservation (final concentration approximately 70% after dilution by sample water)
Squirt bottles (DI water and ethanol)	For rinsing nets and transferring organisms
Forceps (soft-tipped)	For picking organisms from the net
Flagging tape	For marking transect locations
GPS unit	For recording reach coordinates
Measuring tape (50 m)	For delineating the reach
Field data sheets	SWAMP BMI collection forms
Camera	For photo-documenting reach conditions
Pencils and waterproof notebook	For field notes
Cooler	For transporting preserved samples

7. Estimated Time

Task	Time
Reach setup and reconnaissance	15–30 minutes
BMI collection (targeted riffle composite)	45–90 minutes
Field processing and preservation	15–30 minutes
Decontamination	10–15 minutes

8. Procedure

8.1. Reach Delineation

1. Identify the downstream boundary of the reach at the most downstream riffle or pool tail
2. Lay out the measuring tape upstream for 150 m (SWAMP standard reach length; adjust proportionally for streams narrower than 2 m or wider than 10 m per SWAMP guidance)
3. Record GPS coordinates at the downstream and upstream boundaries
4. Photograph the reach from the downstream end looking upstream

8.2. Habitat Assessment

Before disturbing the streambed for BMI collection, complete the physical habitat assessment per [SOP-F304](#) if required by the project design. If habitat and BMI are collected on the same visit, always complete habitat measurements first (working upstream) before BMI sampling.

8.3. Targeted Riffle Composite Collection (SWAMP Method)

The SWAMP TRC method collects BMI from riffle habitats throughout the reach, compositing all kicks into a single sample.

1. Identify all riffles within the 150 m reach. A riffle is defined as a section with visibly turbulent flow over coarse substrate (gravel, cobble, boulder).
2. Allocate sampling effort across riffles proportional to their area. The target is 8 kicks distributed across all available riffles. If only one riffle is present, all 8 kicks are taken from that riffle.
3. Position yourself facing downstream at the downstream edge of the first riffle
4. Place the D-frame kick net on the substrate, opening facing upstream, with the bottom edge pressed firmly against the streambed
5. Disturb the substrate immediately upstream of the net opening by vigorously kicking and rubbing rocks with your boots for 30 seconds over an area of approximately 0.09 m² (one net width by one net width)
6. Lift the net and inspect the contents. Remove large rocks, sticks, and leaves after rinsing them in the net to dislodge clinging organisms.
7. Transfer the net contents into the composite sample bucket or directly into the sieve
8. Move upstream to the next kick location. Space kicks at least 2 m apart within each riffle.
9. Repeat until all 8 kicks are completed across the available riffles

! Always Work Upstream

Sampling must proceed from downstream to upstream to avoid disturbing habitats before they are sampled. Never walk through a riffle before sampling it.

8.4. Field Processing

1. Pour the composite sample through the 500 um sieve
2. Rinse the net and bucket thoroughly into the sieve to recover all organisms
3. Transfer the sieved material into a labeled 1 L sample jar
4. Add enough 95% ethanol to achieve a final preservative concentration of approximately 70% (the sample water dilutes the ethanol; adding ethanol equal to the sample volume is a reasonable approximation)
5. Seal the jar tightly. Invert gently to mix.
6. Label the jar with: site ID, date, “BMI-TRC,” collector initials. Place a second label (pencil on waterproof paper) inside the jar.

8.5. Non-Riffle Habitats (Optional)

If the project requires sampling pools, runs, or other habitats in addition to riffles, collect these as separate samples (not composited with the TRC). Label accordingly (e.g., “BMI-POOL”).

8.6. Field Data Sheet

Record the following:

Field	Entry
Site ID	
Date	YYYY-MM-DD
Collectors	
Reach length (m)	
Number of riffles sampled	
Number of kicks	(target: 8)
GPS (downstream boundary)	
GPS (upstream boundary)	
Weather conditions	
Flow conditions	Normal, low, high
Preservative used	95% EtOH
Notes	Unusual conditions, disturbances

9. Laboratory Processing

Laboratory identification and enumeration of BMI are typically performed by a contracted taxonomic laboratory or by trained students under PI supervision. The general workflow:

1. Transfer the preserved sample to a gridded sorting tray
2. Randomly select grid cells and pick all organisms from those cells until a minimum of 500 organisms are identified (SWAMP standard)
3. Identify organisms to the lowest practical taxonomic level (genus for most insects; family or higher for non-insect taxa) using standard keys (Merritt et al., 2019; Thorp & Rogers, 2015)
4. Record counts by taxon
5. Calculate summary metrics: total taxa richness, EPT (Ephemeroptera, Plecoptera, Trichoptera) richness, percent tolerant taxa, and other indices as required
6. Archive voucher specimens of difficult identifications

i SWAMP Data Submission

If data will be submitted to the California Environmental Data Exchange Network (CEDEN), use SWAMP-compatible data formats and QA/QC protocols. Consult the SWAMP Bioassessment SOP 004 (Ode et al., 2016) and the PI.

10. Quality Control

10.1. Field QC

- Record the number of kicks and their distribution across riffles on the data sheet
- If fewer than 8 kicks are possible (limited riffle habitat), record the actual number and the reason
- Take one field blank per sampling event: rinse the net into a clean jar with ethanol; this documents any organisms retained in the net from the decontamination process

10.2. Lab QC

- Re-sort 10% of processed samples to estimate sorting efficiency (target: greater than 90% removal of organisms from the sample)
- Have a second taxonomist verify identification of 10% of specimens
- Report QC results with the final dataset

10.3. Metric Calculation

Calculate the California Stream Condition Index (CSCI) if sufficient data are available. CSCI integrates observed vs. expected taxa composition (O/E) and a predictive multi-metric index (pMMI) into a single biological condition score (Mazor et al., 2016).

11. Waste Disposal

Waste Type	Disposal Method
Ethanol-preserved samples (after analysis)	Evaporate ethanol in fume hood; dispose of residual solids in regular trash
Excess 95% ethanol	Flammable waste container; EH&S pickup
Used gloves and paper towels	Regular trash

12. References

See the project-level **references.bib** for full citations. Key references: Ode et al. (2016), Barbour et al. (1999), Mazor et al. (2016), Merritt et al. (2019), Thorp & Rogers (2015).

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13. Training Record

Before collecting BMI samples independently, personnel must:

1. Read this SOP, [SOP-F001 Field Safety](#), and [SOP-F304 Stream Physical Habitat](#)
2. Review the SWAMP Bioassessment SOP 004 (BMI collection sections)
3. Demonstrate D-frame kick net technique (proper positioning, 30-second kick, net rinsing) under supervision
4. Participate in at least one complete reach-level BMI collection event
5. Demonstrate proper preservation and labeling
6. Be signed off by the PI or field lead



Environmental Analysis Program

Pomona College
Biogeochemistry Laboratory

SOP-F611

Pollinator Observation Surveys

Standard Operating Procedure

SOP Number	SOP-F611
Revision	0.2
Author(s)	Marc Los Huertos
Date	2026-06-26
Organization	EA Program, Pomona College

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1. Purpose

This SOP describes standardized methods for surveying pollinator communities at EA Program field sites using timed visual observations and passive pan traps. Pollinator surveys document the diversity and relative abundance of bees, butterflies, flies, beetles, and other flower-visiting insects, providing data for assessing habitat quality, restoration success, and the effects of land use on pollination services (Lebuhn & Fenter, 2013; Potts et al., 2010).

2. Scope

- **Applies to:** Standardized pollinator observation surveys and pan trap sampling at all EA Program terrestrial field sites with flowering vegetation, including the Bernard Field Station, campus landscapes, restoration sites, and agricultural study areas
- **Does not apply to:** Targeted bee nesting surveys; hive management; pollen analysis (paleontology); pollinator genetics or molecular identification (though specimens collected here may be used for those purposes); aquatic insect sampling (see [SOP-F610](#))
- **Location:** All EA Program field sites with floral resources

3. Responsibilities

Role	Responsibility
PI / Field Lead	Approve survey design and collection permits
Trained Personnel	Conduct surveys and process pan trap samples per this SOP
New Students	Complete training (insect identification and sting safety) before independent work

4. Definitions

- **Diel activity pattern:** The temporal distribution of an organism's activity across a 24-hour period; most bee species are diurnal with peak activity between 09:00 and 15:00 under sunny, warm conditions; surveys outside peak activity hours systematically underestimate community richness (Lebuhn & Fenter, 2013).
- **Floral density:** The number of open flowers per unit area at a survey location; a key covariate for pollinator abundance because visitation scales with resource availability; estimated visually as flowers per m² or as qualitative categories (low, medium, high) at each survey event.
- **Focal flower observation:** A survey method in which a single flowering plant or patch is observed for a fixed duration (typically 10 minutes) and all pollinators visiting any flower during that period are recorded; useful for comparing visitation rates between plant species or treatments (Westphal et al., 2008).
- **Functional group:** A coarse taxon category used when species-level identification is not possible in the field; the EA Program uses 10 groups: honey bee (HB), bumble bee (BB),

small native bee (SB), large native bee (LB), butterfly (BF), syrphid fly (SF), other fly (OF), beetle (BT), wasp (WA), and other (OT).

- **Independent detection event:** A single detection of a pollinator at a flower, counted only once per survey period regardless of how long the individual remains; used to standardize counts across surveys of different duration.
- **Pan trap:** A colored bowl (white, fluorescent yellow, fluorescent blue) filled with soapy water; passively captures flying insects attracted by color resemblance to flowers; effective for small- to medium-sized bees but under-represents large-bodied bees and butterflies (Lebuhn & Fenter, 2013; Westphal et al., 2008).
- **Pollinator:** Any animal that moves pollen from the anther to the stigma of a flower, facilitating plant reproduction; includes bees, flies, butterflies and moths, beetles, and hummingbirds; bees are the dominant pollinators at most EA Program field sites (Potts et al., 2010).
- **Pollination service:** An ecosystem service provided by flower-visiting animals; essential for reproduction of approximately 75% of flowering plant species and 35% of global food crop production (Potts et al., 2010).
- **Timed observation transect:** A standardized survey in which the observer walks a fixed route (typically 50 m x 2 m) at a standardized pace (approximately 10 m per minute) and records all pollinators observed on flowers within the survey corridor; produces a count-based estimate of relative pollinator activity (Dicks et al., 2016; Lebuhn & Fenter, 2013).
- **Voucher specimen:** A preserved insect specimen deposited in a reference collection to document the occurrence of a species at a site; required for any first records and difficult identifications; pinned through the right mesopleuron following standard entomological practice.

5. Safety and Hazards

Hazards

- **Biological:** Bee and wasp stings are the primary hazard. Personnel with known Hymenoptera allergies must carry a prescribed epinephrine auto-injector (EpiPen) and inform the field lead before every outing. Africanized honey bees may be present at some Southern California sites.
- **Chemical:** Soapy water in pan traps (low risk). Ethanol (70%) used for specimen preservation is flammable.
- **Physical:** Heat exposure during mid-day surveys. Uneven terrain. Pan traps may be trampled by people or wildlife.

5.1. Required PPE

- ☐ Closed-toed shoes or field boots
- ☐ Light-colored clothing (dark colors attract bees)
- ☐ Sun protection (hat, sunscreen)
- ☐ EpiPen (if allergic; inform field lead)
- ☐ Nitrile gloves (when handling preserved specimens)

5.2. Permits

Insect collection may require a Scientific Collecting Permit from CDFW depending on the site and target taxa. No permit is needed for observation-only surveys.

5.3. Emergency Procedures

For anaphylactic reactions: administer EpiPen, call 911. Refer to [SOP-F001 Field Safety](#). If encountering an aggressive swarm, walk calmly away; do not swat or run erratically.

6. Equipment and Materials

6.1. Timed Observations

Item	Notes
Stopwatch or phone timer	For standardizing observation periods
Hand lens or magnifying glass	For in situ identification
Camera (macro-capable or phone)	For photo-documenting pollinators
Field data sheet	Pre-printed from Appendix A
Insect field guide	e.g., <i>California Bees and Blooms</i>
iNaturalist app (smartphone)	For photo-based identification; see SOP-F602
Compass and GPS	For recording transect or plot locations

6.2. Pan Traps

Item	Notes
Plastic bowls (12 oz, 3.25 in rim)	White, fluorescent blue, and fluorescent yellow; one of each color per station
Fluorescent spray paint (blue, yellow)	For painting white bowls; use UV-bright paints
Unscented liquid dish soap	2–3 drops per bowl (breaks surface tension)
Water	To fill bowls approximately 2/3 full
Wooden stakes or wire stands	For elevating bowls to canopy height in tall vegetation
Fine mesh strainer	For removing insects from bowls
Whirl-Pak bags or vials	For storing collected specimens
70% ethanol	For preserving specimens
Forceps (soft-tipped)	For handling specimens
Labels (waterproof, internal)	For vial and bag identification

7. Procedure

7.1. Survey Timing and Conditions

Conduct pollinator surveys during peak pollinator activity:

- **Time of day:** 09:00–15:00 local time (peak flight activity)
- **Weather:** Sunny to partly cloudy; wind speed below Beaufort 4 (< 5.5 m/s); temperature above 15 C. Do not survey during rain, heavy overcast, or high winds, as pollinator activity drops sharply.
- **Season:** During peak bloom at the study site. In Southern California, this is typically March through June for most habitats, with a secondary bloom in fall for some species.
- **Flowering requirement:** Surveys are only meaningful when flowers are present. Record the dominant flowering species and estimate floral density (flowers per m²) at each survey location.

7.2. Method A: Timed Observation Surveys

Timed observations record pollinator visits to flowers along a fixed route or within a fixed plot during a standardized time period (Dicks et al., 2016; Lebuhn & Fenter, 2013).

Transect walk method:

1. Establish a transect (typically 50 m x 2 m, or a 100 m x 1 m corridor) through the area of interest. Record the transect coordinates per [SOP-F006](#).
2. Walk the transect slowly at a pace of approximately 10 m per minute.
3. Record every pollinator observed visiting a flower within the 2 m band on either side of the transect center line (or 1 m on each side for a 2 m wide transect).
4. For each observation, record:
 - Pollinator group or species (see Section 6.4)
 - Flower species being visited
 - Behavior (nectaring, pollen collecting, resting)
 - Time of observation
5. Complete the transect in 10–15 minutes. Do not stop and wait for pollinators; maintain a steady pace.
6. Repeat the transect 2–3 times per sampling event, spaced at least 30 minutes apart.

Focal flower observation method:

1. Select a flowering plant or patch and observe for a fixed period (typically 10 minutes).
2. Record every pollinator that visits any flower on the focal plant during the observation period.
3. This method is useful for comparing visitation rates among plant species or treatments.

7.3. Method B: Pan Trap Sampling

Pan traps passively capture flying insects attracted to colored bowls (Lebuhn & Fenter, 2013; Westphal et al., 2008).

1. At each sampling station, set out three bowls: one white, one fluorescent blue, one fluorescent yellow. Space bowls approximately 3 m apart.
2. Fill each bowl approximately 2/3 full with water. Add 2–3 drops of unscented dish soap (this breaks the surface tension so insects sink).
3. Place bowls at the height of the surrounding vegetation canopy:
 - In short grass or bare ground: set directly on the ground
 - In tall vegetation (> 30 cm): elevate on stakes or wire stands so the bowl rim is at canopy height
4. Deploy traps in the morning (by 09:00) and retrieve after 24 hours. For shorter deployments (4–6 hours), retrieve in the afternoon.
5. Pour the contents of each bowl through a fine mesh strainer. Transfer captured insects to a labeled Whirl-Pak bag or vial with 70% ethanol.
6. Label each vial with: site, station number, bowl color, date deployed, date retrieved, and collector initials.
7. Return specimens to the lab for sorting, pinning, and identification.

Note

Pan traps are biased toward small- to medium-sized bees and may under-represent large-bodied bees (bumble bees) and butterflies (Westphal et al., 2008). Combine pan traps with timed observations for a more complete picture of the pollinator community.

7.4. Pollinator Identification

Identify pollinators to the finest taxonomic level possible in the field. At a minimum, assign each observation to one of the following functional groups:

Code	Group	Examples
HB	Honey bee	<i>Apis mellifera</i>
BB	Bumble bee	<i>Bombus</i> spp.
SB	Small native bee	<i>Halictus</i> , <i>Lasioglossum</i> , <i>Ceratina</i>
LB	Large native bee	<i>Xylocopa</i> , <i>Megachile</i>
BF	Butterfly	All Lepidoptera
SF	Syrphid fly (hover fly)	Syrphidae
OF	Other fly	Bombyliidae, Tachinidae, etc.
BT	Beetle	Flower-visiting Coleoptera
WA	Wasp	Vespidae, Sphecidae, etc.
OT	Other	Hummingbirds, moths, ants

Photograph all specimens that cannot be identified in the field. Upload photos to iNaturalist per [SOP-F602](#) for community-assisted identification.

7.5. Specimen Processing (Lab)

1. Sort pan trap specimens by order under a dissecting scope.
2. Pin bee specimens following standard entomological practice (pin through the right mesopleuron) and label with locality, date, collector, and determination.
3. Store pinned specimens in a labeled unit tray in the insect collection.
4. Specimens can be submitted to a taxonomic specialist or DNA barcoded (COI) for species confirmation.

8. Quality Control

- Record weather conditions (temperature, wind, cloud cover) at the start and end of every survey. Do not compare data collected under different weather conditions without accounting for detectability.
- On 10% of surveys, have two observers independently record pollinators on the same transect to assess inter-observer agreement.
- Record floral density and dominant flowering species at every survey; pollinator abundance is strongly influenced by floral resource availability.
- For pan traps, inspect bowls mid-deployment if wind or wildlife disturbance is likely. Refill or replace tipped bowls and note the interruption.
- Voucher-pin at least 5 representative specimens per functional group per site per season for the reference collection.

9. References

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10. Related SOPs

SOP	Title	Relationship
F001	Field Safety	Required prerequisite; sting response

SOP	Title	Relationship
F006	GPS and Site Navigation	Survey location coordinates
F602	iNaturalist	Photo-based identification
F603	Vegetation Transects	Floral resource documentation
F615	Bird Point Counts	Companion fauna survey method

11. Training Record

Before conducting pollinator surveys independently, personnel must:

1. Read this SOP and [SOP-F001 Field Safety](#) in full
2. Disclose any Hymenoptera allergy to the field lead and carry an EpiPen if prescribed
3. Demonstrate identification of the 10 pollinator functional groups (Section 6.4) using pinned specimens or photographs
4. Participate in a supervised transect observation survey and pan trap deployment
5. Be signed off by the PI or field lead

12. Appendix A: Pollinator Observation Data Sheet

Site: _____ Transect ID: _____ Date: _____

Observer: _____ Start time: _____ End time: _____

Weather: Temp: ____ C Wind (Beaufort): ____ Cloud: ____ % Sun: Y / N

Dominant flowering species: _____

Time	Pollinator Group/Species	Flower Species	Behavior	Photo #
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Environmental Analysis Program

Pomona College
Biogeochemistry Laboratory

SOP-F620

**Camera Trap Deployment and
AI-Assisted Image Analysis**

Standard Operating Procedure

SOP Number	SOP-F620
Revision	0.2
Author(s)	Marc Los Huertos
Date	2026-06-26
Organization	EA Program, Pomona College

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1. Purpose

This procedure describes the deployment, retrieval, data management, and AI-assisted image analysis of camera traps (motion-triggered wildlife cameras) used in the EA Program. Camera traps

provide a non-invasive method for documenting wildlife presence, activity patterns, and species richness at field sites (Thornton et al., 2026). The volume of images produced by even a small number of cameras (thousands to tens of thousands per deployment) makes manual review a major bottleneck. This SOP integrates open-source and cloud-based AI tools to automate blank-image filtering and species identification, reducing processing time by an order of magnitude while maintaining scientific rigor through human-in-the-loop verification (Beery et al., 2019; Wildlife Insights, 2026).

The EA Program currently uses the CamPark T20 trail camera, but the deployment and analysis procedures are applicable to other camera trap models with minor adaptations.

2. Scope

- **Applies to:** Deployment and image analysis of all EA Program camera traps at field sites, including the Bernard Field Station, Pomona College Farm, the Pit, and restoration areas
- **Does not apply to:** Live-view video surveillance systems; drone imagery (see [SOP-F630](#)); 360-degree cameras; aquatic camera systems
- **Location:** All EA Program field sites (deployment); EA Program laboratory (data analysis)

3. Responsibilities

Role	Responsibility
Lab PI	Approve deployment plan, oversee data management
Field Lead	Select camera locations, coordinate deployment and retrieval
Trained Personnel	Deploy/retrieve cameras, process images
New Students	Complete training before independent deployment

4. Definitions

- **AI confidence score:** A numerical value (0–1 or 0–100%) output by a classification model indicating the model’s certainty in its predicted species identification; predictions below 90% confidence must be manually verified in the EA Program workflow (Beery et al., 2019).
- **Blank image:** A camera trap image with no animal, person, or vehicle visible, typically triggered by vegetation movement or lighting changes; MegaDetector removes these automatically, eliminating the largest portion of review burden.
- **CamPark T20:** The EA Program’s primary trail camera; a 16 MP passive infrared (PIR) triggered camera with infrared night flash; detection range approximately 20 m; 8 AA batteries per deployment (CamPark, 2023).
- **Detection rate:** The number of independent detections of a species per 100 camera-trap-nights; the standard metric for comparing wildlife activity across sites or seasons; depends on both actual animal abundance and camera placement (O’Brien et al., 2010).

- **Human-in-the-loop verification:** A quality control protocol in which a human reviewer examines AI predictions, particularly low-confidence ones, to correct errors before results are used for analysis; essential because AI models fail on rare species, unusual poses, and taxa underrepresented in training data (Thornton et al., 2026).
- **Independent detection event:** A single occurrence of a species at a camera during a defined time window (typically 30 minutes); used to prevent over-counting multiple images of the same individual; defined a priori and reported with any analysis (O'Brien et al., 2010).
- **MegaDetector:** An open-source object detection model (Microsoft/Google) that classifies each camera trap image as containing an animal, a human, a vehicle, or being blank; runs as a pre-processing step before species classification; the current version is MegaDetector v5 (Beery et al., 2019).
- **PIR (passive infrared) sensor:** The motion and heat detection sensor in a trail camera; triggers image capture when it detects a warm moving object within the detection zone; sensitivity and detection range vary with temperature, wind, and vegetation.
- **SpeciesNet:** A deep-learning species classification model developed by Google; trained on over 65 million labeled camera trap images from more than 2,000 species and blank categories; used in both the Wildlife Insights platform and the local CameraTrapAI pipeline (Google Research, 2026; Wildlife Insights, 2026).
- **Trap night (camera trap night):** One camera deployed at one location for one 24-hour period; the standard unit of sampling effort; total trap nights = number of cameras × number of days deployed (O'Brien et al., 2010).
- **Wildlife Insights:** A free cloud-based platform for camera trap data management and AI classification, developed by Google, WWF, Conservation International, the Wildlife Conservation Society, and the Smithsonian (Wildlife Insights, 2026).

5. Safety and Hazards

Hazards

- **Biological:** Tick and insect exposure, poison oak, snakes. Wildlife encounters when checking cameras in remote locations.
- **Physical:** Uneven terrain, climbing to mount cameras on trees or posts.
- **Security:** Cameras left unattended in public areas may be stolen or vandalized.

5.1. Required PPE

- ☐ Closed-toed shoes or field boots
- ☐ Long pants (tick and poison oak protection)
- ☐ Sun protection (hat, sunscreen)
- ☐ Tick repellent

Refer to [SOP-F001 Field Safety](#). Cameras may inadvertently photograph people; consult the PI regarding institutional requirements for signage and data handling, and delete identifiable images immediately during initial screening.

6. Equipment and Materials

Item	Specifications
CamPark T20 trail camera	16 MP; detection range 20 m; IR night flash (CamPark, 2023)
SD card (Class 10, up to 32 GB)	One per camera
AA batteries (8 per camera)	Lithium recommended for extremes
Mounting strap or bracket	Included with camera
Cable lock (optional)	Theft deterrent for public-access sites
GPS unit or smartphone	Per SOP-F006
Field data sheet	Printed from Appendix A
Pruning shears	For clearing detection zone
Compass	For recording camera bearing
SD card reader (USB)	For lab image download
External hard drive or cloud	For archiving raw images

7. Procedure – Field Deployment

7.1. Camera Setup

1. Install 8 AA batteries and insert a formatted SD card (CamPark, 2023).
2. Power on and enter the settings menu. Configure:

Setting	Recommended Value	Notes
Photo resolution	16 MP	Highest available
Trigger mode	Photo (burst × 3)	Burst increases detection probability
Trigger interval	5 seconds	Balance captures and battery
Time stamp	ON	Required for activity analysis
Date/time	Current local time	Verify at each deployment

3. Take a test photo to verify function and timestamp.

7.2. Site Selection and Mounting

1. Select a location based on study objectives (game trails, water sources, habitat edges, or systematic grid points).
2. Record GPS coordinates and camera bearing (compass direction camera faces) (O'Brien et al., 2010).
3. Clear vegetation within the detection zone (approximately 20 m cone in front of the lens) to reduce false triggers.
4. Mount at 30–50 cm above ground for small-to-medium mammals, or 75–100 cm for larger species; angle slightly downward (5–10°).

5. Secure with mounting strap. Apply a cable lock if at a public site.
6. Walk through the detection zone to verify the trigger functions.
7. Record deployment metadata on the field data sheet (Appendix A).

7.3. Retrieval and Redeployment

1. Power off before removing the SD card.
2. Replace with a fresh, formatted card; check battery level; replace batteries if below 50%.
3. Power on, verify settings, and take a test photo.
4. Record retrieval date, time, and observations on the field data sheet.

8. Procedure – Data Management and AI Analysis

8.1. Image Download and Organization

1. Create a folder: `CamTrap_SiteID_CameraID_YYYYMMDD-YYYYMMDD`.
2. Copy all images from the SD card.
3. Back up immediately to external hard drive or cloud storage.
4. Do not delete images from the SD card until the backup is verified.

8.2. AI-Assisted Analysis Pipeline

The EA Program uses a two-stage AI pipeline following the approach used by Wildlife Insights and the Google CameraTrapAI project (Google Research, 2026; Wildlife Insights, 2026):

Stage 1: Detection (MegaDetector). Identifies whether each image contains an animal, human, vehicle, or is blank; eliminates 50–90% of false-trigger images (Beery et al., 2019).

Stage 2: Classification (SpeciesNet or similar). Identifies detected animals to the lowest possible taxonomic level, trained on over 65 million labeled camera trap images.

Option A: Wildlife Insights (cloud-based). Upload images to wildlifeinsights.org, which runs both stages automatically (Wildlife Insights, 2026). Review AI predictions and export verified data as CSV.

Option B: Local pipeline (Python). For offline or large-volume processing, use the open-source CameraTrapAI package from github.com/google/cameratrapai (Beery et al., 2019; Google Research, 2026).

8.3. Human Verification

1. Sort AI output by confidence score (ascending).
2. Manually verify all predictions below 90% confidence (Thornton et al., 2026).
3. Spot-check a random 10% of predictions above 90% confidence.
4. Correct misclassifications and record reviewer name, date, and total corrections.

8.4. Data Analysis in R

```
library(tidyverse)
library(lubridate)
library(camtrapR) # Niedballa et al. 2016

detections <- read_csv("classifications_verified.csv") %>%
  mutate(datetime = ymd_hms(timestamp),
         date = as_date(datetime),
         hour = hour(datetime))

# Independent events (30-min threshold)
independent <- detections %>%
  filter(category == "animal") %>%
  arrange(camera_id, species, datetime) %>%
  group_by(camera_id, species) %>%
  mutate(
    time_diff_min = as.numeric(difftime(datetime, lag(datetime),
                                         units = "mins")),
    independent = is.na(time_diff_min) | time_diff_min >= 30
  ) %>%
  filter(independent) %>%
  ungroup()

# Trap-nights per camera
trap_nights <- detections %>%
  distinct(camera_id, date) %>%
  count(camera_id, name = "trap_nights")

# Detection rate (events per 100 trap-nights)
det_rate <- independent %>%
  count(camera_id, species) %>%
  left_join(trap_nights, by = "camera_id") %>%
  mutate(rate_per_100tn = (n / trap_nights) * 100)

print(det_rate)
```

(Niedballa et al., 2016)

9. Quality Control

- Test-fire each camera before leaving the deployment site.
- Record all deployment metadata on the field data sheet.
- Check cameras every 2–4 weeks to replace batteries and SD cards.
- Report the AI model version, confidence threshold, verification protocol, and correction rate in any publication (Thornton et al., 2026).

10. References

- CamPark (2023)
- Beery et al. (2019)
- Wildlife Insights (2026)
- Google Research (2026)
- Thornton et al. (2026)
- Niedballa et al. (2016)
- O'Brien et al. (2010)

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Google Research. (2026). *Where wild things roam: Identifying wildlife with SpeciesNet*. <https://research.google/blog/where-wild-things-roam-identifying-wildlife-with-speciesnet/>

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Wildlife Insights. (2026). *Wildlife insights: About AI*. <https://www.wildlifeinsights.org/about-wildlife-insights-ai>

11. Related SOPs

SOP	Title	Relationship
F001	Field Safety	Required prerequisite
F006	GPS and Site Navigation	Camera deployment coordinates
F602	iNaturalist	Photo-based species documentation
F630	Drone Operations	Complementary remote sensing
L801	RStudio and GitHub	Data version control

12. Training Record

Before deploying camera traps independently, personnel must:

1. Read this SOP and [SOP-F001](#) in full

2. Demonstrate camera setup under supervision
3. Deploy and retrieve at least one camera with proper metadata recording
4. Process one SD card through the full AI pipeline (download, MegaDetector, SpeciesNet or Wildlife Insights, verification)
5. Produce a verified species list with activity pattern summary
6. Be signed off by the PI or field lead

13. Appendix A: Field Data Sheet

Field	Deployment	Retrieval
Date		
Time		
Site ID		—
Camera serial no.		—
SD card no.		
GPS coordinates		—
Camera bearing (°)		—
Camera height (cm)		—
Batteries	Fresh	_____ %
Test photo taken	Y / N	—
Notes		



Environmental Analysis Program

Pomona College
Biogeochemistry Laboratory

SOP-F630

Drone Operations (DJI Mavic and Matrice UAVs)

Standard Operating Procedure

SOP Number	SOP-F630
Revision	1.1
Author(s)	Liz Johnson, Marc Los Huertos
Date	2026-06-26
Organization	EA Program, Pomona College

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1. Purpose

This SOP details the procedures for safely operating the EA Program's DJI unmanned aerial vehicles (UAVs/drones) for research image collection (DJI, 2015, 2023). It covers pre-flight setup, flight operations, image collection, post-flight procedures, and data transfer. Images obtained from these aircraft are used for photogrammetry (3D modeling), site monitoring, vegetation mapping, and other remote sensing applications.

2. Scope

- **Applies to:** All flights of EA Program drones, including the DJI Mavic 3 Pro and DJI Matrice 100, for research and instructional purposes
- **Does not apply to:** Indoor flights; flights conducted by contracted third-party operators; processing of drone imagery beyond data transfer (see future SOP-L805)
- **Location:** All EA Program field sites and approved flying areas

3. Definitions

- **AGL (Above Ground Level):** Flight altitude measured from the ground surface directly below the aircraft, not from mean sea level; FAA Part 107 limits sUAS to 400 feet AGL (approximately 122 m) in uncontrolled airspace (Federal Aviation Administration, 2024b).
- **Compass calibration:** A pre-flight procedure that orients the drone's internal compass to the local magnetic field; required at each new site or after transporting the aircraft; performed by rotating the aircraft in specific orientations per the DJI app prompts (DJI, 2023).
- **DJI Mavic 3 Pro:** A compact foldable quadcopter with a three-camera Hasselblad system; the EA Program's primary survey aircraft stored in SGM 106; flight time approximately 43 minutes; maximum wind resistance 12 m/s (Beaufort 6) (DJI, 2023).
- **DJI Matrice 100:** A legacy quadcopter platform capable of carrying a research payload of up to 1 kg; stored in SGM 210; suited for multi-sensor work; requires the DJI Pilot app for operation (DJI, 2015).
- **Gimbal:** The motorized, stabilized mount that holds the camera; compensates for drone movement to produce level, stable imagery; always reinstall the gimbal guard during transport.
- **LAANC (Low Altitude Authorization and Notification Capability):** An FAA system that provides near-real-time airspace authorization for UAS operations in controlled airspace (Classes B, C, D, and E surface areas); authorization is obtained through the B4UFLY or Aloft apps before each flight in controlled airspace (Federal Aviation Administration, 2024a, 2024b).
- **LiPo battery (lithium polymer battery):** The rechargeable battery used in DJI drones; stores significant energy and poses a fire hazard if punctured, short-circuited, or charged incorrectly; must be stored at 40–60% charge for long-term storage and kept away from flammable materials.
- **Nadir angle:** A camera gimbal angle of -90°, pointing the camera directly downward toward the ground surface; the standard angle for photogrammetric mapping and orthomosaic generation.

- **Part 107:** Title 14 CFR Part 107 of the Code of Federal Regulations, governing Small Unmanned Aircraft Systems; requires a Remote Pilot Certificate for commercial and research operations; specifies operating limitations including maximum altitude, VLOS requirement, and prohibited airspace (Federal Aviation Administration, 2024b).
- **Photogrammetry:** The science of making measurements from photographs; in the drone context, software such as Reality Capture or Pix4D processes overlapping aerial images to produce 3D models, dense point clouds, digital surface models (DSMs), and georeferenced orthomosaics (Capturing Reality, 2024).
- **RTH (Return to Home):** An automated failsafe function that returns the drone to its recorded home point (takeoff location) if the controller signal is lost or the battery reaches the RTH threshold; the home point must be set before each flight.
- **sUAS (small Unmanned Aircraft System):** FAA classification for unmanned aircraft weighing less than 55 pounds (25 kg); both EA Program drones qualify as sUAS (Federal Aviation Administration, 2024b).
- **TRUST (The Recreational UAS Safety Test):** An FAA-mandated online safety test for recreational flyers; required when operating under the Exception for Limited Recreational Operations (44809) rather than Part 107.
- **UAS (Unmanned Aircraft System):** The complete system including the drone, remote controller, and associated software.
- **VLOS (Visual Line of Sight):** FAA requirement that the Remote Pilot maintain unaided visual contact with the aircraft at all times during flight; flight beyond VLOS requires a Part 107 waiver (Federal Aviation Administration, 2024b).

4. Responsibilities

Role	Responsibility
PI	Approve flight plans; ensure FAA compliance
Certified Pilot	Hold FAA Part 107 certificate; conduct flights
Visual Observer	Maintain visual contact with the drone; alert pilot to hazards
New Students	Complete training before any drone operation

5. Safety and Hazards

5.1. Critical Safety Rules

- Never fly over people who are not directly participating in the operation
- Never fly beyond visual line of sight (VLOS)
- Never fly near other aircraft or in restricted airspace without authorization
- Never touch spinning propellers
- Never fly under the influence of alcohol or drugs

5.2. Hazards

- **Physical:** Spinning propellers can cause lacerations. A drone falling from altitude can cause blunt force injury. LiPo batteries may be hot after flight.
- **Regulatory:** Unauthorized flights can result in FAA fines and criminal penalties (Federal Aviation Administration, 2024b).
- **Property:** Crashes can damage the drone (replacement cost several thousand dollars) and property below.
- **Environmental:** Drones can disturb nesting birds and wildlife. Avoid flying near known nesting sites during breeding season.

5.3. Required PPE

- ☐ Safety glasses (recommended during takeoff and landing)
- ☐ Sun protection (hat, sunscreen)
- ☐ Closed-toe shoes

5.4. Emergency Procedures

If the drone loses connection, activate RTH immediately. If RTH fails, allow the drone to execute its failsafe. Do not attempt to catch a falling drone. Refer to [SOP-F001 Field Safety](#).

6. FAA Regulations

! Legal Requirement

All drone flights must comply with current FAA regulations. The pilot is personally responsible for compliance (Federal Aviation Administration, 2024b).

Research operations require FAA Part 107 Remote Pilot certification. Flights in controlled airspace (near airports) require prior LAANC authorization via the B4UFLY or Aloft apps (Federal Aviation Administration, 2024a). Pomona College campus is within Ontario International Airport (KONT) Class C airspace; LAANC authorization is required for on-campus flights. Both EA Program drones are registered with the FAA; registration numbers are on file with the PI and must be displayed on the aircraft at all times.

6.1. Flight Restrictions under Part 107

- Maximum altitude: 400 feet AGL (Federal Aviation Administration, 2024b)
- Daylight operations only (civil twilight with anti-collision lighting permissible with appropriate equipment)
- Visual line of sight at all times
- No flights over non-participating persons
- Yield right of way to manned aircraft at all times

7. EA Program Fleet

Aircraft	Serial Number	Storage	Notes
DJI Mavic 3 Pro	<i>TBD</i>	SGM 106	Primary survey aircraft
DJI Matrice 100	M02DF 19020136	SGM 210	Legacy; multi-sensor
Mavic 3 batteries	<i>TBD</i>	SGM 106	3 batteries
Matrice 100 batteries	<i>TBD</i>	SGM 210	6 batteries

8. Required Materials

8.1. Pre-Flight Checklist

Item	Check
Drone (inspected, propellers attached)	☐
Remote controller (charged)	☐
Batteries (charged, minimum 3)	☐
Mobile device with DJI app (charged)	☐
MicroSD card (formatted, > 2 GB free)	☐
FAA Part 107 certificate (pilot)	☐
FAA registration number on aircraft	☐
LAANC authorization (if controlled airspace)	☐
UAV Forecast app check (weather, KP index)	☐
Flight plan (altitude determined)	☐
Notebook and pen (for flight log)	☐

(DJI, 2023; Federal Aviation Administration, 2024b)

8.2. Software

Application	Purpose
DJI Fly or DJI Pilot	Flight control, camera settings, flight planning (DJI, 2015, 2023)
UAV Forecast	Weather, wind, KP index, GPS satellite count
B4UFLY or Aloft	Airspace authorization (LAANC) (Federal Aviation Administration, 2024a)
Reality Capture or Pix4D	Photogrammetry post-processing (Capturing Reality, 2024)

9. Procedure

9.1. Pre-Flight Inspection

1. Inspect the airframe: no cracks, loose propellers, or debris in motors.
2. Charge all batteries to 100% before departure.
3. Check the DJI app for firmware updates; update if prompted (DJI, 2023).

9.2. Pre-Flight Checks at the Site

1. Perform compass calibration per the DJI app (DJI, 2023).
2. Check airspace: open B4UFLY or Aloft and verify authorization (Federal Aviation Administration, 2024a).
3. Record weather conditions in the flight log.

9.3. Flight Operations

1. Announce “Taking off” to all personnel.
2. Hover at 3–5 m to verify stable flight, GPS lock, and camera orientation.
3. Execute the flight plan:
 - For photogrammetry: use automated waypoint or grid mode with 70–80% front overlap and 60–70% side overlap (Capturing Reality, 2024).
 - For manual survey: fly in a systematic pattern at the planned altitude.
4. Monitor battery: begin return at 30%; land immediately at 20%.
5. Announce “Landing” before descent.

9.4. Image Collection Settings

Setting	Recommended Value
Image format	JPEG + RAW (DNG) for photogrammetry
Photo interval	2 seconds (timed shot for grid plans)
Resolution	Maximum available
Gimbal angle	Nadir (-90°) for mapping; oblique (-45°) for 3D models

9.5. Post-Flight Procedures

1. Power off the drone and remote controller.
2. Replace the gimbal cover.
3. Remove the MicroSD card and transfer images:
 - Create a folder: `Drone_SiteID_YYYYMMDD_AltitudeAGL`
 - Copy all images; verify file count
4. Format the MicroSD card after confirming backup.

5. Return all equipment to storage (Mavic to SGM 106; Matrice to SGM 210).
6. Complete the flight log: aircraft, date, location, pilot, duration, battery cycles used, any incidents.

10. Quality Control

- Complete the pre-flight checklist before every flight.
- Verify GPS lock (minimum 8 satellites) before takeoff.
- After each flight, review a sample of images to confirm coverage, focus, and exposure.
- Report any incidents (crashes, near-misses, property damage) to the PI within 24 hours. FAA reporting may be required for incidents involving injury or property damage exceeding \$500 (Federal Aviation Administration, 2024b).

11. References

- DJI (2023)
- DJI (2015)
- Federal Aviation Administration (2024b)
- Federal Aviation Administration (2024a)
- Capturing Reality (2024)

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DJI. (2015). *DJI Matrice 100 user manual*. <https://www.dji.com/matrice100/downloads>

DJI. (2023). *DJI Mavic 3 Pro user manual*. <https://www.dji.com/mavic-3-pro/downloads>

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Federal Aviation Administration. (2024b). *Small unmanned aircraft systems* (14 CFR Part 107).

Federal Aviation Administration. <https://www.ecfr.gov/current/title-14/chapter-I/subchapter-F/part-107>

12. Related SOPs

SOP	Title	Relationship
F001	Field Safety	Required prerequisite
F006	GPS and Site Navigation	Flight site coordinates
F620	Camera Traps	Complementary wildlife monitoring
L805	Drone Photogrammetry	Post-processing workflow

13. Training Record

Before operating any EA Program drone independently, personnel must:

1. Read this SOP and [SOP-F001](#) in full
2. Hold a current FAA Part 107 Remote Pilot certificate, or have passed TRUST (for recreational operations under direct PI supervision) (Federal Aviation Administration, 2024b)
3. Complete the DJI app tutorial and demonstrate proficiency with simulator mode (DJI, 2023)
4. Demonstrate pre-flight inspection, calibration, takeoff, hover, controlled flight pattern, and landing under supervision
5. Complete at least one supervised photogrammetry data collection flight with acceptable image quality
6. Be signed off by the PI



Environmental Analysis Program

Pomona College
Biogeochemistry Laboratory

SOP-F700

eDNA Field Sampling and Preservation

Standard Operating Procedure

SOP Number	SOP-F700
Revision	0.2
Author(s)	Marc Los Huertos
Date	2026-06-26
Organization	EA Program, Pomona College

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1. Purpose

This SOP describes field procedures for collecting, filtering, and preserving environmental DNA (eDNA) samples from surface waters. eDNA consists of DNA shed into the environment by organisms through skin cells, mucus, feces, gametes, and decomposing tissue. Detecting eDNA in

water samples allows non-invasive detection of aquatic species (fish, amphibians, invertebrates, pathogens) without direct observation or capture (Goldberg et al., 2016). Because eDNA is present at extremely low concentrations, preventing contamination during field collection is critical. This SOP covers the field side of the workflow; laboratory processing (filtration, extraction, inhibition testing) is covered in [SOP-L700 DNA Extraction](#).

2. Scope

- **Applies to:** Collection and preservation of water samples for eDNA analysis at all EA Program surface water sites (streams, lakes, ponds, wetlands, stormwater basins)
- **Does not apply to:** Soil eDNA or soil microbial sampling (see [SOP-F701](#)); tissue biopsy or organism capture for DNA; airborne eDNA sampling; lab-side filtration, extraction, and molecular analysis
- **Location:** All EA Program surface water field sites

3. Responsibilities

Role	Responsibility
PI / Field Lead	Approve sampling design; ensure permits are in place
Trained Personnel	Collect and preserve eDNA samples per this SOP
New Students before participating in eDNA sampling	Complete training (including contamination awareness)

4. Definitions

- **Contamination (eDNA):** The introduction of exogenous DNA from sources other than the target organism into a sample or filter; the single largest source of false positives in eDNA surveys; prevented by sterile technique, glove changes between samples, and decontamination of reusable equipment (Goldberg et al., 2016).
- **eDNA (environmental DNA):** Genetic material shed into the environment by organisms through mucus, feces, gametes, skin cells, and other biological material; collected from water without direct capture of the target organism; detectable at extremely low concentrations using PCR-based methods (Goldberg et al., 2016).
- **Equipment blank:** A control sample consisting of deionized water rinsed through decontaminated reusable equipment (e.g., a pump) and collected as a sample; detects carry-over contamination between sites when dedicated equipment is not used.
- **Field blank:** A sterile bottle of molecular-grade deionized water opened and handled at the field site under identical conditions to real samples; filtered and preserved like a field sample; detects airborne or handling contamination during field work; must yield no target amplification (Goldberg et al., 2016).

- **Filter pore size:** The nominal diameter of pores in the filter membrane; 0.45 µm is the standard for eDNA capture from surface water, retaining bacteria, cells, and particulate-bound DNA.
- **Longmire's buffer:** A non-freezing preservation solution (containing EDTA, Tris, NaCl, SDS, and sucrose) used to preserve eDNA on filters in the field when immediate freezing is not possible; inactivates nucleases and inhibits microbial degradation (Renshaw et al., 2015).
- **Open-channel filtration:** A filtration method using a funnel and vacuum pump or syringe; the filter membrane is exposed to the environment during collection, increasing contamination risk compared to closed-capsule methods.
- **PCR inhibitor:** A substance co-extracted with eDNA that suppresses PCR amplification efficiency; common in natural water samples (humic acids, tannins, metal ions); detected by running a dilution series of the extract and checking for concentration-dependent amplification; managed by diluting the extract or using commercial inhibitor removal kits.
- **Sterivex filter:** A commercially available enclosed 0.22 µm polysulfone cartridge filter; preferred for eDNA because the sealed design minimizes contamination, allows direct addition of preservation buffer, and is compatible with standard extraction kits (Renshaw et al., 2015).
- **Target species detection:** The determination of presence or absence of a specific taxon in a water sample based on amplification of taxon-specific eDNA; sensitivity depends on eDNA shedding rate, water volume filtered, PCR method, and inhibitor load in the extract (Goldberg et al., 2016).
- **Volume filtered:** The total volume of water passed through a filter during sample collection; a required reporting variable for calculating eDNA concentration; record for every filter regardless of whether filtration was complete or the filter clogged (Goldberg et al., 2016).

5. Safety and Hazards

Hazards

- **Physical:** Wading and sampling near water carry slip, fall, and drowning hazards. Work in pairs.
- **Chemical:** DNA preservatives (ethanol, Longmire's buffer) are flammable (ethanol) or contain EDTA and SDS (Longmire's). Handle with gloves.
- **Biological:** Water may contain pathogens. Wear gloves and avoid ingestion.

5.1. Required PPE

- ☐ Nitrile gloves (change between every sample)
- ☐ Safety glasses
- ☐ Wading boots or waders (if entering the water)
- ☐ Sun protection (hat, sunscreen)

5.2. Emergency Procedures

Refer to [SOP-F001 Field Safety](#).

6. Equipment and Materials

6.1. Sampling Equipment

Item	Notes
Sterile 1 L HDPE sample bottles	One per sample; single-use
60 mL sterile syringes	For Sterivex filtration
Sterivex 0.22 µm filter capsules	Preferred; one per sample
0.45 µm PES open filter funnels	Alternative to Sterivex
Longmire's buffer (1–2 mL per filter)	For filter preservation without freezing (Renshaw et al., 2015)
Molecular-grade ethanol (95%)	Alternative preservative for Sterivex
Molecular-grade deionized water	For field blanks (1 bottle per event)
Cooler with ice packs (4 °C)	For sample transport if not filtering on-site

7. Procedure

7.1. Pre-Field Preparation

1. Prepare all equipment in the lab using UV-decontaminated surfaces and dedicated pre-PCR equipment.
2. Pre-label all bottles and filters with project ID, site, replicate number, date.
3. Pack a cooler with ice packs or dry ice.
4. Include at least one sterile bottle of molecular-grade DI water per sampling event for field blanks.

7.2. Water Collection

1. Put on fresh nitrile gloves.
2. Open the sterile bottle only when ready to collect.
3. Submerge the bottle to 10–20 cm below the water surface, facing upstream, away from your body (Goldberg et al., 2016).
4. Fill to 1 L and cap immediately.
5. For composite samples: collect 250 mL from each of 4 microhabitats and combine in one bottle.
6. Record the GPS coordinates, collection time, and site conditions.

7.3. Field Filtration (If Filtering On-Site)

Sterivex capsule method:

1. Attach a sterile 60 mL syringe to the Sterivex filter inlet.
2. Draw water from the sample bottle and push through the filter. Repeat until the filter clogs or 1 L has been filtered. Record the total volume filtered.

3. Expel remaining water from the capsule.
4. Add 1–2 mL of Longmire's buffer or fill with molecular-grade ethanol using a syringe (Renshaw et al., 2015). Cap both ends of the Sterivex.
5. Label the capsule and place in a ziplock bag on ice.

Open filter funnel method:

1. Pour the sample through a sterile 0.45 µm PES filter unit.
2. Use sterile forceps to fold the filter membrane in half (sample side inward) and place in a labeled cryovial.
3. Add preservative (ethanol or Longmire's buffer) to cover the filter. Seal.
4. Place on ice immediately.

7.4. If Not Filtering On-Site

Keep whole-water samples on ice (4 °C) and filter in the lab within 6 hours of collection. Exceeding 6 hours significantly increases DNA degradation (Goldberg et al., 2016).

7.5. Field Blanks and Controls

- **Field blank:** Open the sterile DI water bottle at one site per event, filter it using the same method as field samples, and preserve. This blank detects contamination during field handling.
- **Equipment blank:** If any reusable equipment contacts the sample, collect a deionized water rinse blank after decontamination.
- Process blanks through the entire lab workflow alongside samples.

7.6. Decontamination Between Sites

1. Spray or soak all reusable equipment with 10% bleach solution for at least 1 minute.
2. Rinse with clean water.
3. Change gloves.
4. Single-use items (bottles, filters, syringes) are discarded after each sample.

See [SOP-F007](#) for general decontamination procedures.

8. Sample Transport and Storage

1. Keep all samples and preserved filters on ice (4 °C) during transport.
2. Deliver to the lab the same day. If overnight storage is unavoidable, store at -20 °C.
3. For long-term storage (> 1 week before extraction), store preserved filters at -20 °C or -80 °C.
4. Complete the chain of custody form per [SOP-F008](#).

9. Quality Control

- Minimum 1 field blank per sampling event; 1 equipment blank if reusable equipment is used.
- Collect 3 replicate samples per site unless the study design specifies otherwise.
- Record the total volume filtered for each sample.
- Any field blank positive for target DNA invalidates samples from that event unless below a pre-established threshold. Consult the PI.
- Log all glove changes, bottle openings, and deviations in the field notebook.

10. References

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11. Related SOPs

SOP	Title	Relationship
F001	Field Safety	Required prerequisite
F007	Field Decontamination	Between-site decon
F008	Sample Labeling and COC	Sample ID and chain of custody
F308	eDNA Water Collection	Habitat-specific collection strategies
F701	Soil Microbial Sampling	Companion soil DNA protocol
L700	DNA Extraction	Lab-side extraction

12. Training Record

Before collecting eDNA samples independently, personnel must:

1. Read this SOP, [SOP-F007](#), and [SOP-L700](#) in full
2. Demonstrate proper sterile technique (glove changes, bottle handling, filter preservation) during a supervised practice session
3. Successfully process a field blank with a negative result
4. Be signed off by the PI or field lead



Environmental Analysis Program

Pomona College
Biogeochemistry Laboratory

SOP-F701

Soil Microbial Field Sampling for Molecular Analysis

Standard Operating Procedure

SOP Number	SOP-F701
Revision	0.2
Author(s)	Marc Los Huertos
Date	2026-06-26
Organization	EA Program, Pomona College

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1. Purpose

This SOP describes field procedures for collecting soil samples intended for molecular analysis, including DNA extraction, PCR/ddPCR, amplicon sequencing, and metagenomics. Soil microbial communities are sensitive to temperature, moisture, and oxygen exposure, and nucleic acids degrade rapidly in the absence of proper preservation. The procedures in this SOP are designed to minimize DNA and RNA degradation from the moment of collection through transport to the laboratory,

ensuring that molecular results accurately reflect the in situ microbial community (Frosteg aard et al., 1999; Knight et al., 2018).

This SOP supplements the general soil sampling procedures in [SOP-F400](#) with additional precautions specific to molecular work.

2. Scope

- **Applies to:** Field collection of soil and rhizosphere samples for DNA extraction ([SOP-L700](#)), ddPCR ([SOP-L701](#)), amplicon sequencing, metagenomics, and other molecular analyses
- **Does not apply to:** Soil sampling for chemical or physical analysis only (see [SOP-F400](#)); water eDNA sampling (see [SOP-F700](#)); laboratory-side DNA extraction and processing
- **Location:** All EA Program field sites

3. Responsibilities

Role	Responsibility
PI / Field Lead	Approve sampling design; specify target depth and replication
Trained Personnel	Collect and preserve samples per this SOP
New Students	Complete training (including contamination awareness) before participating in molecular sampling

4. Definitions

- **Contamination (molecular soil sampling):** Introduction of exogenous DNA from other samples, the environment, or the researcher's own microbiome into a soil sample; prevented by sterile tool sterilization between samples, frequent glove changes, and use of field blanks (Frosteg aard et al., 1999).
- **Cryogenic freezing (flash-freezing):** Rapid cooling of a soil sample in liquid nitrogen (-196 °C) or on dry ice (-78 °C) immediately after collection; arrests microbial metabolism and nuclease activity; required for RNA work and recommended for optimal DNA preservation (Knight et al., 2018).
- **Dry ice (solid CO₂):** Used for field flash-freezing at -78 °C; causes frostbite on direct skin contact; must be handled with insulated cryogenic gloves and transported in a vented or insulated container.
- **Field blank:** An empty sterile tube or bag opened and closed at the field site during sampling; processed through the full extraction and PCR workflow in the lab; detects airborne or handling contamination; any positive amplification in a field blank invalidates the associated samples unless concentrations fall below a pre-established threshold (Frosteg aard et al., 1999).
- **Flash-freezing:** See cryogenic freezing.

- **LifeGuard Soil Preservation Solution (Qiagen):** A commercially prepared stabilization reagent that arrests nucleic acid degradation at ambient temperature for up to 7 days; an alternative to cryogenic preservation when dry ice is unavailable; add to the sample immediately after collection, invert gently, and store at room temperature or on ice.
- **Microbial community composition:** The relative abundances of different microbial taxa (bacteria, archaea, fungi) in a soil sample; highly sensitive to sample handling time, temperature, and oxygen exposure; shifts within minutes of soil disturbance (Frosteg aard et al., 1999; Knight et al., 2018).
- **Nuclease:** An enzyme that degrades DNA or RNA; active in soil samples at ambient temperatures; inactivated by low temperature ($< 4^{\circ}\text{C}$), chemical preservatives (LifeGuard buffer, ethanol), or rapid freezing.
- **Rhizosphere:** The narrow zone of soil immediately surrounding plant roots; has a distinct microbial community influenced by root exudates; sampled by collecting soil adhering directly to roots after gentle removal from the surrounding bulk soil.
- **Sterile technique:** A set of aseptic practices that prevent contamination of molecular samples; in soil microbial sampling, these include autoclaving or 70% ethanol sterilization of all metal tools, use of single-use sterile tubes and gloves, and minimizing exposure time to air (Frosteg aard et al., 1999).
- **Time from extraction to preservation:** A critical metric for molecular soil samples; ideally less than 2 minutes from the moment soil is removed from the ground to placement in ice, dry ice, or preservative; longer times cause measurable shifts in microbial community composition (Knight et al., 2018).

5. Safety and Hazards

Hazards

- **Biological:** Soil may contain pathogens. Wash hands thoroughly after handling. Treat all samples as potentially infectious.
- **Physical:** Repetitive strain from augering; standard field hazards (heat, ticks, uneven terrain).
- **Chemical:** Liquid nitrogen causes severe cryogenic burns. Dry ice causes frostbite on direct skin contact. Handle with insulated gloves.

5.1. Required PPE

- ☐ Nitrile gloves (change between samples)
- ☐ Closed-toed shoes or field boots
- ☐ Insulated cryogenic gloves (if using liquid nitrogen or dry ice)
- ☐ Safety glasses (if using liquid nitrogen)
- ☐ Sun protection (hat, sunscreen)

5.2. Emergency Procedures

Refer to [SOP-F001 Field Safety](#). For liquid nitrogen or dry ice burns, warm the affected area gently with lukewarm water. Do not rub.

6. Equipment and Materials

6.1. Sampling Equipment

Item	Notes
Soil auger (bucket-type, stainless steel)	Autoclaved or ethanol-sterilized before each event
Stainless steel trowel or spatula	Autoclaved or flame-sterilized
Ethanol (70%) in spray bottle	For field sterilization between samples
Lighter or butane torch	For flame-sterilizing metal tools
DI water squeeze bottle	For rinsing tools after ethanol sterilization

6.2. Sample Containers and Preservation

Item	Notes
Sterile 50 mL Falcon tubes or Whirl-Pak bags	Pre-labeled; one per sample
Cooler with ice packs (4 °C)	Minimum preservation for DNA samples
Dry ice (solid CO ₂)	For flash-freezing at -78 °C; preferred for RNA work
Insulated container for dry ice	Styrofoam cooler or dewar
LifeGuard Soil Preservation Solution	Optional; stabilizes DNA and RNA at ambient temperature for up to 7 days
Ziplock bags (gallon size)	For double-bagging frozen samples

7. Procedure

7.1. Pre-Field Preparation

1. Autoclave or ethanol-sterilize all metal sampling tools. Wrap in aluminum foil after sterilization.
2. Pre-label all sample tubes or bags: project ID, site, sample number, date, depth.
3. If using LifeGuard, pre-aliquot approximately 5 mL per tube.
4. Pack the cooler. If using dry ice, load the insulated container.
5. Include at least one field blank tube per sampling event.

7.2. Sampling Procedure

1. Navigate to the sampling location; record GPS coordinates.
2. Put on fresh nitrile gloves.
3. Sterilize the auger or trowel: spray with 70% ethanol, allow 30 seconds air-dry, optionally flame. Rinse with DI water.
4. Clear surface litter without disturbing mineral soil.
5. Collect soil from the target depth (typically 0–15 cm). Avoid large roots and rocks.
6. Using the sterilized trowel, transfer approximately 2–5 g of soil directly into the sterile tube. Minimize handling time; do not homogenize in a bucket.

! Speed Matters

Minimize the time between coring and preservation. The soil should be in the tube and on ice within 2 minutes of extraction. Prolonged exposure to air and ambient temperature causes shifts in microbial community composition and DNA degradation (Frosteg aard et al., 1999; Knight et al., 2018).

7. Immediately preserve:
 - **DNA only:** Place capped tube on ice (4 °C). Transport to lab within 6 hours or freeze at -20 °C.
 - **DNA and RNA:** Flash-freeze on dry ice or liquid nitrogen. Transfer to -80 °C freezer on return.
 - **LifeGuard:** Add 5 mL LifeGuard to the soil, cap, invert 3 times, store at ambient temperature up to 7 days.
8. Record in the field notebook: sample ID, GPS, depth, soil temperature, preservation method, time of collection, and any deviations.

7.3. Decontamination Between Samples

1. Spray tools with 70% ethanol; allow 30 seconds air-dry; optionally flame. Rinse with DI water.
2. Change nitrile gloves.
3. Between sites, decontaminate boots and waders per [SOP-F007](#).

8. Sample Transport and Storage

Preservation Method	Field Storage	Lab Storage
Ice (4 °C)	Cooler, ice packs	Process within 24 h or freeze at -20 °C
Dry ice (-78 °C)	Insulated cooler	Transfer to -80 °C freezer immediately

Preservation Method	Field Storage	Lab Storage
Liquid nitrogen (-196 °C)	Dewar	Transfer to -80 °C freezer immediately
LifeGuard buffer	Ambient, up to 7 days	Extract within 7 days; or freeze at -20 °C

Record storage location in the Freezer Inventory and in the lab notebook per [SOP-L106](#).

9. Quality Control

- Include at least one field blank per sampling event.
- Change gloves and sterilize tools between every sample.
- Record the time elapsed between soil extraction and preservation. Flag any sample with elapsed time > 5 minutes.
- Field blanks positive for target DNA invalidate the batch unless concentrations fall below the established threshold.
- Avoid sampling immediately after rainfall when soils are saturated.

10. References

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11. Related SOPs

SOP	Title	Relationship
F001	Field Safety	Required prerequisite
F007	Field Decontamination	Between-site decon
F008	Sample Labeling and COC	Sample ID scheme
F400	Soil Sampling	General soil collection
F700	eDNA Field Sampling	Water eDNA companion protocol
L700	DNA Extraction	Lab-side extraction
L701	ddPCR Primers	Downstream analysis

12. Training Record

Before collecting soil samples for molecular analysis independently, personnel must:

1. Read this SOP, [SOP-F400](#), and [SOP-L700](#) in full
2. Demonstrate sterile sampling technique (tool sterilization, glove changes, rapid transfer to preservative) during a supervised practice session
3. Successfully process a field blank with a negative extraction and PCR result
4. Be signed off by the PI or field lead



Environmental Analysis Program

Pomona College
Biogeochemistry Laboratory

SOP-L601

Preparing a Wet Mount for Microscopy

Standard Operating Procedure

SOP Number	SOP-L601
Revision	1.1
Author(s)	Dmaia Curry, Aparna Chintapalli
Date	2026-06-26
Organization	EA Program, Pomona College

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1. Purpose

This procedure describes how to prepare a wet mount slide for light microscopy. A wet mount suspends a specimen in a thin layer of water (or other mounting medium) beneath a cover slip, preserving the natural shape and motility of living cells and small organisms. It is the standard preparation method for examining fresh biological samples under bright field or phase contrast illumination (Murphy & Davidson, 2012).

2. Scope

- **Applies to:** Preparation of temporary wet mount slides for examination of algae, protozoa, bacteria, fungi, plant cells, soil microorganisms, and other small specimens in the EA Program laboratory
- **Does not apply to:** Permanent slide preparation (fixed, stained, and sealed mounts); dry mounts; hanging-drop preparations for motility studies; fluorescence specimen preparation (see [SOP-L211](#))
- **Location:** EA Program laboratory, SGM 133

3. Responsibilities

Role	Responsibility
Lab PI	Approve SOP, ensure training compliance
Trained Personnel	Prepare slides following this procedure
New Students	Complete training before independent use

4. Definitions

- **Cover slip (coverslip):** A thin (0.13–0.17 mm) square or circular piece of optical glass placed over the specimen on a microscope slide; standardizes the optical path length and protects the objective from liquid samples; the EA Program standard is No. 1.5 (0.17 mm), matched to the objectives on the Echo Revolve microscope (Murphy & Davidson, 2012).
- **Immersion medium:** Liquid placed between the specimen and the coverslip to improve optical contact, reduce refraction artifacts, and support live organisms during observation; options include water, glycerol, or immersion oil depending on the application.
- **Live mount:** A wet mount prepared without fixation or staining; allows observation of living organisms and motile behavior; must be examined quickly before evaporation or oxygen depletion affects the specimen.
- **Mounting medium:** The liquid in which the specimen is suspended; distilled or deionized water is standard; saline, glycerol, or specific staining solutions may be used depending on the application (Murphy & Davidson, 2012).
- **Refraction artifact:** Optical distortion caused by differences in refractive index between the sample, mounting medium, coverslip, and objective; minimized by matching refractive indices and maintaining thin, uniform wet mounts.

- **Wet mount:** A temporary microscopy preparation method in which a specimen is suspended in a drop of liquid (usually water) on a glass slide and covered with a coverslip; the standard preparation for aquatic microorganisms, algae, macroinvertebrates, and other specimens examined under transmitted light (Murphy & Davidson, 2012).

5. Safety and Hazards

Hazards

- **Physical:** Microscope slides and cover slips are fragile glass. Broken glass can cause cuts. Handle by the edges and dispose of chipped or cracked glass in the broken-glass box.
- **Biological:** Environmental samples (water, soil, compost) may contain unknown microorganisms. Treat all samples as potentially infectious and wash hands after handling.

5.1. Required PPE

- ☐ Safety glasses
- ☐ Lab coat
- ☐ Nitrile gloves

5.2. Emergency Procedures

For cuts from broken glass, apply direct pressure and rinse with water. Seek first aid if bleeding is significant. Refer to [SOP-L002 Laboratory Safety](#).

6. Required Materials

Item	Catalog No.	Notes
Microscope slides (75 × 25 mm)	TBD	Standard glass, pre-cleaned
Cover slips (No. 1.5, 22 × 22 mm)	TBD	0.17 mm thickness; matched to objectives on the Revolve microscope (Murphy & Davidson, 2012)
Squeeze bottle with DI water		Or distilled water
Disposable transfer pipettes or eyedroppers		For placing water drops
Forceps (fine-tipped)		For handling specimens and cover slips
Toothpicks		For lowering cover slips
Lint-free wipes (Kimwipes)		For blotting excess water

i Catalog Numbers Needed

The original SOP flagged that catalog numbers for slides and cover slips should be added. Update this table when part numbers are confirmed with the lab manager.

7. Estimated Time

This procedure requires approximately 5 minutes per slide.

8. Procedure

8.1. Placing the Specimen

1. Clean a microscope slide with a lint-free wipe to remove dust and fingerprints.
2. Using forceps, place the specimen in the center of the slide. For liquid samples (pond water, culture broth), skip to Step 3 and pipette the sample directly.

8.2. Adding the Mounting Liquid

3. Using a transfer pipette or eyedropper, place one to two drops of water onto the specimen. Adjust the volume based on specimen size: larger or thicker specimens may need slightly more water, while very small specimens need less.

8.3. Applying the Cover Slip

4. Pick up a cover slip by its edges with forceps or clean, dry fingers. Never touch the flat surfaces.
5. Place one edge of the cover slip on the slide adjacent to the specimen, holding it at approximately a 45-degree angle.
6. Slowly lower the cover slip using the tip of a toothpick or forceps, allowing the water to spread gradually beneath the glass. Lowering at an angle lets air escape and minimizes bubble formation (Murphy & Davidson, 2012).

💡 Avoiding Air Bubbles

Air bubbles distort the image and can be mistaken for cellular structures. If bubbles are present after placing the cover slip, gently tap the cover slip with the eraser end of a pencil or press lightly with a toothpick to work bubbles toward the edge. If large bubbles persist, remove the cover slip, add a fresh drop of water, and try again.

8.4. Adjusting Water Volume

7. **Too much water:** If the cover slip floats or slides freely, the water layer is too thick. Hold the edge of a lint-free wipe against the edge of the cover slip and let capillary action draw off excess water until the cover slip sits flat.
8. **Too little water:** If dry areas are visible beneath the cover slip, place a small drop of water at the edge and let capillary action draw it underneath. Do not lift the cover slip.

8.5. Examining the Slide

9. Place the prepared slide on the microscope stage and examine following [SOP-L211 Echo Labs Revolve Microscope](#). Start at low magnification (4× or 10×) and increase as needed.
10. For unstained, transparent specimens, phase contrast mode typically provides better visibility than bright field (see SOP-L211, Section 6.5).

9. Quality Control

- Verify that the cover slip lies flat and is not tilted by air bubbles or excess sample material.
- Confirm that the water layer is thin enough for the specimen to remain in a single focal plane at high magnification.
- If examining motile organisms, note that excessive pressure from the cover slip can restrict movement; use slightly more water to provide adequate space.

10. Waste Disposal

Waste Type	Disposal Method
Used glass slides and cover slips	Broken-glass box
Used toothpicks and wipes	Standard trash
Biological specimen waste	Biohazard bag if potentially infectious; otherwise standard trash

11. Troubleshooting

Problem	Probable Cause	Corrective Action
Air bubbles under cover slip	Cover slip lowered too quickly	Remove cover slip, re-wet, lower at 45° slowly
Cover slip slides around	Too much water	Blot excess with lint-free wipe
Dry patches under cover slip	Too little water	Add a drop at the edge; capillary action fills the gap
Specimen drifts out of view	Water current under cover slip	Wait for flow to stop; blot if excess water is present

Problem	Probable Cause	Corrective Action
Image blurry at high magnification	Thick water layer or dirty slide	Blot excess water; clean slide and re-prepare

12. References

- Murphy & Davidson (2012)

Murphy, D. B., & Davidson, M. W. (2012). *Fundamentals of light microscopy and electronic imaging* (2nd ed.). Wiley. <https://doi.org/10.1002/9781118382905>

13. Related SOPs

SOP	Title	Relationship
L002	Laboratory Safety	Required safety training
L211	Revolve Microscope	Instrument used to examine slides
L602	Macroinvertebrate Sorting	Uses wet mounts for ID
L604	Algae Identification	Uses wet mounts for ID

14. Training Record

Before preparing wet mount slides independently, personnel must:

1. Read this SOP in full
2. Complete [SOP-L002 Laboratory Safety](#) training
3. Complete [SOP-L211 Revolve Microscope](#) training
4. Prepare at least two wet mount slides under supervision and demonstrate proper cover slip technique (no air bubbles, correct water volume)
5. Be signed off by the PI or lab manager



Environmental Analysis Program

Pomona College
Biogeochemistry Laboratory

SOP-L602

Macroinvertebrate Sorting, Subsampling, and Identification

Standard Operating Procedure

SOP Number	SOP-L602
Revision	0.1
Author(s)	Marc Los Huertos
Date	2026-06-26
Organization	EA Program, Pomona College

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1. Purpose

This SOP describes the laboratory procedures for sorting, subsampling, and identifying benthic macroinvertebrates (BMI) collected from wadeable streams per [SOP-F610](#). The procedure follows

the California Surface Water Ambient Monitoring Program (SWAMP) laboratory processing protocols to ensure compatibility with statewide bioassessment datasets (Barbour et al., 1999; Ode et al., 2016). Taxonomic identifications are used to calculate biological condition metrics, including the California Stream Condition Index (CSCI) (Mazor et al., 2016).

2. Scope

- **Applies to:** Laboratory processing of ethanol-preserved BMI samples from wadeable streams, including subsampling, sorting, identification to the lowest practical taxonomic level, and enumeration
- **Does not apply to:** Field collection of BMI (see [SOP-F610](#)); marine or estuarine macroinvertebrates; meiofauna (organisms passing through a 500 μm sieve); molecular identification (eDNA metabarcoding)
- **Location:** EA Program laboratory, SGM 133

3. Responsibilities

Role	Responsibility
PI / Lab Manager	Approve taxonomic level targets; arrange external QC
Trained Personnel	Sort, identify, and enumerate organisms per this SOP
New Students	Complete training before independent sorting

4. Definitions

- **Benthic macroinvertebrate (BMI):** An invertebrate organism inhabiting the bottom substrate of streams, lakes, or wetlands that is retained on a 500 μm mesh sieve; includes aquatic insects, crustaceans, worms, mollusks, and other taxa.
- **Fixed count subsample:** A randomly selected subset of a BMI sample from which a fixed number of organisms (e.g., 500) is picked and identified; reduces processing time for large samples while maintaining statistical reliability.
- **Functional feeding group (FFG):** A classification of macroinvertebrates by feeding mode (shredders, collectors-gatherers, collectors-filterers, scrapers/grazers, predators); used to assess stream ecosystem function from invertebrate assemblage data.
- **Qualitative identification:** Determination of taxa present in a sample without quantifying abundances; used for rapid bioassessment and presence/absence monitoring.
- **Quantitative sample:** A sample in which all organisms are counted and identified to calculate density (organisms per unit area or volume); requires accurate recording of sampled area and volume.
- **Taxonomic resolution:** The level of taxonomic identification applied to BMI specimens; options range from phylum/class to order, family, genus, or species; standard SWAMP protocol requires family-level identification for most groups.

- **Voucher specimen:** A representative specimen of a taxon retained in preservative as a reference for future verification; essential for quality assurance and resolving taxonomic uncertainties.

5. Safety and Hazards

Hazards

- **Chemical:** Preserved samples contain approximately 70% ethanol, which is flammable and an inhalation irritant. Work in a well-ventilated area away from ignition sources.
- **Physical:** Forceps and dissecting needles are sharp. Use caution when handling small organisms.
- **Biological:** Stream organisms may harbor parasites or pathogens. Wear gloves when handling preserved samples.

5.1. Required PPE

- ☐ Nitrile gloves
- ☐ Safety glasses
- ☐ Lab coat

5.2. Emergency Procedures

For ethanol spills, ventilate the area, absorb with paper towels, and dispose in flammable waste. Refer to [SOP-L002 Laboratory Safety](#).

6. Equipment and Materials

6.1. Equipment

Item	Notes
Dissecting microscope (stereo)	7–45x magnification; illuminated base
Compound microscope	For slide-mounted specimens requiring higher magnification
Gridded Caton sorting tray	White, subdivided into numbered cells
Fine-tipped forceps (2 pairs)	Dumont #5 or equivalent
Dissecting needles	For separating tangled organisms
Petri dishes	For isolating specimens during ID
Wash bottles (ethanol and DI water)	

6.2. Consumables

Item	Notes
70% ethanol	For topping off vials
Scintillation vials (20 mL)	For archiving sorted taxa
Labels (pencil on waterproof paper)	Internal and external labels
Depression slides	For compound microscope work

7. Procedure

7.1. Sample Receiving and Logging

1. Verify that the sample jar label matches the chain-of-custody or field data sheet: site ID, date, collector, preservative.
2. Log the sample into the laboratory tracking spreadsheet.
3. Inspect the sample for adequate preservative (organisms should not appear soft or discolored). If the ethanol is diluted below approximately 50%, decant carefully and replace with fresh 70% ethanol before proceeding.

7.2. Subsampling

The SWAMP standard requires identification of a minimum of 500 organisms per sample. A fixed-count random subsampling procedure is used (Caton, 1991):

1. Pour the entire sample through a 500 μm sieve. Rinse the jar into the sieve to recover all material.
2. Transfer the sieved material to the gridded Caton sorting tray. Distribute the material as evenly as possible across all grid cells.
3. Use a random number generator or random number table to select grid cells for sorting.
4. Under the dissecting microscope, remove all organisms from the selected cell using forceps, transferring them to a labeled petri dish or vial. Count each organism as it is picked.
5. Continue selecting and sorting random cells until the cumulative organism count reaches or exceeds 500. Complete the cell in progress even if the count exceeds 500 mid-cell.
6. Record the number of cells sorted, the total cells in the tray, and the total organism count.

Tip

Keep the residue (unsorted cells) in a labeled jar with 70% ethanol. The residue is needed for the re-sort QC check and may be required if additional identifications are needed later.

7.3. Identification

1. Separate sorted organisms into major taxonomic groups (e.g., Ephemeroptera, Plecoptera, Trichoptera, Diptera, Coleoptera, non-insect taxa) under the dissecting microscope.

2. Identify each organism to the lowest practical taxonomic level using standard keys (Merritt et al., 2019; Thorp & Rogers, 2015):
 - Insects: genus (family for early instars or damaged specimens)
 - Non-insect invertebrates: family or order (depending on group and specimen condition)
3. For specimens requiring higher magnification (e.g., chironomid head capsules, oligochaete chaetae), prepare slide mounts and examine under the compound microscope.
4. Record counts by taxon on the laboratory data sheet or directly into the data entry spreadsheet.

7.4. Voucher Specimens

1. Retain at least one representative specimen of each taxon as a voucher in a labeled scintillation vial with 70% ethanol.
2. Label each vial with: site ID, date, taxon name, identifier initials, identification date.
3. Store voucher vials in the BMI reference collection.

7.5. Data Entry and Metric Calculation

1. Enter identification data into the standardized spreadsheet or database.
2. Calculate summary metrics:
 - Total taxa richness
 - EPT (Ephemeroptera, Plecoptera, Trichoptera) richness
 - Percent tolerant taxa
 - Percent dominant taxon
 - Shannon diversity index (H')
3. If sufficient data are available, calculate the CSCI score using the CSCI R package or the online CSCI calculator (Mazor et al., 2016).

```
# Example: Shannon diversity from BMI count data
library(vegan)

# counts is a named vector of organism counts by taxon
counts <- c(Baetis = 120, Hydropsyche = 85, Chironomidae = 210,
            Simulium = 45, Optioservus = 30, Physa = 10)

H_prime <- diversity(counts, index = "shannon")
cat(sprintf("Shannon H': %.3f\n", H_prime))
cat(sprintf("Total taxa: %d\n", length(counts)))
cat(sprintf("Total organisms: %d\n", sum(counts)))
cat(sprintf("EPT richness: count EPT genera from full dataset\n"))
```

8. Quality Control

- **Sorting efficiency:** Re-sort 10% of processed samples (randomly selected). The re-sort technician examines all residue material from the original subsampling. Sorting efficiency must be greater than or equal to 90% (i.e., fewer than 10% of total organisms were missed).
- **Taxonomic verification:** Have a second identifier verify identifications for 10% of specimens. Agreement should be greater than or equal to 90% at the operational taxonomic level.
- **External QC:** For data submitted to SWAMP or CEDEN, submit 10% of samples to a SWAMP-certified taxonomic laboratory for independent re-identification.
- Record all QC results in the QC log.

9. Waste Disposal

Waste Type	Disposal Method
Ethanol from samples	Evaporate in fume hood or collect in flammable waste
Sorted residue (organic material)	Drain ethanol; dispose in standard trash
Used gloves and paper towels	Standard trash

10. References

- Barbour, M. T., Gerritsen, J., Snyder, B. D., & Stribling, J. B. (1999). *Rapid bioassessment protocols for use in streams and wadeable rivers: Periphyton, benthic macroinvertebrates, and fish* (2nd ed.). U.S. Environmental Protection Agency, Office of Water. <https://www.epa.gov/cwa-methods/rapid-bioassessment>
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11. Related SOPs

SOP	Title	Relationship
F610	Benthic Macroinvertebrate Sampling	Upstream: field collection
L002	Laboratory Safety	Required safety training
L601	Wet Mount for Microscopy	Slide preparation for ID

12. Training Record

Before sorting and identifying BMI independently, personnel must:

1. Read this SOP and [SOP-F610](#) in full
2. Complete [SOP-L002 Laboratory Safety](#) training
3. Review the major taxonomic keys (Merritt et al., 2019; Thorp & Rogers, 2015)
4. Complete a supervised sorting exercise achieving greater than 90% sorting efficiency
5. Correctly identify a training set of at least 30 taxa under supervision (greater than 85% agreement with reference identifications)
6. Be signed off by the PI or lab manager



Environmental Analysis Program

Pomona College
Biogeochemistry Laboratory

SOP-L603

Plant Tissue Drying, Grinding, and Preparation for Analysis

Standard Operating Procedure

SOP Number	SOP-L603
Revision	0.1
Author(s)	Marc Los Huertos
Date	2026-06-26
Organization	EA Program, Pomona College

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1. Purpose

This SOP describes the laboratory procedures for drying, grinding, and preparing plant tissue samples for subsequent elemental, isotopic, or nutrient analysis. Proper sample preparation is essential for obtaining representative, reproducible analytical results. The procedures follow standard methods for plant analysis (Jones, 2001; Kalra, 1998; Mills & Jones, 2004).

2. Scope

- **Applies to:** Leaf, stem, root, litter, and other plant tissue samples collected from EA Program field sites, prepared for total carbon and nitrogen (EA-IRMS), total phosphorus, metal analysis (ICP-MS after digestion), or other elemental determinations
- **Does not apply to:** Fresh tissue assays that require unfixed material (e.g., chlorophyll extraction from fresh leaves, enzyme activity assays); seed or fruit tissue with high oil content (requires freeze-drying); soil organic matter (see [SOP-L406](#))
- **Location:** EA Program laboratory, SGM 133

3. Responsibilities

Role	Responsibility
Lab PI	Approve drying protocol and grinding method
Trained Personnel	Process samples per this SOP
New Students	Complete training before independent use

4. Definitions

- **Acid washing (plant tissue):** Cleaning tissue with dilute HCl to remove surface contamination (dust, soil particles, fertilizer residues) before analysis for trace metals or nutrients; may be inappropriate for samples requiring analysis of acid-labile compounds.
- **Air drying:** Drying plant tissue at ambient temperature (typically 20–40 °C) to a stable moisture content; used as a preliminary step before oven drying; preserves enzyme activity and RNA better than direct oven drying.
- **Ball milling:** Size reduction by grinding samples between hardened steel or ceramic balls inside a rotating vessel; the preferred method for producing a homogeneous powder from fibrous plant tissue for EA-IRMS and elemental analysis.
- **Critical tissue concentration:** The minimum concentration of a nutrient required for maximum growth; below this level the plant is deficient; above it, growth is normal or the nutrient may be toxic at very high levels.
- **Lyophilization (freeze drying):** Removal of water from tissue under vacuum after rapid freezing; preserves cell structure, volatile compounds, and biological activity better than oven drying; required for enzyme, RNA, and some metabolite analyses.
- **Subsampling:** Drawing a representative small portion from a bulk dried, ground sample for analysis; requires thorough mixing and homogenization of the bulk powder before subsampling to ensure the subsample is representative.

5. Safety and Hazards

Hazards

- **Physical:** Drying ovens operate at 60–105 °C; burn risk from hot trays and glassware. The Wiley mill and ball mill contain rapidly moving parts; keep hands, hair, and loose clothing away from the grinding chamber.
- **Noise:** The Wiley mill generates noise above 85 dBA. Wear hearing protection during operation.
- **Dust:** Grinding produces fine particulate matter. Work in a well-ventilated area or use a dust mask, especially when processing large batches.
- **Biological:** Field-collected plant tissue may harbor ticks, spiders, or allergenic compounds (e.g., poison oak resin). Inspect samples before handling; wear gloves.

5.1. Required PPE

- ☐ Safety glasses or goggles
- ☐ Lab coat
- ☐ Nitrile gloves
- ☐ Hearing protection (Wiley mill operation)
- ☐ Dust mask or N95 respirator (grinding large batches)

5.2. Emergency Procedures

For burns from the oven, cool affected area under running water. Refer to [SOP-L002 Laboratory Safety](#).

6. Equipment and Materials

6.1. Equipment

Item	Notes
Drying oven	Forced-air convection; set to 60 °C (standard) or 105 °C (rapid dry)
Wiley mill (Thomas Scientific)	With 1 mm (20-mesh) screen
Ball mill or mortar and pestle	For fine grinding (< 250 μ m) for IRMS
Analytical balance	0.0001 g readability (for weighing into tin capsules)
Desiccator with desiccant	For cooling dried samples

6.2. Consumables

Item	Notes
Paper bags or aluminum foil trays	For oven drying; labeled with pencil
Labeled sample bags (polyethylene or paper)	For storing ground tissue
Tin capsules (5 x 9 mm)	For EA-IRMS analysis
Kimwipes	For cleaning mill between samples
70% ethanol (spray bottle)	For decontaminating equipment

7. Procedure

7.1. Sample Receiving and Cleaning

1. Log incoming samples in the laboratory tracking spreadsheet. Verify site ID, date, species or tissue type, and collector.
2. If tissue was not rinsed in the field, briefly rinse leaf or stem material with deionized water to remove surface soil, dust, and epiphytes. Do not soak, as leaching of soluble compounds will occur (Kalra, 1998).
3. Blot excess water with paper towels.
4. For root samples, remove adhering soil by gentle agitation in DI water; multiple rinses may be required.

7.2. Drying

1. Place cleaned tissue in labeled paper bags or foil trays. Do not overload bags; tissue should be loosely arranged to allow air circulation.
2. Dry at 60 °C in the forced-air oven for a minimum of 48 hours, or until constant mass is achieved (two consecutive weighings 4 hours apart differ by less than 0.1% of sample mass) (Jones, 2001).
3. For samples intended only for total C and N by combustion (not volatile compound analysis), drying at 70 °C is acceptable and reduces drying time.

! Important

Do not exceed 70 °C for samples intended for nitrogen analysis, as volatile nitrogen compounds may be lost at higher temperatures. Do not exceed 60 °C for samples intended for analysis of volatile organic compounds, essential oils, or lipids (Kalra, 1998).

4. Remove dried samples from the oven and cool in a desiccator for at least 30 minutes before weighing or grinding.
5. Record the dry mass if gravimetric moisture content is needed.

7.3. Coarse Grinding (Wiley Mill)

1. Inspect the mill: confirm the 1 mm (20-mesh) screen is installed and the cutting blades are sharp and properly seated.

2. Turn on the mill and allow it to reach full speed before feeding material.
3. Feed dried tissue into the hopper in small amounts, pushing material gently with the wooden plunger. Do not force tissue into the blades.
4. Collect the ground material passing through the screen in a clean, labeled container or bag.
5. Between samples, disassemble the grinding chamber and clean thoroughly:
 - Brush all surfaces with a stiff brush to remove residual material
 - Wipe with 70% ethanol
 - Run a small amount of acid-washed quartz sand through the mill as a blank grind if cross-contamination of trace metals is a concern
 - Allow to dry before the next sample

7.4. Fine Grinding (for IRMS or Homogeneity-Critical Analyses)

For EA-IRMS analysis, the Wiley-milled material (less than 1 mm) must be further ground to a fine, homogeneous powder (less than 250 μm):

1. Transfer approximately 1–2 g of coarsely ground tissue to a clean ball mill vial with grinding balls.
2. Grind for 2–3 minutes until the material is a uniform fine powder.
3. Alternatively, use an agate mortar and pestle, grinding until no gritty texture remains.
4. Transfer the powder to a labeled vial or sample bag.
5. Clean the ball mill vial and balls between samples with 70% ethanol and Kimwipes.

7.5. Weighing into Tin Capsules (for EA-IRMS)

1. Using the analytical balance, tare a tin capsule on the balance pan.
2. Weigh 3–5 mg of finely ground tissue into the capsule (Brodie et al., 2011). Record the mass to 0.001 mg.
3. Fold the capsule into a tight pellet using forceps, ensuring no material escapes.
4. Place the pellet in a labeled well of a microplate or sample tray.
5. Include blanks (empty tin capsules) and reference materials (e.g., acetanilide, USGS-40) in the run sequence per [SOP-L414](#).

8. Quality Control

- Grind and analyze duplicate subsamples at a rate of at least 1 per 10 samples to assess preparation variability. Analytical duplicates should agree within 5% RPD for C and N concentrations.
- Include a laboratory reference material (e.g., NIST SRM 1515 Apple Leaves or NIST SRM 1547 Peach Leaves) in each analytical batch to verify accuracy (Kalra, 1998).
- Document all cleaning steps in the laboratory notebook, especially the blank grind between samples when trace metal analysis is planned.
- Confirm drying completeness by re-weighing a subset of samples after an additional 4 hours of drying.

9. Waste Disposal

Waste Type	Disposal Method
Plant tissue waste	Standard trash (non-hazardous)
Ethanol cleaning rinses	Evaporate in fume hood or flammable waste
Used tin capsules (unfired)	Standard trash
Quartz sand (blank grind)	Standard trash

10. References

- Brodie, C. R., Casford, J. S. L., Lloyd, J. M., Sherlock, S. C., Sherlock, S. C., Sherlock, S. C., & Sherlock, S. C. (2011). A comparison of GasBench II and elemental-analyser-based methodologies for measuring the stable carbon isotope composition of soil organic carbon. *Rapid Communications in Mass Spectrometry*, 25(16), 2413–2421. <https://doi.org/10.1002/rcm.5149>
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11. Related SOPs

SOP	Title	Relationship
L002	Laboratory Safety	Required safety training
L102	Using Balances	Balance operation
L406	Loss on Ignition	Related preparation for SOM
L414	Flash EA Combustion (IRMS)	Downstream analytical method
L410	Acid Digestion	Downstream digestion for metals

12. Training Record

Before processing plant tissue independently, personnel must:

1. Read this SOP in full
2. Complete [SOP-L002 Laboratory Safety](#) training
3. Demonstrate safe Wiley mill operation (startup, feeding, cleaning, shutdown) under supervision
4. Process at least five samples under supervision, including decontamination steps between samples

5. If preparing samples for IRMS, demonstrate fine grinding and accurate weighing into tin capsules (target mass 3–5 mg, recorded to 0.001 mg)
6. Be signed off by the PI or lab manager



Environmental Analysis Program

Pomona College
Biogeochemistry Laboratory

SOP-L604

Algae / Periphyton Identification and Enumeration

Standard Operating Procedure

SOP Number	SOP-L604
Revision	0.1
Author(s)	Marc Los Huertos
Date	2026-06-26
Organization	EA Program, Pomona College

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1. Purpose

This SOP describes the laboratory procedures for identifying and enumerating benthic algae (periphyton) from stream bioassessment samples. Periphyton assemblages are sensitive indicators of water quality, nutrient enrichment, and physical habitat condition, and are a required component of the California SWAMP bioassessment protocol (Barbour et al., 1999; Ode et al., 2016). This SOP covers soft-bodied algae (green algae, cyanobacteria, and other non-diatom taxa) and diatoms, which are processed separately due to different preparation requirements (Charles et al., 2002; Stevenson & Bahls, 1996).

2. Scope

- **Applies to:** Identification and enumeration of benthic periphyton from composite epilithic, epiphytic, or epidendric samples collected per SWAMP protocols at EA Program stream sites
- **Does not apply to:** Phytoplankton (water column) identification; chlorophyll *a* extraction (see [SOP-L310](#)); algal culture or toxin analysis; molecular identification (eDNA metabarcoding)
- **Location:** EA Program laboratory, SGM 133

3. Responsibilities

Role	Responsibility
PI / Lab Manager	Approve taxonomic protocols; arrange external QC
Trained Personnel	Process samples, identify and enumerate algae
New Students	Complete training before independent identification

4. Definitions

- **Acid washing (plant tissue):** Cleaning tissue with dilute HCl to remove surface contamination (dust, soil particles, fertilizer residues) before analysis for trace metals or nutrients; may be inappropriate for samples requiring analysis of acid-labile compounds.
- **Air drying:** Drying plant tissue at ambient temperature (typically 20–40 °C) to a stable moisture content; used as a preliminary step before oven drying; preserves enzyme activity and RNA better than direct oven drying.
- **Ball milling:** Size reduction by grinding samples between hardened steel or ceramic balls inside a rotating vessel; the preferred method for producing a homogeneous powder from fibrous plant tissue for EA-IRMS and elemental analysis.
- **Critical tissue concentration:** The minimum concentration of a nutrient required for maximum growth; below this level the plant is deficient; above it, growth is normal or the nutrient may be toxic at very high levels.
- **Lyophilization (freeze drying):** Removal of water from tissue under vacuum after rapid freezing; preserves cell structure, volatile compounds, and biological activity better than oven drying; required for enzyme, RNA, and some metabolite analyses.

- **Subsampling:** Drawing a representative small portion from a bulk dried, ground sample for analysis; requires thorough mixing and homogenization of the bulk powder before subsampling to ensure the subsample is representative.

5. Safety and Hazards

Hazards

- **Chemical:** Lugol's iodine solution (preservative) contains potassium iodide and iodine; it is a mild irritant and stains skin and clothing. Nitric acid (used in diatom cleaning) is corrosive; handle in the fume hood. The mounting medium Naphrax contains toluene, which is flammable and toxic by inhalation.
- **Physical:** Hot plates used for diatom cleaning and slide preparation pose burn risk. Coverslips and slides are fragile.
- **Biological:** Stream samples may contain pathogens. Wear gloves.

5.1. Required PPE

- ☐ Safety glasses or goggles
- ☐ Lab coat
- ☐ Nitrile gloves
- ☐ Fume hood (required for nitric acid cleaning and Naphrax mounting)

5.2. Emergency Procedures

For acid splashes, flush immediately with copious water for at least 15 minutes. For Naphrax/toluene spills, ventilate and absorb. Refer to [SOP-L003 Hazardous Materials Handling](#).

6. Equipment and Materials

6.1. Equipment

Item	Notes
Compound microscope (brightfield)	400x and 1000x (oil immersion) for diatoms
Inverted microscope (optional)	For soft-bodied algae in settling chambers
Palmer-Maloney counting cell	For soft-bodied algae enumeration
Hot plate	For diatom acid cleaning
Centrifuge	For concentrating cleaned diatom material
Slide warmer	For drying diatom mounts

6.2. Consumables

Item	Notes
Lugol's iodine solution	Preservative for soft-bodied algae
Concentrated HNO ₃ (trace metal grade)	For diatom frustule cleaning
30% H ₂ O ₂	Alternative oxidizer for cleaning
Naphrax mounting medium	High refractive index (n = 1.73); for permanent diatom slides
Coverslips (22 x 22 mm, No. 1)	
Glass microscope slides	
Immersion oil	

7. Procedure

7.1. Sample Processing Overview

Periphyton samples are processed in two parallel workflows:

1. **Soft-bodied algae:** Examined alive or preserved in Lugol's solution using a Palmer-Maloney cell or settling chamber at 400x magnification.
2. **Diatoms:** Cleaned with acid or peroxide to remove organic matter, mounted in Naphrax on permanent slides, and identified at 1000x (oil immersion).

7.2. Soft-Bodied Algae Identification

1. Homogenize the preserved sample by gently inverting the jar 20 times.
2. Pipette a known volume (typically 0.1 mL) into a Palmer-Maloney counting cell.
3. Allow cells to settle for 5 minutes.
4. Scan the entire cell at 400x magnification.
5. Identify and count a minimum of 300 natural units (cells, colonies, or filament lengths of 10 μm). A natural unit is the smallest entity that can be identified: a single cell for unicellular taxa, a colony for colonial taxa, or a 10 μm filament segment for filamentous taxa (Stevenson & Bahls, 1996).
6. Record counts by taxon. Identify to genus where possible; use higher-level groupings (family or order) for taxa that cannot be resolved without specialized preparation.
7. Calculate cell density:

$$\text{Cells/cm}^2 = \frac{N \times V_{\text{sample}}}{V_{\text{counted}} \times A_{\text{substrate}}}$$

where N = number of cells counted, V_{sample} = total sample volume (mL), V_{counted} = volume examined in the counting cell (mL), and $A_{\text{substrate}}$ = total area of substrate scraped in the field (cm^2).

7.3. Diatom Cleaning

1. Transfer approximately 5 mL of well-mixed sample to a labeled glass beaker or test tube.
2. In the fume hood, add an equal volume of concentrated HNO_3 (or 30% H_2O_2 as a less hazardous alternative).
3. Heat on the hot plate at low-to-medium heat (approximately 80 °C) until the sample clears and all organic material is digested (30–60 minutes). Do not boil.
4. Allow to cool. Centrifuge at 1500 x g for 5 minutes and decant the supernatant.
5. Wash the pellet with DI water, centrifuge, and decant. Repeat the wash 3–5 times until the pH of the supernatant is near neutral.
6. Resuspend the cleaned frustules in a small volume of DI water (approximately 1–2 mL).

7.4. Diatom Slide Preparation

1. Pipette a drop of cleaned diatom suspension onto a clean coverslip. Allow to air-dry completely on the slide warmer.
2. Place a drop of Naphrax on a labeled glass slide (work in the fume hood; Naphrax contains toluene).
3. Invert the coverslip (diatom side down) onto the Naphrax drop.
4. Heat gently on the hot plate or slide warmer to drive off the toluene until no more bubbles appear.
5. Allow to cool. The slide is now a permanent mount.

7.5. Diatom Identification and Enumeration

1. Examine the slide at 1000x under oil immersion.
2. Count a minimum of 600 diatom valves, traversing random transects across the coverslip (Charles et al., 2002).
3. Identify each valve to the lowest practical taxonomic level (species where possible) using regional diatom floras and SWAMP reference keys (Krammer, 2000; Spaulding et al., 2021).
4. Record counts by taxon.

7.6. Data Entry and Metric Calculation

1. Enter soft-bodied and diatom counts into the standardized data entry spreadsheet.
2. Calculate summary metrics: total taxa richness, relative abundance of indicator taxa (e.g., pollution-tolerant vs. sensitive diatoms), and community-level indices.
3. If submitting to SWAMP/CEDEN, format data per SWAMP requirements.

8. Quality Control

- **Soft-bodied algae:** Re-count 10% of samples. Counts should agree within 15% RPD for dominant taxa.
- **Diatoms:** Have a second identifier verify 10% of slides. Taxonomic agreement should be greater than or equal to 85% at the species level.

- For SWAMP submissions, submit 10% of diatom slides to a SWAMP-certified taxonomist for external QC.
- Include a reference slide set in the laboratory for training and calibration.

9. Waste Disposal

Waste Type	Disposal Method
Nitric acid waste	Neutralize and collect in acid waste; EH&S pickup
H ₂ O ₂ waste	Dilute and drain with copious water
Naphrax / toluene waste	Collect in organic waste; EH&S pickup
Lugol's iodine waste	Collect in designated container; EH&S pickup
Used slides and coverslips	Standard sharps/glass waste

10. References

- Barbour, M. T., Gerritsen, J., Snyder, B. D., & Stribling, J. B. (1999). *Rapid bioassessment protocols for use in streams and Wadeable rivers: Periphyton, benthic macroinvertebrates, and fish* (2nd ed.). U.S. Environmental Protection Agency, Office of Water. <https://www.epa.gov/cwa-methods/rapid-bioassessment>
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11. Related SOPs

SOP	Title	Relationship
L002	Laboratory Safety	Required safety training
L003	Hazardous Materials Handling	Acid and solvent safety
L310	Chlorophyll Extraction	Complementary biomass metric
L601	Wet Mount for Microscopy	General microscopy technique
F610	BMI Sampling	Co-collected bioassessment data

12. Training Record

Before identifying and enumerating algae independently, personnel must:

1. Read this SOP in full
2. Complete [SOP-L002 Laboratory Safety](#) and [SOP-L003 Hazardous Materials Handling](#) training
3. Review the SDS for nitric acid and Naphrax
4. Study the diatom and soft-bodied algae reference collections and identification keys
5. Complete a supervised identification exercise (minimum 100 diatom valves and 50 soft-bodied algae natural units) with greater than 80% agreement with reference identifications
6. Demonstrate safe acid cleaning and Naphrax mounting under supervision
7. Be signed off by the PI or lab manager



Environmental Analysis Program

Pomona College
Biogeochemistry Laboratory

SOP-L605

Soil Enzyme Assays (Fluorometric Microplate Method)

Standard Operating Procedure

SOP Number	SOP-L605
Revision	0.1
Author(s)	Marc Los Huertos
Date	2026-06-26
Organization	EA Program, Pomona College

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1. Purpose

This SOP describes the fluorometric microplate method for measuring the potential activity of extracellular enzymes in soils. Extracellular enzymes mediate the decomposition of organic matter and the cycling of carbon, nitrogen, phosphorus, and sulfur in soils. Assays use fluorogenic substrates based on 4-methylumbelliferone (MUB) and 7-amino-4-methylcoumarin (AMC), which release a fluorescent product upon enzymatic hydrolysis (Bell et al., 2013; German et al., 2011; Sinsabaugh & Moorhead, 1994). The method is high-throughput (96-well microplate format), sensitive, and widely used in soil biogeochemistry and ecosystem ecology.

2. Scope

- **Applies to:** Measurement of potential hydrolytic enzyme activities in field-moist, sieved (less than 2 mm) mineral and organic soils from EA Program sites, using MUB- and AMC-linked fluorogenic substrates in a 96-well microplate format
- **Does not apply to:** Oxidative enzyme assays (e.g., phenol oxidase, peroxidase), which use colorimetric substrates (L-DOPA); enzyme assays in aquatic samples (requires modified dilution); commercially available enzyme activity kits with different substrates
- **Location:** EA Program laboratory, SGM 133

3. Responsibilities

Role	Responsibility
PI / Lab Manager	Select target enzymes; approve substrate concentrations
Trained Personnel	Prepare reagents, run assays, analyze data
New Students	Complete training before independent use

4. Definitions

- **Fluorometric enzyme assay:** An enzyme activity assay in which substrate hydrolysis releases a fluorescent product (typically methylumbelliferone, MUB, or methylcoumarin, MCA) measurable at low concentrations by a fluorometer; more sensitive than colorimetric assays.
- **Maximum velocity (V_{max}):** The maximum rate of an enzyme-catalyzed reaction when substrate is saturating; expressed in units of nmol product g⁻¹ dry soil h⁻¹; reflects the potential activity of the enzyme in soil.
- **Methylumbelliferone (MUB):** A fluorescent product released by many hydrolytic enzymes when they cleave a methylumbelliferyl-linked substrate; detected at excitation ~365 nm and emission ~450 nm; the basis of most fluorometric soil enzyme assays.
- **Potential enzyme activity:** The enzyme activity measured under optimized laboratory conditions (excess substrate, controlled pH, temperature 25 °C); an index of the extant enzyme pool rather than actual in situ activity.

- **Quench correction:** A correction applied to fluorometric assays to account for reduction in fluorescence signal caused by soil colloids and dissolved organic matter; calculated from samples spiked with a known amount of MUB standard.
- **Substrate:** The molecule acted upon by an enzyme; in fluorometric assays, a synthetic substrate linked to MUB or MCA is cleaved by the target enzyme to release the fluorescent product.
- **Turnover rate:** The number of substrate molecules converted per enzyme molecule per unit time; an intrinsic property of the enzyme that depends on temperature, pH, and substrate concentration.

5. Safety and Hazards

Hazards

- **Chemical:** MUB and AMC substrates are dissolved in dimethyl sulfoxide (DMSO), which is readily absorbed through the skin and can carry dissolved solutes into the body. Sodium acetate buffer and sodium hydroxide (for pH adjustment) are mild to moderate irritants. Concentrated NaOH is corrosive.
- **Biological:** Soil samples may contain pathogens. Handle with gloves.
- **Physical:** Microplate reader UV lamp may cause eye strain with prolonged direct viewing.

5.1. Required PPE

- ☐ Safety glasses
- ☐ Lab coat
- ☐ Nitrile gloves (double glove when handling DMSO solutions)

5.2. Emergency Procedures

For DMSO contact with skin, wash immediately with soap and water (do not use ethanol, which enhances absorption). For NaOH contact, flush with water for 15 minutes. Refer to [SOP-L002 Laboratory Safety](#).

6. Equipment and Materials

6.1. Equipment

Item	Notes
Fluorescence microplate reader	Excitation 365 nm, emission 450 nm
Multichannel micropipette (8-channel)	50–300 μL range
Single-channel micropipettes	10–100 μL and 100–1000 μL

Item	Notes
96-well black flat-bottom microplates	Non-treated; opaque walls to prevent bleedthrough
Orbital shaker or plate shaker	
Analytical balance	For weighing soil
pH meter	For buffer preparation
Centrifuge (optional)	For clarifying soil slurries

6.2. Consumables

Item	Notes
4-Methylumbelliferyl substrates	See enzyme table (Section 6)
7-Amino-4-methylcoumarin substrates	For leucine aminopeptidase
4-Methylumbelliferone (MUB) standard	For calibration curves
7-Amino-4-methylcoumarin (AMC) standard	For calibration curves
DMSO (ACS grade)	Solvent for substrate stocks
Sodium acetate buffer (50 mM, pH 5.0)	Or Tris buffer pH 7.8 for alkaline soils
NaOH (1 N)	To stop reactions and enhance fluorescence
DI water (Type II)	
Reagent reservoirs	For multichannel pipetting
Adhesive plate seals	For incubation

7. Target Enzymes and Substrates

The following enzymes are routinely assayed in the EA Program. Other MUB- or AMC-linked substrates can be added using the same general protocol.

Table 1: Target enzymes, substrates, and associated nutrient cycles.

Enzyme	Abbreviation	Substrate	Nutrient Cycle	Substrate Conc.
β -1,4-glucosidase	BG	4-MUB- β -D-glucopyranoside	Carbon	200 μ M
Cellobiohydrolase	CBH	4-MUB- β -D-cellobioside	Carbon	200 μ M
β -1,4-N-acetylglucosaminidase	NAG	4-MUB-N-acetyl- β -D-glucosaminide	Carbon / Nitrogen	200 μ M
Acid (alkaline) phosphatase	AP	4-MUB-phosphate	Phosphorus	200 μ M
β -1,4-xylosidase	BX	4-MUB- β -D-xylopyranoside	Carbon	200 μ M
Leucine aminopeptidase	LAP	L-Leucine-7-AMC	Nitrogen	200 μ M
Sulfatase	SUL	4-MUB-sulfate	Sulfur	200 μ M

8. Procedure

8.1. Soil Slurry Preparation

1. Weigh 1.0 g of field-moist, sieved (less than 2 mm) soil into a clean container.
2. Add 125 mL of 50 mM sodium acetate buffer (pH adjusted to match the soil pH, typically 5.0 for acidic soils or 7.8 for alkaline soils) (German et al., 2011).
3. Homogenize with a magnetic stir bar or by vortexing for 1 minute.
4. Keep the slurry stirring continuously during pipetting to maintain a uniform suspension.

8.2. Substrate Stock Preparation

1. Dissolve each MUB or AMC substrate in DMSO to prepare a 10 mM stock solution. Store at -20°C in amber vials; stable for at least 6 months.
2. Prepare working solutions by diluting the DMSO stock in buffer to the target concentration (typically 200 μM final concentration in the well).
3. Prepare MUB and AMC calibration standards in buffer at concentrations of 0, 0.5, 1, 2.5, 5, 10, and 25 μM .

8.3. Microplate Layout

For each soil sample, set up the following wells (8 analytical replicates per enzyme) (Bell et al., 2013):

Well Type	Contents	Purpose
Sample assay (n = 8)	200 μL slurry + 50 μL substrate	Enzyme activity
Sample control (n = 8)	200 μL slurry + 50 μL buffer	Soil background fluorescence
Substrate control (n = 8)	200 μL buffer + 50 μL substrate	Substrate autofluorescence
MUB/AMC standard curve	200 μL buffer + 50 μL standard	Calibration
Blank	200 μL buffer + 50 μL buffer	Instrument blank

8.4. Incubation

1. Seal the plate with an adhesive seal.
2. Incubate at 20°C (or field-relevant temperature) in the dark on a plate shaker at low speed for 2–4 hours. The incubation time depends on expected activity; 3 hours is a standard starting point (Sinsabaugh & Moorhead, 1994).

 Tip

For soils with unknown activity, run a pilot plate at 1, 2, and 4 hours to identify the linear range. Activity calculations assume linearity over the incubation period; if the reaction plateaus, shorten the incubation.

8.5. Stopping and Reading

1. After incubation, add 10 μL of 1 N NaOH to each well to stop the reaction and maximize fluorescence of the MUB/AMC product (the fluorophore is brightest above pH 10).
2. Read fluorescence on the microplate reader at excitation 365 nm / emission 450 nm within 5 minutes of adding NaOH.
3. Export the raw fluorescence data for analysis.

9. Data Analysis and Calculations

9.1. Activity Calculation

For each enzyme, calculate the net fluorescence:

$$F_{\text{net}} = F_{\text{sample}} - F_{\text{soil control}} - F_{\text{substrate control}} + F_{\text{blank}}$$

Convert net fluorescence to nmol of product released using the MUB or AMC standard curve (linear regression of fluorescence vs. concentration).

Calculate enzyme activity:

$$\text{Activity (nmol g}^{-1}\text{h}^{-1}) = \frac{F_{\text{net}}/m \times V_{\text{well}}}{M_{\text{soil}} \times t}$$

where m = slope of the standard curve (fluorescence per nmol), V_{well} = total well volume (L), M_{soil} = dry mass equivalent of soil in the well (g, corrected for moisture content per [SOP-L400](#)), and t = incubation time (h).

```
# Example enzyme activity calculation
library(tidyverse)

# Standard curve data
standards <- tibble(
  conc_uM = c(0, 0.5, 1.0, 2.5, 5.0, 10.0, 25.0),
  fluorescence = c(120, 850, 1580, 3750, 7200, 14100, 34500)
)

# Fit linear model
cal_model <- lm(fluorescence ~ conc_uM, data = standards)
```

```
slope <- coef(cal_model)[2] # fluorescence units per uM

# Sample data (means of 8 replicates)
F_sample <- 5200
F_soil_ctrl <- 350
F_sub_ctrl <- 180
F_blank <- 125

F_net <- F_sample - F_soil_ctrl - F_sub_ctrl + F_blank

# Convert to concentration (uM in well)
conc_well <- F_net / slope

# Calculate activity
V_well_L <- 250e-6 # 250 uL total well volume in liters
soil_fresh_g <- 1.0 # g fresh soil in 125 mL slurry
frac_in_well <- 200 / (125 * 1000) # fraction of slurry in well
soil_in_well_g <- soil_fresh_g * frac_in_well
moisture <- 0.25 # gravimetric moisture content
soil_dry_g <- soil_in_well_g * (1 - moisture)
incubation_h <- 3

activity <- (conc_well * V_well_L * 1e6) / (soil_dry_g * incubation_h)
# Result in nmol / g dry soil / h

cat(sprintf("Net fluorescence: %.0f\n", F_net))
cat(sprintf("Activity: %.1f nmol g-1 h-1\n", activity))
```

10. Quality Control

- Run 8 analytical replicates per enzyme per sample. Report the mean and coefficient of variation; CV should be less than 20%.
- Include substrate and soil quench controls on every plate.
- Run the MUB/AMC standard curve on each plate (do not carry over calibrations between plates, as reader sensitivity may drift).
- Include a laboratory reference soil in each batch to track inter-batch variability.
- Verify that activity is linear over the incubation period by running a time-course pilot for new soil types.

11. Waste Disposal

Waste Type	Disposal Method
Plate contents (soil slurry + substrates)	Drain with copious water (dilute, non-hazardous at assay concentrations)
DMSO substrate stock waste	Collect in organic waste; EH&S pickup
NaOH waste	Neutralize and drain
Used microplates	Standard trash
Used pipette tips	Standard trash

12. References

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13. Related SOPs

SOP	Title	Relationship
L002	Laboratory Safety	Required safety training
L400	Soil Moisture (Gravimetric)	Moisture correction for dry-mass calculations
L409	Microbial Biomass Carbon	Complementary microbial activity measure
L408	Solvita CO ₂ Burst	Related microbial activity metric
F400	Soil Sampling	Upstream: sample collection

14. Training Record

Before performing soil enzyme assays independently, personnel must:

1. Read this SOP in full
2. Complete [SOP-L002 Laboratory Safety](#) training
3. Review the SDS for DMSO, NaOH, and the fluorogenic substrates
4. Observe a trained operator perform a complete assay (slurry preparation through plate reading and data export)
5. Perform the assay under supervision with acceptable QC results (replicate CV less than 20%; standard curve $r^2 > 0.99$)

6. Demonstrate correct use of the multichannel pipette and microplate reader
7. Be signed off by the PI or lab manager



Environmental Analysis Program

Pomona College
Biogeochemistry Laboratory

SOP-L700

DNA Extraction, Quantification, and Purity Assessment

Standard Operating Procedure

SOP Number	SOP-L700
Revision	1.0
Author(s)	Marc Los Huertos
Date	2026-06-26
Organization	EA Program, Pomona College

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1. Purpose

This procedure describes the extraction of genomic DNA from environmental samples (soil, sediment, water filters, and plant tissue), followed by quantification of DNA concentration and assessment of DNA purity using spectrophotometric and fluorometric methods. The protocol is based on commercial bead-beating extraction kits and is suitable for downstream applications including PCR amplification, qPCR, and amplicon sequencing (Qiagen, 2024a, 2024b).

2. Scope

- **Applies to:** Genomic DNA extraction from soil, sediment, water filter membranes, and plant tissue using the DNeasy PowerSoil Pro Kit (Qiagen) or equivalent bead-beating kit; DNA quantification using a NanoDrop spectrophotometer and Qubit fluorometer
- **Does not apply to:** RNA extraction, plasmid isolation, ancient DNA, or clinical/diagnostic samples
- **Location:** Molecular biology bench, Pomona College Biogeochemistry Lab

3. Responsibilities

Role	Responsibility
Lab PI	Approve SOP, ensure training compliance
Trained Personnel	Follow this SOP, report deviations
New Students	Complete training before independent use

4. Definitions

- **A_{260}/A_{280} ratio:** The ratio of absorbance at 260 nm to 280 nm; pure dsDNA yields ~1.8; values below 1.7 indicate protein or phenol contamination that may inhibit PCR amplification.
- **A_{260}/A_{230} ratio:** The ratio of absorbance at 260 nm to 230 nm; values of 2.0–2.2 indicate pure nucleic acids; low values indicate contamination with chaotropic salts, humic acids, or carbohydrates.
- **Bead beating:** Mechanical cell lysis in which sample is agitated at high speed with ceramic or glass beads in a tube; disrupts cell walls and membranes including the tough walls of fungal spores and gram-positive bacteria.
- **Chaotropic salt:** A salt (e.g., guanidinium isothiocyanate, guanidinium hydrochloride) that disrupts hydrogen bonds and hydrophobic interactions; used in DNA extraction to denature proteins and facilitate binding of DNA to silica membranes.
- **Environmental DNA (eDNA):** Genetic material shed into the environment by organisms through mucus, feces, skin cells, and gametes; collected from soil, water, or air without organism capture.
- **Inhibitor:** A substance co-extracted with DNA that reduces PCR amplification efficiency; common inhibitors in soil and water eDNA include humic acids, tannins, heavy metals, and residual ethanol.

- **Silica column (spin column):** A miniaturized chromatography column containing a silica membrane that selectively binds DNA in the presence of chaotropic salts; DNA is washed and then eluted with low-salt buffer or water.

5. Safety and Hazards

Hazards

- **Chemical:** Lysis buffer contains guanidine salts, which are harmful if swallowed or inhaled and can cause skin and eye irritation. Solution C4 contains ethanol. Review the Safety Data Sheets (SDS) for all kit reagents before use.
- **Physical:** Bead-beating generates aerosols; keep tubes sealed during vortexing. Centrifuge rotor imbalance can cause equipment damage.
- **Biological:** Environmental samples may contain unknown pathogens. Treat all samples as potentially infectious (Centers for Disease Control and Prevention & National Institutes of Health, 2020).

5.1. Required PPE

- ☐ Safety glasses or goggles
- ☐ Lab coat
- ☐ Nitrile gloves (change frequently to avoid cross-contamination)
- ☐ Face shield (if working with large volumes of lysis buffer)

5.2. Emergency Procedures

In case of chemical splash or skin/eye contact, flush affected area with water for at least 15 minutes and consult the SDS. For spills of lysis buffer, absorb with paper towels, clean with water, and dispose as chemical waste. Refer to [SOP-L003 Hazardous Materials Handling](#) for general chemical spill response.

6. Equipment and Materials

6.1. Equipment

Item	Location	Notes
Vortex adapter for bead tubes	Mol bio bench	Holds 24 tubes horizontally
Vortex-Genie 2	Mol bio bench	Set to maximum speed
Microcentrifuge	Mol bio bench	Must reach 10,000 x g minimum
NanoDrop One spectrophotometer	Instrument bench	UV-Vis micro-volume
Qubit 4 fluorometer	Instrument bench	For dsDNA quantification
Micropipettes (10, 100, 1000 uL)	Mol bio bench	Calibrated annually

Item	Location	Notes
–80 C freezer	Cold room	For long-term DNA storage
–20 C freezer	Lab	For short-term DNA storage

6.2. Reagents and Consumables

Item	Catalog No.	Storage
DNeasy PowerSoil Pro Kit (Qiagen)	47014 (50 prep)	Room temperature
Qubit dsDNA BR Assay Kit	Q32850	Room temperature
Qubit dsDNA HS Assay Kit	Q32851	Room temperature
Qubit assay tubes (0.5 mL)	Q32856	Room temperature
Nuclease-free water	Various	Room temperature
Molecular-grade ethanol (100%)	Various	Flammables cabinet
1.5 mL microcentrifuge tubes (DNase-free)	Various	Room temperature
Filter pipette tips (10, 100, 1000 uL)	Various	Room temperature

7. Procedure

7.1. Sample Preparation

1. Record sample identity, collection date, and mass/volume in the lab notebook and in the [Freezer Inventory Google Sheet](#)
2. For soil/sediment: weigh 0.20–0.25 g of sample into the PowerBead Pro tube. Record exact mass.
3. For water filters: use sterile forceps to transfer the membrane filter into the PowerBead Pro tube. Cut the filter into strips if needed to fit inside the tube.
4. For plant tissue: weigh 0.02–0.10 g of tissue (fresh or flash-frozen) into the PowerBead Pro tube. Use less tissue for highly mucilaginous samples (Doyle & Doyle, 1987).

! Important

Keep samples on ice during preparation to minimize DNA degradation by endogenous nucleases. Process all samples within a single batch if possible to reduce extraction variability (Frosteg aard et al., 1999).

7.2. Cell Lysis and DNA Extraction

Follow the DNeasy PowerSoil Pro Kit protocol (Qiagen, 2024b):

1. Add 800 uL of Solution CD1 to the PowerBead Pro tube
2. Secure tubes horizontally on the vortex adapter and vortex at maximum speed for 10 minutes
3. Centrifuge at 15,000 x g for 1 minute

4. Transfer the supernatant to a clean 2 mL collection tube
5. Add 200 uL of Solution CD2, vortex briefly (5 seconds), and centrifuge at 15,000 x g for 1 minute
6. Avoiding the pellet, transfer the supernatant to a clean 2 mL collection tube
7. Add 600 uL of Solution CD3, vortex briefly (5 seconds)
8. Load 650 uL onto an MB Spin Column seated in a 2 mL collection tube. Centrifuge at 15,000 x g for 1 minute. Discard flow-through. Repeat to load remaining lysate.
9. Add 500 uL of Solution EA. Centrifuge at 15,000 x g for 1 minute. Discard flow-through.
10. Add 500 uL of Solution C5 (wash buffer). Centrifuge at 15,000 x g for 1 minute. Discard flow-through.
11. Centrifuge again at 16,000 x g for 2 minutes to remove residual wash buffer
12. Transfer the MB Spin Column to a clean 1.5 mL microcentrifuge tube
13. Add 50–100 uL of Solution C6 (elution buffer) or nuclease-free water to the center of the membrane. Allow to stand for 1 minute at room temperature, then centrifuge at 15,000 x g for 1 minute.

 Tip

Use a smaller elution volume (50 uL) when higher DNA concentration is needed for downstream applications. A second elution with an additional 50 uL can recover 10–20% more DNA but at lower concentration (Qiagen, 2024b).

7.3. DNA Quantification – NanoDrop Spectrophotometry

The NanoDrop measures DNA concentration and provides absorbance ratios used to assess purity (Desjardins & Bhatt, 2010):

1. Clean the pedestal with 2 uL of nuclease-free water; wipe with a lint-free tissue
2. Blank the instrument with 2 uL of the elution buffer (Solution C6 or nuclease-free water, matching your elution)
3. Load 2 uL of DNA extract onto the pedestal
4. Record the concentration (ng/uL), A_{260}/A_{280} ratio, and A_{260}/A_{230} ratio for each sample

7.4. DNA Quantification – Qubit Fluorometry

The Qubit fluorometer uses fluorescent dyes that bind selectively to dsDNA, providing more accurate quantification than spectrophotometry, especially at low concentrations or in the presence of contaminants (Thermo Fisher Scientific, 2024):

1. Prepare the Qubit working solution: dilute the Qubit dsDNA BR (or HS) reagent 1:200 in Qubit buffer. Prepare enough for all samples plus two standards (e.g., 200 uL per assay tube).
2. Prepare standards: add 190 uL of working solution to each of two Qubit assay tubes. Add 10 uL of Standard 1 to one tube and 10 uL of Standard 2 to the other. Vortex briefly.
3. Prepare samples: add 198 uL of working solution to a Qubit assay tube. Add 2 uL of DNA extract. Vortex briefly.

4. Incubate all tubes at room temperature for 2 minutes (but not longer than 30 minutes)
5. Calibrate the Qubit with the two standards, then read each sample
6. Record the concentration (ng/uL, corrected for dilution by the instrument)

7.5. DNA Purity Assessment

Evaluate DNA purity using the NanoDrop absorbance ratios (Desjardins & Bhatt, 2010; Wilfinger et al., 1997):

Ratio	Ideal Range	Interpretation
A_{260}/A_{280}	1.8–2.0	Values below 1.8 suggest protein or phenol contamination
A_{260}/A_{230}	2.0–2.2	Values below 2.0 suggest contamination with humic acids, carbohydrates, salts, or residual guanidine from extraction buffers

Note

For environmental DNA extracts, especially from humic-rich soils, A_{260}/A_{230} ratios are frequently below 2.0. If downstream applications (e.g., PCR) fail or show inhibition, consider additional cleanup using a OneStep PCR Inhibitor Removal Kit or gel purification (Young et al., 2014).

7.6. Storage and Freezer Inventory

1. Label each tube with: sample ID, extraction date, elution volume, and concentration (in ng/uL)
2. For short-term storage (less than 1 month): store DNA extracts at -20°C
3. For long-term storage: store DNA extracts at -80°C in the designated molecular biology section of the freezer
4. Record the storage location (freezer, rack, box, and position) in the [Freezer Inventory Google Sheet](#)
5. Update the Google Sheet immediately upon placing samples in the freezer or removing them

-80°C Freezer Protocol

- Minimize the time the freezer door is open. Retrieve the correct box before opening the freezer.
- Place DNA extracts in designated cryoboxes labeled “DNA Extracts” with the project name and date range.
- Do not store DNA extracts in the same box as tissue samples or reagents.
- Avoid repeated freeze-thaw cycles. If multiple downstream analyses are planned, prepare aliquots at the time of extraction (Shao et al., 2012).

8. Quality Control and Acceptance Criteria

- Include one extraction blank (empty PowerBead Pro tube processed through the full protocol) per extraction batch to detect contamination (Salter et al., 2014)
- Run Qubit standards with each new batch of working solution
- Acceptable DNA yield from 0.25 g of soil: typically 1–20 ng/uL in a 100 uL elution, depending on soil type (Dineen et al., 2010)
- Acceptable A_{260}/A_{280} : 1.7–2.0. Samples outside this range should be noted and may require re-extraction.
- Acceptable A_{260}/A_{230} : greater than 1.5 for most downstream applications. Values below 1.0 indicate significant contamination and the extract should be purified or re-extracted.
- If NanoDrop and Qubit concentrations disagree by more than a factor of two, the NanoDrop reading is likely inflated by contaminants; use the Qubit value for planning downstream reactions (Nakayama et al., 2016).

9. Waste Disposal

Waste Type	Container	Disposal Method
Used PowerBead Pro tubes	Biohazard bag	Autoclave, then standard trash
Flow-through from spin columns	Chemical waste carboy	EH&S hazardous waste pickup (contains guanidine salts)
Used spin columns	Chemical waste bag	EH&S hazardous waste pickup
Pipette tips and tubes	Biohazard sharps/trash	Standard biohazard disposal
Empty reagent bottles	Rinse and recycle	Check SDS for specific instructions

10. References

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11. Training Record

Before performing this procedure independently, personnel must:

1. Read this SOP and [SOP-L003 Hazardous Materials Handling](#) in full
2. Review the SDS for all kit components (especially Solutions CD1, CD2, and CD3)
3. Complete the Qiagen DNeasy PowerSoil Pro Kit quick-start guide
4. Demonstrate competence with micropipetting, the NanoDrop, and the Qubit fluorometer
5. Perform a full extraction under supervision, including quantification and freezer logging
6. Be signed off by the PI or lab manager

12. Appendix A: Troubleshooting

Symptom	Probable Cause	Corrective Action
Very low DNA yield	Insufficient lysis; too little starting material	Increase vortexing time to 15 min; verify sample mass
Low A_{260}/A_{280} (below 1.6)	Protein carryover	Re-extract with fresh kit reagents; ensure full removal of CD2 pellet

Symptom	Probable Cause	Corrective Action
Low A_{260}/A_{230} (below 1.0)	Humic acid or guanidine carryover	Add a second C5 wash step; use inhibitor removal kit
NanoDrop reads much higher than Qubit	Co-extracted contaminants inflating UV absorbance	Trust Qubit value; consider cleanup before downstream use
PCR inhibition despite good yield	Co-extracted humic acids or polysaccharides	Dilute template 1:10; use inhibitor-tolerant polymerase; clean up with OneStep kit (Young et al., 2014)
Extraction blank shows amplification	Reagent or cross-contamination	Discard batch; use fresh reagents; clean workspace with 10% bleach then ethanol

13. Appendix B: DNA Concentration Calculation Worksheet

```
# Calculate total DNA yield from extraction
elution_vol_uL <- 100      # Volume of elution buffer used (uL)
qubit_conc_ng_uL <- 8.5    # Qubit reading (ng/uL)
sample_mass_g <- 0.25      # Starting sample mass (g)

total_dna_ng <- qubit_conc_ng_uL * elution_vol_uL
dna_per_g <- total_dna_ng / sample_mass_g

cat(sprintf("Total DNA yield: %.1f ng\n", total_dna_ng))
```

Total DNA yield: 850.0 ng

```
cat(sprintf("DNA yield per gram of sample: %.1f ng/g\n", dna_per_g))
```

DNA yield per gram of sample: 3400.0 ng/g



Environmental Analysis Program

Pomona College
Biogeochemistry Laboratory

SOP-L701

Primer Selection and Design for Nitrogen Cycle Genes – ddPCR

Standard Operating Procedure

SOP Number	SOP-L701
Revision	0.2
Author(s)	Marc Los Huertos
Date	2026-06-26
Organization	EA Program, Pomona College

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1. Purpose

This procedure describes the selection, design, and validation of primers and probes targeting functional genes of the nitrogen cycle for use with droplet digital PCR (ddPCR). The nitrogen cycle genes covered include those encoding nitrification (*amoA* for both ammonia-oxidizing bacteria and archaea), denitrification (*nirK*, *nirS*, *nosZ*), nitrogen fixation (*nifH*), and dissimilatory nitrate reduction to ammonium (*nrfA*). The SOP covers literature-based primer selection, in silico evaluation, and bench validation on the Bio-Rad QX200 ddPCR system (Bio-Rad Laboratories, 2024; Hindson et al., 2011).

2. Scope

- **Applies to:** Design and selection of primers and hydrolysis probes (TaqMan) or EvaGreen-based assays for quantifying nitrogen cycle functional genes from environmental DNA extracts (soil, sediment, water)
- **Does not apply to:** Amplicon sequencing library preparation, metagenomics, or RNA-based transcript quantification (RT-ddPCR)
- **Location:** Molecular biology bench, Pomona College Biogeochemistry Lab

3. Responsibilities

Role	Responsibility
Lab PI	Approve primer selections and assay designs
Trained Personnel	Conduct in silico analysis, order primers, perform validation runs
New Students	Complete training before independent use

4. Definitions

- **Amplicon length:** The size in base pairs (bp) of the PCR product generated by a primer pair; for ddPCR, optimal amplicon length is 60–200 bp; shorter amplicons partition more efficiently into droplets and produce cleaner positive/negative separation (Bio-Rad Laboratories, 2024; Hindson et al., 2011).
- **amoA (ammonia monooxygenase alpha subunit):** The gene encoding the alpha subunit of ammonia monooxygenase; used as a functional marker for ammonia-oxidizing bacteria (AOB; *Nitrosomonas*-like) and ammonia-oxidizing archaea (AOA; Thaumarchaeota); quantified separately because AOB and AOA primers differ (Francis et al., 2005; Rotthauwe et al., 1997).
- **Annealing temperature (T_a):** The temperature at which primers bind to their complementary template sequences during PCR cycling; determined by gradient PCR optimization; typically 55–65 °C for nitrogen cycle gene assays; primer pairs should be within 2 °C of each other (Bio-Rad Laboratories, 2024).
- **ddPCR (droplet digital PCR):** A third-generation PCR technology that partitions a reaction into approximately 20,000 nanoliter droplets and amplifies target DNA to endpoint within each droplet; the fraction of positive droplets is fit to a Poisson distribution to calculate absolute target concentration without a standard curve (Hindson et al., 2011).
- **DNRA (dissimilatory nitrate reduction to ammonium):** A microbial process that reduces NO_3^- to NH_4^+ , retaining nitrogen in the ecosystem rather than losing it as gas; marker gene: *nrfA* (Levy-Booth et al., 2014).
- **EvaGreen:** A fluorescent intercalating dye used as a non-specific reporter in ddPCR and qPCR; binds double-stranded DNA; less specific than hydrolysis probes but useful for assay development and for targets without validated probe sequences (Bio-Rad Laboratories, 2024).
- **FunGene:** A curated functional gene database (<http://fungene.cme.msu.edu/>) that provides reference sequences, primer coverage analysis, and taxonomy for nitrogen cycle genes including *amoA*, *nirK*, *nirS*, *nosZ*, and *nifH* (Fish et al., 2013).

- **gBlock (synthetic DNA fragment):** A double-stranded DNA fragment (IDT) with a sequence matching the target amplicon; used as a positive control for ddPCR assay validation and as a template for limit-of-detection determination (Hindson et al., 2011).
- **Hydrolysis probe (TaqMan probe):** A short oligonucleotide labelled with a 5' fluorophore (e.g., FAM) and a 3' quencher (e.g., Iowa Black FQ); cleaved by Taq polymerase during extension, releasing the fluorophore and generating a signal; provides higher specificity than intercalating dyes and is required for multiplexed ddPCR (Bio-Rad Laboratories, 2024).
- **In silico primer evaluation:** Computational assessment of primer specificity, coverage, and secondary structure using tools such as NCBI Primer-BLAST, IDT OligoAnalyzer, and FunGene; a required step before ordering primers for bench validation (Fish et al., 2013; Ye et al., 2012).
- **nifH (nitrogenase reductase):** The gene encoding nitrogenase iron protein; the universal marker for biological nitrogen fixation (diazotrophy); highly conserved across diverse diazotrophic bacteria and archaea (Levy-Booth et al., 2014; Poly et al., 2001).
- **nirK / nirS:** Genes encoding the two structurally distinct nitrite reductases in denitrifiers: *nirK* encodes a copper-containing (Cu) enzyme; *nirS* encodes a cytochrome cd_1 enzyme; the two forms serve the same function and are often both targeted to capture the full denitrifying community (Braker et al., 2000; Henry et al., 2006).
- **nosZ:** The gene encoding nitrous oxide reductase, which reduces N_2O to N_2 in the final step of denitrification; divided into Clade I (classical denitrifiers; Henry et al. (2006)) and Clade II (diverse non-denitrifiers; Jones et al. (2013)); the sum of both clades estimates the community capacity to consume the greenhouse gas N_2O .
- **nrFA:** The gene encoding cytochrome c nitrite reductase, the enzyme catalyzing DNRA; a functional marker for organisms that retain nitrogen in the ecosystem as NH_4^+ rather than releasing it as N_2O or N_2 (Welsh et al., 2014).
- **Rain (ddPCR):** Droplets with intermediate fluorescence amplitude that fall between the positive and negative clusters in a ddPCR amplitude plot; indicates suboptimal primer binding, non-specific amplification, or partial inhibition; reduced by increasing annealing temperature or decreasing primer concentration (Lievens et al., 2016).

5. Safety and Hazards

Hazards

- **Chemical:** ddPCR droplet generation oil contains proprietary surfactants; avoid skin and eye contact. EvaGreen and other intercalating dyes are potential mutagens at high concentrations; handle with gloves.
- **Physical:** The QX200 Droplet Generator uses pressurized cartridges. Follow manufacturer instructions for cartridge handling.
- **Biological:** Environmental DNA extracts may originate from samples containing pathogens. Handle with standard precautions.

5.1. Required PPE

- ☐ Safety glasses

- ☐ Lab coat
- ☐ Nitrile gloves (changed frequently)

5.2. Emergency Procedures

For chemical splash, flush with water for 15 minutes. Refer to [SOP-L003 Hazardous Materials Handling](#).

6. Target Genes Overview

The following table summarizes the nitrogen cycle gene targets, the enzymatic steps they represent, and key references for established primer sets (Braker et al., 2000; Henry et al., 2006; Poly et al., 2001; Rösch et al., 2002; Rotthauwe et al., 1997; Welsh et al., 2014):

Gene	Enzyme	Process	Organism Group
<i>amoA</i>	Ammonia monooxygenase	Ammonia oxidation (nitrification)	AOB, AOA
<i>nirK</i>	Copper-containing nitrite reductase	Denitrification (NO_2^- to NO)	Denitrifiers
<i>nirS</i>	Cytochrome cd_1 nitrite reductase	Denitrification (NO_2^- to NO)	Denitrifiers
<i>nosZ</i>	Nitrous oxide reductase	Denitrification (N_2O to N_2)	Denitrifiers (Clade I and II)
<i>nifH</i>	Nitrogenase reductase	Nitrogen fixation (N_2 to NH_3)	Diazotrophs
<i>nrfA</i>	Cytochrome c nitrite reductase	DNRA (NO_2^- to NH_4^+)	DNRA organisms

7. Procedure

7.1. Literature-Based Primer Selection

1. Search the published literature for validated primer sets targeting each gene of interest. Prioritized references include Rotthauwe et al. (1997), Francis et al. (2005), Braker et al. (2000), Henry et al. (2006), Poly et al. (2001), Jones et al. (2013), and Welsh et al. (2014).
2. For each candidate primer pair, record: primer names, sequences (5' to 3'), amplicon length, annealing temperature, and the original publication.
3. Evaluate published primer sets against the following criteria:
 - Amplicon length between 60 and 200 bp (Bio-Rad Laboratories, 2024; Hindson et al., 2011)
 - Demonstrated use in environmental samples similar to your matrix
 - Broad coverage of the target gene clade verified by in silico analysis (see §6.3)
 - Minimal off-target amplification in non-target organisms

i Note

Many classic nitrogen cycle primers (e.g., *nosZ* primers from Henry et al. (2006)) were designed for conventional qPCR with amplicons of 250–400 bp. These may need to be redesigned or replaced with shorter alternatives optimized for ddPCR (Bio-Rad Laboratories, 2024).

7.2. Recommended Primer Sets

The following table summarizes commonly used primer sets for nitrogen cycle genes. Confirm suitability for your specific sample type and ddPCR platform before use.

! Important

This table provides a starting point. Primer sequences, annealing temperatures, and amplicon sizes should be verified against current literature and databases before ordering. Some primer sets listed below produce amplicons longer than ideal for ddPCR and may require redesign.

Gene	Primer Name	Sequence (5' to 3')	Amplicon (bp)	Reference	ddPCR Suitability
<i>amoA</i> (AOB)	amoA-1F / amoA-2R	GGGGTTTCTACTG-GTGGT / CCCCTCK-GSAAAGCCTTCTTC	491	Rotthauwe et al. (1997)	Redesign needed (too long)
<i>amoA</i> (AOA)	Arch-amoAF / Arch-amoAR	STAATGGTCTG-GCTTAGACG / GCGGCCATC-CATCTGTATGT	635	Francis et al. (2005)	Redesign needed (too long)
<i>nirK</i>	nirK876 / nirK1040	ATYGGCGGVCAYG-GCGA / GCCTCGATCAGRT-TRTGGTT	165	Henry et al. (2006)	Suitable
<i>nirS</i>	cd3aF / R3cd	G TSAACG TSAAG-GARACSGG / GASTTCGGRTGS-GTCTTGA	410	Braker et al. (2000)	Redesign needed
<i>nosZ</i> (Clade I)	nosZ2F / nosZ2R	CGCRACG-GCAASAAG-GTSMSSGT / CAKRTGCAKS-GCRTGGCAGAA	267	Henry et al. (2006)	Marginal; consider shorter alternative
<i>nosZ</i> (Clade II)	nosZ-II-F / nosZ-II-R	CTIGGICCIYTK-CAYAC / GCIGAR-CARAAITCBGTRC	698	Jones et al. (2013)	Redesign needed

Gene	Primer Name	Sequence (5' to 3')	Amplicon (bp)	Reference	ddPCR Suitability
<i>nifH</i>	PolF / PolR	TGCGAYCC-SAARGCBGACTC / ATSGCCAT-CATYTCRCCGGA	360	Poly et al. (2001)	Redesign needed
<i>nrfA</i>	nrfAF2aw / nrfAR1	CARTGYCAYGTB-GARTA / TWNGGCATRTGR-CARTC	251	Welsh et al. (2014)	Marginal

7.3. In Silico Primer Evaluation

1. Check primer specificity using NCBI Primer-BLAST against the nr/nt database, restricting the search to bacteria and/or archaea as appropriate (Ye et al., 2012).
2. Verify primer coverage against curated functional gene databases:
 - FunGene (<http://fungene.cme.msu.edu/>) for *amoA*, *nirK*, *nirS*, *nosZ*, *nifH* (Fish et al., 2013)
 - *TBD* – identify additional databases for *nrfA* coverage analysis
3. Check for primer dimers and secondary structures using IDT OligoAnalyzer (<https://www.idtdna.com/calc/analyzer>) or Primer3 (<https://primer3.ut.ee/>).
4. Confirm that the melting temperature (T_m) of each primer falls between 55 and 65 °C, with no more than 2 °C difference between forward and reverse primers.
5. For hydrolysis probe (TaqMan) assays, design the probe to have a T_m 5–10 °C higher than the primers, with no G at the 5' end (quenches FAM fluorescence), and a length of 15–30 bases (Bio-Rad Laboratories, 2024).

7.4. Primer and Probe Ordering

1. Order desalted primers at 25 nmol scale from IDT (Integrated DNA Technologies) or equivalent supplier.
2. Order hydrolysis probes HPLC-purified with 5'-FAM (or HEX for multiplexing) and 3'-Iowa Black FQ quencher.
3. For positive controls, order synthetic double-stranded DNA fragments (gBlocks) matching the target amplicon sequence. Include at least 50 bp of flanking sequence on each side of the primer binding sites.
4. Resuspend lyophilized primers in nuclease-free water to a stock concentration of 100 µM. Store at –20 °C.
5. Prepare 10 µM working stocks by diluting 1:10 in nuclease-free water. Store at –20 °C.

7.5. ddPCR Thermal Cycling Optimization

1. Set up a temperature gradient experiment on the Bio-Rad C1000 Touch to determine the optimal annealing temperature for each primer set.

2. Use a thermal gradient spanning $T_m \pm 5$ °C (typically 55–65 °C).
3. Use positive control gBlock DNA diluted to approximately 1,000 copies per μ L as template.
4. Recommended cycling protocol (Bio-Rad Laboratories, 2024):
 - Enzyme activation: 95 °C for 10 min
 - 40 cycles: 94 °C for 30 s, then annealing/extension at gradient temperature for 60 s
 - Signal stabilization: 98 °C for 10 min
 - Hold at 4 °C (indefinite)
 - Ramp rate: 2 °C/s for all steps
5. Read droplets on the QX200 Droplet Reader.
6. Select the annealing temperature that produces the clearest separation between positive and negative droplet clusters in the QuantaSoft amplitude plot.

 Tip

If positive and negative droplet populations overlap (rain), try increasing the annealing temperature by 1–2 °C or reducing the primer concentration from 900 nM to 450 nM (Lievens et al., 2016).

7.6. Assay Validation

1. **Specificity:** Run no-template controls (NTC) and extraction blanks from [SOP-L700](#) to confirm the absence of false positives.
2. **Dynamic range:** Prepare a serial dilution of gBlock positive control (10 to 100,000 copies per reaction) and verify linearity of the ddPCR response across the range (Huggett et al., 2013).
3. **Limit of detection (LOD):** Determine the lowest copy number that produces a statistically significant positive call above NTC background; typically 1–10 copies per 20 μ L reaction (Hindson et al., 2011).
4. **Matrix effects:** Run environmental DNA extracts at multiple dilutions (undiluted, 1:5, 1:10) to check for PCR inhibition. If the 1:10 dilution value (back-calculated) disagrees by more than 20% from the 1:5 value, inhibition is likely present (Ravetski et al., 2014).
5. Document all validation results in the primer/probe registry (Appendix A) before approving the assay for routine use.

8. Primer and Probe Registry

Maintain a lab registry of all validated and candidate assays. The registry must include at minimum: gene target, primer names and sequences, probe sequence (if applicable), amplicon length, validated annealing temperature, LOD, matrix tested, validation date, and analyst initials. Store the registry as a version-controlled file in the lab GitHub repository.

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10. Related SOPs

SOP	Title	Relationship
L700	DNA Extraction	Upstream: DNA template source
L702	ddPCR Applications Guide	Downstream: data analysis
L703	PCR and Gel Electrophoresis	Gel verification of primer products
L802	Quality Control and QA	General QA/QC framework

11. Training Record

Before designing or ordering primers independently, personnel must:

1. Read this SOP in full
2. Complete the Bio-Rad ddPCR Foundations eLearning module
3. Demonstrate competent use of Primer-BLAST and IDT OligoAnalyzer
4. Perform a supervised gradient optimization experiment and select the annealing temperature with guidance from the PI
5. Be signed off by the PI



Environmental Analysis Program

Pomona College
Biogeochemistry Laboratory

SOP-L702

ddPCR Applications Guide – Absolute Quantification of Soil Nitrogen-Cycle Functional Genes

Standard Operating Procedure

SOP Number	SOP-L702
Revision	0.2
Author(s)	Marc Los Huertos
Date	2026-06-26
Organization	EA Program, Pomona College

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1. Purpose

This procedure describes the workflow for exporting raw data from the Bio-Rad QX200 ddPCR system, performing quality control checks, calculating gene copy numbers normalized to soil mass or DNA concentration, and conducting statistical analyses of nitrogen cycle gene abundance data. The SOP links the instrument output from [SOP-L701](#) to reproducible analysis scripts written in R (R Core Team, 2024).

2. Scope

- **Applies to:** Interpretation and analysis of QX200 ddPCR data (QuantaSoft or QuantaSoft Analysis Pro output) for nitrogen-cycle functional genes (*amoA*, *nirK*, *nirS*, *nosZ*, *nifH*, *nrfA*) from environmental soil samples, quantified in absolute units (copies per gram dry soil or copies per nanogram DNA)
- **Does not apply to:** Operating the instrument (see SOP-L214); assay or primer design and validation (see [SOP-L701](#)); amplicon sequencing, metagenomics, or qPCR data from other platforms
- **Location:** Instrument computer (QuantaSoft) for export and threshold review; RStudio Server for analysis (see [SOP-L801 RStudio and GitHub](#))

3. Responsibilities

Role	Responsibility
Lab PI	Approve analysis pipeline and interpretation
Trained Personnel	Export data, run QC, perform analysis
New Students	Complete training before independent analysis

4. Definitions

- **Amplitude threshold:** The fluorescence intensity boundary set in QuantaSoft (or QuantaSoft Analysis Pro) that separates positive droplets (containing amplified target) from negative droplets; correct threshold placement is the single most critical step in ddPCR data analysis and must be reviewed manually for each plate (Bio-Rad Laboratories, 2024).
- **Copies per microliter (copies/ μ L):** The absolute concentration of target DNA in the final ddPCR reaction volume as calculated by Poisson statistics from the fraction of positive droplets; must be back-calculated through all dilution steps to report concentration in the original sample (Hindson et al., 2011).
- **Droplet count filter:** A minimum threshold of accepted droplets per well (10,000 recommended) below which the Poisson estimate is unreliable; wells below this threshold must be excluded from analysis (Bio-Rad Laboratories, 2024).
- **DMINQ (Digital Minimum Information for Publication of Quantitative Digital PCR Experiments):** The community standard for reporting ddPCR data (The dMIQE Group & Huggett, 2020); specifies required metadata including droplet count, threshold method, NTC results, and normalization approach.
- **NTC (no-template control):** A reaction well containing all reagents except DNA template; run on every plate to detect reagent or consumable contamination; must show 0 or fewer than 3 positive droplets per well (Bio-Rad Laboratories, 2018).
- **Normalization:** The process of expressing gene copy numbers relative to a common denominator (total DNA concentration in ng/ μ L, or dry soil mass in g) to allow comparison across samples and studies; both normalizations should be reported when possible.

- **Poisson correction:** The statistical calculation applied by QuantaSoft to convert the fraction of positive droplets to an absolute copy number per microliter, correcting for the probability that a single droplet may contain more than one target molecule (Hindson et al., 2011).
- **QLp file:** The proprietary binary data file format produced by the Bio-Rad QX200 system; contains raw droplet amplitude data, QuantaSoft threshold settings, and computed results; must be archived unmodified before any downstream analysis.
- **Rain:** Droplets with intermediate fluorescence amplitude falling between the positive and negative clusters in the amplitude plot; indicates suboptimal amplification, partial inhibition, or primer issues; quantified as the percentage of events in the intermediate cluster and should be documented (Bio-Rad Laboratories, 2018; Lievens et al., 2016).

5. Why ddPCR for This Application

Quantitative PCR (qPCR) infers target abundance from amplification kinetics against a standard curve, so its accuracy depends on amplification efficiency and on curve quality. ddPCR instead partitions each reaction into roughly 20,000 nanoliter droplets, amplifies to endpoint within each droplet, and counts droplets as positive or negative. The fraction of positive droplets is fit to a Poisson distribution to recover the starting concentration in copies per microliter, with no standard curve required (Bio-Rad Laboratories, 2018; Hindson et al., 2011).

For soil nitrogen-cycle work, this matters for several reasons:

- **Absolute, comparable counts.** Copy numbers are returned directly in copies per microliter, which can be normalized to soil mass or DNA and compared across plates, runs, and studies without a shared standard curve.
- **Tolerance of inhibitors.** Soil DNA extracts carry humic acids and other PCR inhibitors. Endpoint partitioning is less sensitive to partial inhibition than the kinetic measurement that qPCR depends on, although inhibition should still be checked (§6) (Hindson et al., 2011).
- **Precision for small differences.** The large number of partitions lets ddPCR resolve modest fold-changes in gene abundance between treatments (Hindson et al., 2011).
- **Low-abundance targets.** Genes such as *nosZ* or *nifH* are often present at low copy numbers relative to bulk DNA; partitioning improves the signal-to-noise ratio for rare targets.

These properties are the reason functional-gene abundance is increasingly reported in absolute units when relating microbial communities to nitrogen cycling processes (Levy-Booth et al., 2014).

6. The Soil Nitrogen Cycle and Functional Gene Markers

Soil nitrogen transformations are carried out by distinct microbial guilds, each defined by the enzymes (and therefore the genes) it carries. Quantifying these marker genes provides a direct, culture-independent estimate of the genetic potential for each process (Kuypers et al., 2018; Levy-Booth et al., 2014).

Gene	Process / guild	What the abundance indicates	Primary reference
<i>amoA</i> (AOB)	Ammonia oxidation, bacterial	Bacterial ammonia-oxidizer potential; first, rate-limiting step of nitrification	Rotthauwe et al. (1997)
<i>amoA</i> (AOA)	Ammonia oxidation, archaeal	Archaeal ammonia-oxidizer potential; often dominant in low-N, acidic soils	Francis et al. (2005)
<i>nirK</i>	Nitrite reduction (denitrification)	Cu-type nitrite-reductase potential	Henry et al. (2006)
<i>nirS</i>	Nitrite reduction (denitrification)	cd ₁ -type nitrite-reductase potential	Braker et al. (2000)
<i>nosZ</i>	Nitrous oxide reduction	N ₂ O-reductase potential; capacity to reduce N ₂ O to N ₂	Henry et al. (2006)
<i>nifH</i>	Nitrogen fixation	Diazotroph potential; biological N ₂ fixation	Levy-Booth et al. (2014)
<i>nrfA</i>	DNRA	Retains N in system as NH ₄ ⁺	Levy-Booth et al. (2014)

7. From Instrument Output to Quality-Controlled Data

7.1. Reviewing and Exporting Data

Instrument operation and the QuantaSoft interface are covered in SOP-L214. For analysis, the entry point is the exported well-level data.

1. Open the completed experiment file (.qlp) in QuantaSoft and confirm the positive/negative threshold for each well. Re-apply thresholds manually in QuantaSoft Analysis Pro if needed, and record any change in the lab notebook.
2. Export with **Options > Export CSV** to produce a well-level results file. Key columns include Well, Sample, Target, Concentration (Poisson-corrected copies/μL (Hindson et al., 2011)), Positives, Negatives, AcceptedDroplets, MeanAmplitudeOfPositives, and MeanAmplitudeOfNegatives.
3. Name the file YYYY-MM-DD_gene_project_plate.csv.
4. Archive both the .qlp and the exported .csv to the lab shared drive under ddPCR_raw_data/ before any analysis begins.

Data Integrity

- Never overwrite raw exported data files. Work only with copies.
- Back up raw .q1p files immediately after each run.
- All analysis scripts must be version-controlled in the lab GitHub repository.

7.2. Quality Control and Acceptance Criteria

Apply these checks before any analysis, following the data-quality expectations set out for digital PCR reporting (Bio-Rad Laboratories, 2018; The dMIQE Group & Huggett, 2020).

1. **Droplet count filter.** Exclude wells with fewer than 10,000 accepted droplets; below this, the Poisson estimate is unreliable (Bio-Rad Laboratories, 2024).
2. **No-template controls (NTC).** NTC wells must show 0 or fewer than 3 positive droplets. More than 3 positives indicates contamination; rerun the affected target or plate.
3. **Extraction blanks.** Blanks carried from DNA extraction ([SOP-L700](#)) must fall below the NTC threshold. Elevated counts point to contamination in the extraction workflow. Note that direct soil extraction is itself a known source of quantitative bias (Frosteg aard et al., 1999), so blanks and recovery checks matter for absolute numbers.
4. **Positive controls.** gBlock serial dilution positive controls must return concentrations within 20% of expected values across the linear range (Huggett et al., 2013).
5. **Rain.** Document the percentage of rain droplets per well. Assays with persistent high rain (> 10–15% of events) should be flagged and the primer or cycling conditions reviewed (Lievens et al., 2016).

8. Calculating Gene Copy Numbers

8.1. From Reaction to Sample

The concentration reported by QuantaSoft is in copies per microliter of the final ddPCR reaction. To convert to copies per gram dry soil:

$$N_{\text{soil}} = C_{\text{rxn}} \times V_{\text{rxn}} \times D \times \frac{V_{\text{elution}}}{V_{\text{rxn input}}} \times \frac{1}{m_{\text{dry soil}}}$$

where:

- C_{rxn} = QuantaSoft-reported concentration (copies/μL)
- V_{rxn} = total ddPCR reaction volume (typically 20 μL)
- D = dilution factor applied to the extract before the reaction
- V_{elution} = total DNA extract volume (μL)
- $V_{\text{rxn input}}$ = volume of extract added to the reaction (μL)
- $m_{\text{dry soil}}$ = mass of dry soil extracted (g)

8.2. R Implementation

```
# =====
# ddPCR copy number normalization (SOP-L702)
# =====

library(tidyverse)

# Import well-level QuantaSoft export
raw <- read_csv("2025-05-14_amoA_wetland_plate1.csv")

# Parameters (adjust per run)
rxn_vol      <- 20      # µL total ddPCR reaction volume
input_vol    <- 5       # µL extract added to reaction
elution_vol  <- 100    # µL total DNA extract
dilution     <- 10     # dilution factor applied to extract before reaction

# Dry soil mass (g) per sample -- join from sample metadata
metadata <- read_csv("sample_metadata.csv") # cols: Sample, dry_soil_g

results <- raw |>
  filter(AcceptedDroplets >= 10000) |>      # droplet count filter
  left_join(metadata, by = "Sample") |>
  mutate(
    copies_per_g = Concentration *          # copies/µL in reaction
      rxn_vol *                             # total reaction volume
      dilution *                           # dilution correction
      (elution_vol / input_vol) *          # extraction recovery
      (1 / dry_soil_g)                     # normalize to dry soil
  )

# Flag QC issues
results <- results |>
  mutate(
    qc_flag = case_when(
      AcceptedDroplets < 10000 ~ "low_droplets",
      Concentration == 0 & !is.na(copies_per_g) ~ "no_signal",
      TRUE ~ "OK"
    )
  )

write_csv(results, "2025-05-14_amoA_wetland_plate1_processed.csv")
```

9. Interpreting Relative Gene Abundances

Individual gene copy numbers are most informative when examined in relation to each other and to the soil's total microbial biomass (often estimated as 16S rRNA gene copies) (Kuypers et al., 2018; Levy-Booth et al., 2014).

Suggested derived metrics:

Metric	Formula	Ecological interpretation
AOB : AOA ratio	$\frac{amoA(AOB)}{amoA(AOA)}$	Relative dominance of bacterial vs. archaeal nitrifiers
Denitrification potential	$nirK + nirS + nosZ$	Combined capacity for N loss as gas
N ₂ O consumer : producer	$\frac{nosZ}{nirK + nirS}$	Capacity to complete denitrification; low values predict N ₂ O accumulation
Diazotroph index	$nifH / 16S$	Fraction of community with N fixation capacity
DNRA : denitrification	$\frac{nrfA}{nirK + nirS}$	N retention vs. N loss balance

10. References

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11. Related SOPs

SOP	Title	Relationship
L700	DNA Extraction	Upstream: DNA template source
L701	ddPCR Primer Design	Primer validation and assay registry
L801	RStudio and GitHub	Version-controlled analysis
L802	Quality Control and QA	General QA/QC framework

12. Training Record

Before performing ddPCR data analysis independently, personnel must:

1. Read this SOP and [SOP-L701](#) in full
2. Review the DMINQ reporting standard (The dMIQE Group & Huggett, 2020)

3. Demonstrate correct QuantaSoft threshold setting on at least two plates under supervision
4. Run the copy number normalization R script on a test dataset and reproduce the trainer's results
5. Be signed off by the PI or lab manager

13. Appendix A: DMINQ Checklist

When reporting ddPCR data from this workflow, include the following metadata per The dMIQE Group & Huggett (2020):

Item	Required by DMINQ	Notes
Instrument model and serial	Yes	Bio-Rad QX200
Software version	Yes	QuantaSoft version
Droplet count per well	Yes	Must be 10,000
Threshold setting method	Yes	Manual or automated; document both
NTC result	Yes	Copies/ μ L; must be 0 or < 3 positive droplets
Positive control	Yes	gBlock; expected and measured concentration
Rain percentage	Yes	Document even if low
Dilution factor	Yes	
Normalization denominator	Yes	Dry soil mass (g) and/or DNA (ng)
Inhibition check result	Yes	1:5 vs. 1:10 dilution agreement
Primer/probe sequences	Yes	Or cite SOP-L701 registry

Item	Required by DMINQ	Notes
Cycling conditions	Yes	Annealing temperature, cycles
Reference for target gene	Yes	Cite from SOP-L701 registry



Environmental Analysis Program

Pomona College
Biogeochemistry Laboratory

SOP-L702

ddPCR Data Export and Analysis – Nitrogen Cycle Gene Abundance

Standard Operating Procedure

SOP Number	SOP-L702
Revision	0.1
Author(s)	Marc Los Huertos
Date	2026-06-26
Organization	EA Program, Pomona College

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10

1. Purpose

This procedure describes the workflow for exporting raw data from the Bio-Rad QX200 ddPCR system, performing quality control checks, calculating gene copy numbers normalized to soil mass or DNA concentration, and conducting statistical analyses of nitrogen cycle gene abundance data. The SOP links the instrument output from [SOP-L701](#) to reproducible analysis scripts written in R (R Core Team, 2024).

2. Scope

- **Applies to:** Processing and analysis of ddPCR data (QuantaSoft or QuantaSoft Analysis Pro output) for nitrogen cycle functional genes (*amoA*, *nirK*, *nirS*, *nosZ*, *nifH*, *nrfA*) from environmental samples
- **Does not apply to:** Amplicon sequencing data, metagenomics, or qPCR data processed with different software
- **Location:** Instrument computer (QuantaSoft) and RStudio Server (see [SOP-L801 RStudio and GitHub](#))

3. Responsibilities

Role	Responsibility
Lab PI	Approve analysis pipeline and interpretation
Trained Personnel	Export data, run QC, perform analysis
New Students	Complete training before independent analysis

4. Definitions

- **Amplitude threshold:** The fluorescence intensity boundary set in DropletQuant (or equivalent software) that separates positive droplets (containing amplified target) from negative droplets; correct threshold placement is the single most critical step in ddPCR data analysis.
- **Copies per microliter (copies/ μ L):** The absolute concentration of target DNA in the final ddPCR reaction volume as calculated by Poisson statistics from the fraction of positive droplets; must be back-calculated through all dilution steps to report concentration in the original sample.
- **DropletQuant / QuantaSoft:** Bio-Rad software used to read the QX200 droplet reader output, set amplitude thresholds, and calculate target concentrations; raw .qfp files must be archived unmodified.
- **Duplex assay:** A ddPCR assay in which two targets (e.g., a gene of interest and a reference gene) are quantified simultaneously using two spectrally distinct fluorescent probes (FAM and HEX/VIC) in the same reaction; requires careful threshold setting for each channel.

- **Fractional abundance:** The proportion of positive droplets relative to total accepted droplets: $\lambda = -\ln(1 - p)$ where p is the positive fraction; used by Poisson correction to calculate copies/ μL .
- **No-template control (NTC):** A reaction containing all ddPCR components except sample DNA; must yield zero or near-zero positive droplets; persistent positive droplets in NTCs indicate reagent contamination.
- **Poisson correction:** The statistical adjustment applied by DropletQuant to account for the probability that some droplets contain more than one target molecule; assumes random partitioning and is essential for accurate absolute quantification.
- **Rain:** Droplets with intermediate fluorescence amplitude between the positive and negative clusters; caused by partial amplification, degraded DNA, or probe competition; must be assigned to positive or negative by threshold adjustment and noted in data records.
- **Rejected droplets:** Droplets excluded from analysis by the instrument because they fall outside acceptable size or fluorescence quality criteria; runs with fewer than 10,000 accepted droplets per well should be flagged and potentially repeated.

5. Safety and Hazards

No physical or chemical hazards are associated with this procedure. Data integrity and reproducibility are the primary concerns.

Data Integrity

- Never overwrite raw exported data files. Work only with copies.
- All analysis scripts must be version-controlled in the lab GitHub repository.
- Back up raw .qlp files from QuantaSoft to the lab shared drive immediately after each run.

6. Equipment and Materials

6.1. Equipment

Item	Location	Notes
QX200 instrument computer	Instrument room	Runs QuantaSoft or QuantaSoft Analysis Pro
RStudio Server	Remote (see SOP-L801)	For R-based analysis
USB drive or network share	Lab	For transferring .qlp and .csv files

6.2. Software

Software	Version	Purpose
QuantaSoft or QuantaSoft Analysis Pro	1.7+ or 1.0+	Threshold setting, data export
R	4.3+	Statistical analysis
RStudio	Current	IDE for R scripts
tidyverse R package	Current	Data wrangling and visualization
ggplot2 R package	Current	Plotting
<i>TBD – identify additional R packages for ddPCR analysis (e.g., ddpccr, twoddpccr)</i>		

7. Procedure

7.1. Data Export from QuantaSoft

1. Open the completed experiment file (.q1p) in QuantaSoft
2. Review the droplet plots for each well. Confirm that the positive/negative threshold was set appropriately during the run (see SOP-L701, Section 6.6)
3. If threshold adjustment is needed, use QuantaSoft Analysis Pro to re-apply thresholds manually. Document any threshold changes in the lab notebook.
4. Export data using **Options > Export CSV** to generate a comma-separated file containing well-level results
5. The exported CSV includes the following key columns:
 - **Well:** plate position (e.g., A01)
 - **Sample:** sample name as entered during plate setup
 - **Target:** gene target name
 - **Concentration:** copies per uL of the final ddPCR reaction (Poisson-corrected) (Hindson et al., 2011)
 - **Positives:** number of positive droplets
 - **Negatives:** number of negative droplets
 - **AcceptedDroplets:** total accepted droplets
 - **MeanAmplitudeOfPositives:** mean fluorescence of positive cluster
 - **MeanAmplitudeOfNegatives:** mean fluorescence of negative cluster
6. Save the CSV with the naming convention: YYYY-MM-DD_gene_project_plate.csv (e.g., 2025-05-14_amoA_wetland_plate1.csv)
7. Copy the .q1p file and the exported .csv to the lab shared drive under ddPCR_raw_data/

7.2. Quality Control Checks

Perform the following checks on the exported data before proceeding to analysis. These can be scripted in R for reproducibility.

1. **Droplet count filter:** Exclude any well with fewer than 10,000 accepted droplets. Wells below this threshold have unreliable Poisson statistics (Bio-Rad Laboratories, 2024).
2. **No-template controls (NTC):** NTC wells must show 0 or fewer than 3 positive droplets. If NTC wells show more than 3 positives, the entire plate or the affected target should be considered contaminated and rerun.
3. **Extraction blanks:** Extraction blanks (from SOP-L80) must show fewer positive droplets than the NTC acceptance threshold. Elevated counts in extraction blanks indicate contamination in the DNA extraction workflow.
4. **Positive controls:** Positive control gBlock wells should fall within 20% of the expected concentration based on the dilution prepared.
5. **Technical replicate agreement:** If technical replicates were run, calculate the coefficient of variation (CV) across replicates. A CV greater than 25% suggests pipetting error or sample heterogeneity.

 Tip

Create a QC report template in R Markdown or Quarto that automatically flags wells failing any of the criteria above. This makes batch QC efficient and ensures nothing is missed.

7.3. Calculating Gene Copy Numbers

The QuantaSoft **Concentration** output is in copies per uL of the ddPCR reaction mixture. To express results per gram of dry soil (or per unit of extracted DNA), apply the following conversions:

$$\text{Copies per g dry soil} = \frac{C_{\text{ddPCR}} \times V_{\text{reaction}} \times DF \times V_{\text{elution}}}{V_{\text{template}} \times m_{\text{soil}} \times (1 - \theta_g)}$$

where:

- C_{ddPCR} = concentration from QuantaSoft (copies/uL)
- V_{reaction} = total ddPCR reaction volume (typically 20 uL)
- DF = dilution factor of the DNA template (e.g., 10 if diluted 1:10)
- V_{elution} = total DNA elution volume from extraction (uL; see SOP-L80)
- V_{template} = volume of DNA template added to the ddPCR reaction (uL)
- m_{soil} = mass of soil extracted (g)
- θ_g = gravimetric soil moisture (as a decimal; see [SOP-L400](#))

To express results per ng of extracted DNA instead:

$$\text{Copies per ng DNA} = \frac{C_{\text{ddPCR}} \times V_{\text{reaction}}}{V_{\text{template}} \times C_{\text{DNA}}}$$

where C_{DNA} is the Qubit DNA concentration (ng/uL) from SOP-L80.

```
# Example calculation: copies per g dry soil
c_ddpcr <- 52.3          # copies/uL (from QuantaSoft)
v_reaction <- 20         # uL
dilution_factor <- 10   # 1:10 dilution of template
v_elution <- 100        # uL (from SOP-L80)
v_template <- 2          # uL added to ddPCR reaction
soil_mass_g <- 0.25       # g soil extracted
moisture_decimal <- 0.35 # gravimetric moisture as decimal

copies_per_g <- (c_ddpcr * v_reaction * dilution_factor * v_elution) /
  (v_template * soil_mass_g * (1 - moisture_decimal))

cat(sprintf("Gene copies per g dry soil: %.0f\n", copies_per_g))
```

Gene copies per g dry soil: 3218462

```
cat(sprintf("Gene copies per g dry soil (log10): %.2f\n", log10(copies_per_g)))
```

Gene copies per g dry soil (log10): 6.51

7.4. Data Organization and Merging

1. Merge the QC-filtered ddPCR results with the sample metadata (site, treatment, depth, date, soil moisture, DNA concentration) using a shared sample ID column
2. Organize the merged data in a tidy (long) format with one row per sample-by-gene combination (Wickham, 2014)
3. Save the merged analysis-ready dataset as a .csv in the project GitHub repository under data/processed/
4. TBD – define the standard column naming convention for the lab's ddPCR datasets (e.g., *sample_id*, *gene*, *copies_per_g_dry*, *copies_per_ng_dna*, *site*, *treatment*, *depth_cm*, *date*)

7.5. Statistical Analysis

Note

The choice of statistical approach depends on the experimental design (e.g., paired vs. independent sites, factorial treatments, time series). The guidance below covers common analyses for nitrogen cycle gene abundance. Consult the PI before selecting a final analytical approach.

7.5.1. Data Exploration and Visualization

1. Plot gene copy numbers ($\log_{10}$ -transformed) by treatment, site, or depth using boxplots or dot plots

2. Assess normality of log-transformed data using Shapiro-Wilk tests and Q-Q plots
3. Check for outliers and investigate whether they represent true biological variation or analytical artifacts

7.5.2. Comparing Gene Abundance Across Treatments

- For two-group comparisons (e.g., amended vs. control): use Welch's t-test on $\log_{10}$ -transformed copies per g dry soil, or the non-parametric Wilcoxon rank-sum test if normality assumptions are not met (Frosteg aard et al., 1999)
- For multi-group comparisons: use one-way ANOVA on $\log_{10}$ -transformed data with Tukey HSD post hoc tests, or Kruskal-Wallis with Dunn's test for non-parametric data
- For factorial designs (e.g., treatment x depth): use two-way ANOVA or linear mixed-effects models with site as a random effect (Pinheiro & Bates, 2000)

7.5.3. Gene Ratios

Ratios of functional gene abundances can provide insight into the balance of nitrogen cycling processes (Hallin et al., 2009):

- *nirK/nirS*: relative contribution of the two nitrite reductase pathways to denitrification
- *nosZ/(nirK + nirS)*: completeness of denitrification (higher values suggest greater N_2O reduction capacity)
- *amoA* AOA/*amoA* AOB: relative dominance of archaeal vs. bacterial ammonia oxidation
- *nrfA/(nirK + nirS)*: relative importance of DNRA vs. denitrification

TBD – determine which gene ratios are most relevant for the lab's current research questions and establish standard visualization formats.

7.5.4. Correlations with Environmental Variables

- Correlate gene abundances with soil physicochemical data (pH, moisture, total C, total N, nitrate, ammonium) using Pearson or Spearman correlations
- *TBD – evaluate whether multivariate approaches (e.g., PCA, redundancy analysis) are appropriate given the number of samples and variables*
- *TBD – determine whether gene abundance data should be related to process rate measurements (e.g., N_2O flux, nitrification potential) and define the analytical framework for doing so*

7.5.5. Reporting

- Report gene abundance as copies per g dry soil (mean +/- standard error) and/or copies per ng DNA
- Present data on a $\log_{10}$ scale in figures; report back-transformed means and confidence intervals in tables where appropriate (Bolker et al., 2009)
- Include all QC metrics (droplet counts, NTC results, positive control recovery) as supplementary tables
- *TBD – create a standardized R Markdown/Quarto reporting template for ddPCR results that the lab can reuse across projects*

8. Quality Control and Acceptance Criteria

- Raw .q1p files must be archived on the lab shared drive before any analysis proceeds
- All R scripts must be committed to the lab GitHub repository with meaningful commit messages
- QC filtering must be applied before any statistical analysis; unfiltered data should never be reported
- Positive control recovery within 20% of expected value
- TBD – define minimum sample sizes required for each statistical test based on power analysis
- TBD – establish whether and how to handle values below the limit of detection (e.g., substitution with LOD/2, censored regression, or Tobit models) (Helsel, 2011)

9. Waste Disposal

No physical waste is generated by this procedure. Data management practices are described in Section 6.4.

10. References

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11. Training Record

Before performing this procedure independently, personnel must:

1. Read this SOP, [SOP-L701 Primer Design](#), and [SOP-L700 DNA Extraction](#)
2. Complete [SOP-L801 RStudio and GitHub](#) training
3. Demonstrate competence exporting data from QuantaSoft
4. Successfully run the QC and analysis pipeline on a practice dataset under supervision
5. Be signed off by the PI or lab manager

12. Appendix A: Example QC Filtering Script

```
library(tidyverse)

# Read exported QuantaSoft CSV
raw <- read_csv("data/raw/2025-05-14_amoA_wetland_plate1.csv")

# QC filtering
qc_filtered <- raw %>%
  filter(
    AcceptedDroplets >= 10000      # Minimum droplet threshold
  ) %>%
  mutate(
    qc_flag = case_when(
      Sample == "NTC" & Positives > 3 ~ "NTC_contamination",
      Sample == "Extraction_Blank" & Positives > 3 ~ "Extraction_contamination",
      TRUE ~ "Pass"
    )
  )

# Flag and report QC failures
qc_failures <- qc_filtered %>%
  filter(qc_flag != "Pass")

if (nrow(qc_failures) > 0) {
  warning("QC failures detected:")
  print(qc_failures %>% select(Well, Sample, Target, Positives, qc_flag))
}

# Retain only passing sample wells
analysis_ready <- qc_filtered %>%
```

```
filter(qc_flag == "Pass",
       !Sample %in% c("NTC", "Extraction_Blank", "Positive_Control"))
```

13. Appendix B: Visualization Template

```
library(tidyverse)

# Assumes analysis_ready has columns: sample_id, gene, copies_per_g_dry, treatment
analysis_ready %>%
  ggplot(aes(x = treatment, y = log10(copies_per_g_dry), fill = treatment)) +
  geom_boxplot(alpha = 0.7, outlier.shape = NA) +
  geom_jitter(width = 0.2, alpha = 0.5, size = 1.5) +
  facet_wrap(~ gene, scales = "free_y") +
  labs(
    x = "Treatment",
    y = expression(log[10]~"(gene copies g"^-1}~"dry soil)"),
    title = "Nitrogen Cycle Gene Abundance by Treatment"
  ) +
  theme_minimal() +
  theme(legend.position = "none")
```

14. Appendix C: Sending Samples to External Laboratories

TBD – if ddPCR analysis is outsourced to an external service provider (e.g., university core facility, commercial lab), document the following:

- Preferred service provider(s) and contact information
- Sample submission requirements (volume, concentration, format)
- Data format and file types returned by the provider
- Turnaround time expectations
- Cost per sample and funding source/account number
- How to reconcile externally generated data with in-house QC standards



Environmental Analysis Program

Pomona College
Biogeochemistry Laboratory

SOP-L703

PCR Amplification and Gel Electrophoresis

Standard Operating Procedure

SOP Number	SOP-L703
Revision	0.1
Author(s)	Marc Los Huertos
Date	2026-06-26
Organization	EA Program, Pomona College

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1. Purpose

This SOP describes standard polymerase chain reaction (PCR) amplification of target DNA fragments and visualization of PCR products by agarose gel electrophoresis. PCR is used in the EA Program for confirming DNA extraction success, screening for target genes before ddPCR or sequencing, and verifying amplicon size and specificity prior to library preparation (Lorenz, 2012; Mullis, 1990). Gel electrophoresis separates DNA fragments by size, providing a visual confirmation that amplification produced the expected product.

2. Scope

- **Applies to:** Standard endpoint PCR amplification of environmental DNA extracts (soil, water, tissue) and visualization of products on agarose gels; applicable to gene-specific screening, amplicon verification, and colony PCR
- **Does not apply to:** Quantitative PCR (qPCR) or digital PCR (ddPCR; see [SOP-L701](#) and [SOP-L702](#)); RT-PCR for RNA targets; polyacrylamide gel electrophoresis (PAGE)
- **Location:** Molecular biology bench, SGM 133

3. Responsibilities

Role	Responsibility
Lab PI	Approve primer selections and cycling protocols
Trained Personnel	Set up PCR reactions and run gels per this SOP
New Students	Complete training before independent use

4. Definitions

- **Agarose gel:** A porous polymer matrix cast from dissolved agarose in TAE or TBE buffer; DNA fragments migrate through the gel under an electric field and are separated by size; typical concentrations are 1–2% (w/v).
- **Amplicon:** The specific DNA fragment produced by PCR between a defined pair of primers; its length (in base pairs) is determined by the primer binding positions and is used to verify PCR specificity on a gel.
- **Annealing temperature (T_a):** The temperature during the PCR extension cycle at which primers bind to the template; typically 3–5 °C below the calculated primer melting temperature (T_m); incorrect T_a causes non-specific amplification or failed reactions.
- **DNA ladder (size standard):** A mixture of DNA fragments of known sizes run alongside samples on a gel; used to estimate the size of amplified products.
- **Ethidium bromide (EtBr) / SYBR Safe:** Fluorescent intercalating dyes added to agarose gels or post-staining baths to visualize DNA under UV light; EtBr is a potent mutagen requiring dedicated waste disposal; SYBR Safe is a lower-hazard alternative.

- **Master mix:** A pre-mixed reagent solution containing DNA polymerase, dNTPs, buffer, and MgCl_2 in optimized concentrations; reduces pipetting error and variability between replicate reactions.
- **Melting temperature (T_m):** The temperature at which 50% of a primer-template duplex is dissociated; calculated from primer sequence and salt concentration; the basis for selecting annealing temperature.
- **Non-specific amplification:** PCR products of unexpected size caused by primer mis-binding; detected as extra bands on a gel; remedied by increasing annealing temperature or redesigning primers.
- **Primer:** A short (18–25 bp) single-stranded oligonucleotide that binds to a complementary sequence on the template DNA and defines the start point for DNA polymerase extension.
- **TAE / TBE buffer:** Running buffers for agarose gel electrophoresis; TAE (Tris-acetate-EDTA) gives sharper bands for larger fragments; TBE (Tris-borate-EDTA) provides better resolution for smaller fragments and is more resistant to overheating.

5. Safety and Hazards

Hazards

- **Chemical:** Ethidium bromide (EtBr) and SYBR Safe are nucleic acid stains. EtBr is a suspected mutagen; handle with gloves at all times and dispose of gels and buffers as hazardous waste. SYBR Safe is a safer alternative but should still be handled with gloves.
- **Electrical:** Gel electrophoresis uses high voltage (80–120 V). Do not touch the buffer or gel while the power supply is on. Always disconnect the leads before opening the gel box.
- **UV radiation:** Transilluminators emit UV light that can cause skin burns and eye damage. Use a UV shield, wear UV-blocking safety glasses, and minimize exposure time. Blue-light transilluminators are preferred when used with SYBR Safe.
- **Biological:** Environmental DNA extracts may originate from samples containing pathogens. Handle with standard precautions.

5.1. Required PPE

- ☐ Safety glasses (UV-blocking if using a UV transilluminator)
- ☐ Lab coat
- ☐ Nitrile gloves (change frequently; required at all times when handling gels, stains, or PCR products)

5.2. Emergency Procedures

For EtBr contact with skin, wash immediately with soap and water. For UV exposure, flush eyes with water and seek medical attention. Refer to [SOP-L003 Hazardous Materials Handling](#).

6. Equipment and Materials

6.1. Equipment

Item	Location	Notes
Thermal cycler (Bio-Rad C1000 Touch or equivalent)	Mol bio bench	Verify block type matches tube format
Gel electrophoresis system (tank, comb, casting tray)	Mol bio bench	Mini-gel (7 x 10 cm) or midi-gel
Power supply	Mol bio bench	Capable of 10–300 V
Gel imaging system (transilluminator + camera)	Mol bio bench	UV or blue-light; with gel documentation software
Micropipettes (2, 10, 100, 1000 uL)	Mol bio bench	Calibrated annually
Microwave	Lab	For melting agarose
Microcentrifuge	Mol bio bench	For brief spin-downs

6.2. Reagents and Consumables

Item	Notes
Taq DNA polymerase or high-fidelity polymerase	e.g., GoTaq (Promega), Q5 (NEB), or OneTaq (NEB)
5x or 10x PCR buffer (with MgCl ₂)	Supplied with polymerase
dNTP mix (10 mM each)	Store at –20 C
Forward and reverse primers (10 uM working stocks)	Store at –20 C
Nuclease-free water	For master mix and negative controls
PCR tubes or plates (0.2 mL, thin-wall)	Matched to thermal cycler block
Agarose (molecular biology grade)	For gel preparation
1x TAE or TBE buffer	Running buffer; prepare from 50x stock
DNA stain (SYBR Safe or ethidium bromide)	SYBR Safe preferred
DNA ladder (100 bp and/or 1 kb)	e.g., NEB 100 bp or 1 kb Plus
6x gel loading dye	Often included with ladder or polymerase
Filter pipette tips (10, 100, 1000 uL)	Barrier tips mandatory to prevent cross-contamination

7. Procedure

7.1. PCR Workspace Preparation

! Contamination Prevention

PCR is extremely sensitive to contamination. Follow these rules:

- Set up reactions in a designated pre-PCR area, physically separated from post-PCR work (gel loading, product analysis).
- Never bring PCR products, gels, or gel imaging equipment into the pre-PCR area.
- Use dedicated pipettes for pre-PCR work. Use filter tips exclusively.
- Wipe bench surfaces with 10% bleach followed by 70% ethanol before setting up reactions.
- Change gloves frequently, especially after touching any surface outside the pre-PCR area.

7.2. Master Mix Preparation

Prepare all components on ice. Calculate volumes for the number of reactions plus 10% overage to account for pipetting losses.

Standard 25 uL reaction (GoTaq example):

Component	Volume per reaction	Final concentration
5x GoTaq Reaction Buffer	5.0 uL	1x
dNTP mix (10 mM each)	0.5 uL	200 uM each
Forward primer (10 uM)	1.25 uL	500 nM
Reverse primer (10 uM)	1.25 uL	500 nM
GoTaq DNA Polymerase (5 U/uL)	0.125 uL	0.625 U
Nuclease-free water	14.875 uL	–
Subtotal (master mix)	23.0 uL	
Template DNA	2.0 uL	Variable (1–10 ng)
Total	25.0 uL	

1. Thaw buffer, dNTPs, and primers on ice. Vortex briefly and spin down.
2. Prepare the master mix in a sterile 1.5 mL tube, adding polymerase last. Mix by pipetting; do not vortex.
3. Aliquot 23 uL of master mix into each labeled PCR tube.
4. Add 2 uL of template DNA to each tube. Include controls:
 - **Positive control:** DNA known to amplify with these primers
 - **Negative control (NTC):** 2 uL of nuclease-free water
 - **Extraction blank:** DNA extract from [SOP-L700](#) extraction blank
5. Cap tubes, vortex briefly, and spin down.

7.3. Thermal Cycling

Program the thermal cycler with the following general protocol. Adjust the annealing temperature for your specific primer set.

Step	Temperature	Time	Cycles
Initial denaturation	95 C	2 min	1
Denaturation	95 C	30 s	30–35
Annealing	T_a C	30 s	30–35
Extension	72 C	1 min/kb	30–35
Final extension	72 C	5 min	1
Hold	4 C	Hold	–

where T_a is the annealing temperature, typically 3–5 C below the lower primer T_m . For primers with T_m of 55–60 C, start at 55 C. Optimize by running a gradient if needed (Lorenz, 2012).

Record the cycling parameters, primer names, and annealing temperature in the lab notebook.

7.4. Agarose Gel Preparation

1. Weigh agarose for a 1–2% gel (w/v), depending on the expected amplicon size:

Agarose %	Effective separation range
0.8%	500–10,000 bp
1.0%	250–7,000 bp
1.5%	100–3,000 bp
2.0%	50–1,500 bp

2. Add agarose to 1x TAE (or TBE) buffer in an Erlenmeyer flask.
3. Microwave in 30-second intervals, swirling between intervals, until the agarose is fully dissolved and the solution is clear.
4. Cool to approximately 55 C (comfortable to hold).
5. If using SYBR Safe, add 5 uL per 50 mL of gel. Swirl gently.
6. Pour into the casting tray with the comb in place. Allow to solidify (approximately 20–30 minutes at room temperature).

7.5. Loading and Running the Gel

1. Remove the comb carefully. Place the gel in the electrophoresis tank and fill with 1x TAE (or TBE) buffer until the gel is submerged by 2–3 mm.
2. Load 5 uL of DNA ladder into the first well.
3. Add loading dye to each PCR product (if not already included in the PCR buffer). Load 5–10 uL per well.
4. Record the loading order in the lab notebook.

5. Connect the leads: DNA migrates toward the positive (red) electrode. Confirm the orientation.
6. Run at 80–120 V for 30–45 minutes, or until the leading dye front has migrated approximately two-thirds of the gel length.
7. Turn off the power supply and disconnect the leads before removing the gel.

7.6. Gel Imaging

1. Place the gel on the transilluminator (blue light for SYBR Safe; UV for EtBr).
2. Capture an image using the gel documentation system.
3. Save the image with the naming convention: YYYY-MM-DD_primers_project_gel.tif
4. Evaluate the image:
 - Positive control: visible band at the expected size
 - NTC and extraction blank: no visible band
 - Samples: band at the expected size indicates successful amplification
5. Upload the image to the project GitHub repository under **results/gels/**.

8. Troubleshooting

Symptom	Probable Cause	Corrective Action
No bands in any lane	Failed reaction; wrong cycling parameters	Check primer and dNTP stocks; verify cycling program
Bands in NTC	Contamination	Discard reagents; clean workspace with bleach; use fresh aliquots
Multiple bands	Non-specific amplification	Increase annealing temperature; reduce primer concentration; redesign primers
Smear instead of band	DNA degradation or overloaded gel	Reduce template amount; check DNA quality on NanoDrop
Bands too faint	Insufficient template or cycles	Increase template to 5–10 ng; increase to 35 cycles
Bands in samples but not positive control	Positive control degraded	Prepare fresh positive control DNA

9. Quality Control

- Every PCR run must include: positive control, NTC, and extraction blank
- No bands should appear in the NTC or extraction blank. Any amplification in controls invalidates the run (Salter et al., 2014).
- Band size must match the expected amplicon size (compare to the DNA ladder)
- For quantitative downstream applications (library prep, sequencing), confirm band intensity is roughly uniform across samples

10. Waste Disposal

Waste Type	Container	Disposal Method
EtBr-stained gels and buffer	Designated EtBr waste container	EH&S hazardous waste pickup
SYBR Safe-stained gels	Standard trash (check local EH&S policy)	Non-hazardous per manufacturer
Used PCR tubes and tips	Standard trash	Non-hazardous after thermal cycling
Unused EtBr stock solution	Chemical waste	EH&S hazardous waste pickup

11. References

- Lorenz, T. C. (2012). Polymerase chain reaction: Basic protocol plus troubleshooting and optimization strategies. *Journal of Visualized Experiments*, (63), e3998. <https://doi.org/10.3791/3998>
- Mullis, K. B. (1990). The unusual origin of the polymerase chain reaction. *Scientific American*, 262(4), 56–65. <https://doi.org/10.1038/scientificamerican0490-56>
- Salter, S. J., Cox, M. J., Sherrill-Mix, S., Turner, R. M., Sherrill-Mix, S., Sherrill-Mix, S., & Walker, A. W. (2014). Reagent and laboratory contamination can critically impact sequence-based microbiome analyses. *BMC Biology*, 12, 87. <https://doi.org/10.1186/s12915-014-0087-z>

12. Related SOPs

SOP	Title	Relationship
L003	Hazardous Materials	EtBr and UV safety
L700	DNA Extraction	Upstream: provides template DNA
L701	ddPCR Primers	Primer design and validation
L704	Amplicon Library Prep	Downstream: library preparation
L106	Freezers	Reagent and DNA storage

13. Training Record

Before performing PCR and gel electrophoresis independently, personnel must:

1. Read this SOP and [SOP-L700](#) in full
2. Demonstrate master mix preparation with correct volumes and controls
3. Run a supervised PCR and gel, producing a clean positive control band with no NTC contamination
4. Demonstrate safe handling of DNA stains and proper gel disposal
5. Be signed off by the PI or lab manager



Environmental Analysis Program

Pomona College
Biogeochemistry Laboratory

SOP-L704

Amplicon Library Preparation for Sequencing

Standard Operating Procedure

SOP Number	SOP-L704
Revision	0.1
Author(s)	Marc Los Huertos
Date	2026-06-26
Organization	EA Program, Pomona College

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1. Purpose

This SOP describes the preparation of amplicon sequencing libraries for Illumina platforms, following a two-step PCR approach. The first PCR amplifies the target marker gene (e.g., 16S rRNA for bacteria, ITS for fungi) with gene-specific primers tailed with Illumina adapter overhangs. The second PCR attaches unique dual-index barcodes and the full Illumina adapter sequences, enabling multiplexed sequencing of many samples per run. This protocol is based on the Illumina 16S Metagenomic Sequencing Library Preparation guide and the Earth Microbiome Project (EMP) standards (Caporaso et al., 2012; Illumina, Inc., 2013; Thompson et al., 2017).

2. Scope

- **Applies to:** Preparation of amplicon libraries targeting 16S rRNA (V4 or V3–V4 region), ITS (ITS1 or ITS2 region), 18S rRNA, or other marker genes from environmental DNA extracts for paired-end sequencing on Illumina MiSeq or NextSeq platforms
- **Does not apply to:** Shotgun metagenomic library preparation; long-read amplicon sequencing (PacBio, Nanopore); ddPCR (see [SOP-L701](#)); eDNA metabarcoding using non-Illumina workflows
- **Location:** Molecular biology bench, SGM 133

3. Responsibilities

Role	Responsibility
Lab PI	Approve target region, primer choice, and sequencing facility
Trained Personnel	Prepare libraries per this SOP; submit to sequencing facility
New Students	Complete training before independent use

4. Definitions

- **Adapter:** A short double-stranded DNA sequence ligated to both ends of a library fragment; contains the sequencing primer binding site, an index sequence, and the flow cell binding sequence required for Illumina cluster generation.
- **Amplicon library:** A sequencing library prepared by PCR amplification of a target region (e.g., 16S rRNA gene V4 region or ITS2) using primers that incorporate Illumina adapter sequences; represents the diversity of that locus in a community sample.
- **Index (barcode):** A unique 6–12 bp sequence incorporated into the adapter of each sample's library; allows multiple samples to be pooled and sequenced together (multiplexed) and computationally separated (demultiplexed) after sequencing.
- **Library normalization:** Adjustment of each sample's library to the same molar concentration before pooling; ensures approximately equal sequencing depth across samples; performed by quantification (Qubit or qPCR) followed by dilution.

- **Library pooling:** Combining equimolar amounts of individually indexed libraries from multiple samples into a single tube for multiplexed sequencing; the total library pool is loaded onto the sequencer.
- **Paired-end sequencing:** A sequencing strategy in which both ends of a DNA insert are sequenced, producing two reads (R1 and R2) per cluster; paired reads overlap for amplicons shorter than twice the read length, enabling error correction by merging.
- **PCR inhibitor:** A substance carried over from DNA extraction that suppresses amplification efficiency (e.g., humic acids, tannins, residual ethanol); detected by inhibition testing and remedied by further purification or dilution of the template.
- **Size selection:** A step in library preparation that removes off-target fragments (primer dimers, non-specific products) using bead-based cleanup (e.g., AMPure XP beads) or gel excision; improves sequencing quality and reduces wasted reads.

5. Safety and Hazards

Hazards

- **Chemical:** AMPure XP beads (or equivalent SPRI beads) contain polyethylene glycol (PEG) and carboxylated magnetic particles; low toxicity but handle with gloves. Molecular-grade ethanol is flammable.
- **Biological:** Environmental DNA extracts may contain pathogen-derived DNA. Handle with standard precautions.

5.1. Required PPE

- ☐ Safety glasses
- ☐ Lab coat
- ☐ Nitrile gloves (changed frequently)

5.2. Emergency Procedures

Refer to [SOP-L003 Hazardous Materials Handling](#).

6. Equipment and Materials

6.1. Equipment

Item	Location	Notes
Thermal cycler	Mol bio bench	For both PCR1 and PCR2
Magnetic stand (for 96-well plates or strip tubes)	Mol bio bench	For SPRI bead cleanup
Qubit 4 fluorometer	Instrument bench	For library quantification

Item	Location	Notes
NanoDrop One	Instrument bench	Optional purity check
Gel electrophoresis system	Mol bio bench	For amplicon verification; see SOP-L703
Multichannel pipette (optional)	Mol bio bench	For high-throughput plate work
Plate vortex mixer	Mol bio bench	For mixing bead suspensions
Microcentrifuge and plate spinner	Mol bio bench	For brief spin-downs

6.2. Reagents and Consumables

Item	Notes
High-fidelity DNA polymerase	e.g., KAPA HiFi HotStart (Roche), Q5 (NEB), or Phusion (Thermo Fisher); high-fidelity polymerase is required to minimize PCR-introduced errors (Schirmer et al., 2015)
Gene-specific primers with Illumina overhangs (PCR1)	See Section 6.1
Nextera XT Index Kit v2 (or equivalent dual-index primers)	For PCR2 barcoding
AMPure XP beads (Beckman Coulter) or equivalent SPRI beads	For size selection and cleanup
Molecular-grade ethanol (80%, freshly prepared)	For bead washes
Nuclease-free water	For elution and dilutions
Qubit dsDNA HS Assay Kit	For library quantification
Agarose and gel supplies	For amplicon verification
PCR plates or strip tubes (0.2 mL)	Low-bind preferred
Plate sealing film or strip caps	
Filter pipette tips	Barrier tips mandatory

7. Procedure

7.1. Primer Design (PCR1: Gene-Specific Amplification)

Primers for the first PCR consist of the gene-specific sequence plus the Illumina adapter overhang. The overhang sequences are:

- Forward overhang: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-[gene-specific forward]-3'
- Reverse overhang: 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-[gene-specific reverse]-3'

Commonly used gene-specific primers:

Target	Primers	Region	Amplicon	Reference
16S bacteria/archaea	515F / 806R	V4	~290 bp	Caporaso et al. (2012); Parada et al. (2016)
16S bacteria	341F / 785R	V3–V4	~460 bp	Klindworth et al. (2013)
ITS fungi	ITS1f / ITS2	ITS1	~250–400 bp	Smith & Peay (2014); White et al. (1990)
ITS fungi	ITS7 / ITS4	ITS2	~250–400 bp	Ihrmark et al. (2012)
18S eukaryotes	Euk1391f / EukBr	V9	~130 bp	Amaral-Zettler et al. (2009)

i Note

The EMP standard primers for 16S V4 are 515F (Parada) / 806R (Apprill), which include degeneracies for improved archaeal coverage (Apprill et al., 2015; Parada et al., 2016). Use these unless the project specifically requires a different region.

7.2. PCR1: Target Amplification

1. Prepare the master mix on ice. Use a high-fidelity polymerase.

Component	Volume per reaction	Final concentration
2x KAPA HiFi HotStart ReadyMix	12.5 uL	1x
Forward primer with overhang (10 uM)	1.0 uL	400 nM
Reverse primer with overhang (10 uM)	1.0 uL	400 nM
Nuclease-free water	8.5 uL	–
Template DNA (1–10 ng)	2.0 uL	Variable
Total	25.0 uL	

2. Include controls: positive control, NTC, and extraction blank.
3. Cycling parameters (adjust annealing temperature per primer set):

Step	Temperature	Time	Cycles
Initial denaturation	95 C	3 min	1
Denaturation	95 C	30 s	25–28
Annealing	55 C	30 s	25–28
Extension	72 C	30 s	25–28
Final extension	72 C	5 min	1
Hold	4 C	Hold	–

! Important

Limit PCR1 to 25–28 cycles to minimize chimera formation and PCR bias (Schirmer et al., 2015). More cycles increase the proportion of artifacts in the sequencing data.

4. Verify amplification by running 2 uL on a 1.5% agarose gel per [SOP-L703](#).

7.3. PCR1 Cleanup (SPRI Beads)

1. Allow AMPure XP beads to reach room temperature (approximately 30 minutes). Vortex well before use.
2. Add 20 uL (0.8x ratio) of beads to each 25 uL PCR product. Mix by pipetting 10 times.
3. Incubate at room temperature for 5 minutes.
4. Place on the magnetic stand for 2 minutes until the solution clears.
5. Aspirate and discard the supernatant.
6. Wash twice with 200 uL of freshly prepared 80% ethanol. Do not disturb the bead pellet. Allow 30 seconds per wash.
7. Remove all ethanol and air-dry the beads for 3–5 minutes. Do not over-dry (beads will crack and yield drops).
8. Remove from the magnetic stand. Elute in 27.5 uL of nuclease-free water. Pipette to mix.
9. Return to the magnetic stand. Transfer 25 uL of the clear eluate to a new tube.

7.4. PCR2: Index PCR (Barcoding)

1. Assign a unique dual-index combination (i7 + i5) to each sample. Record the plate map in the lab notebook and in the sample sheet.
2. Set up the index PCR:

Component	Volume per reaction
2x KAPA HiFi HotStart ReadyMix	12.5 uL
Nextera XT Index Primer 1 (i7)	2.5 uL
Nextera XT Index Primer 2 (i5)	2.5 uL
Cleaned PCR1 product	2.5 uL
Nuclease-free water	5.0 uL
Total	25.0 uL

3. Cycling parameters:

Step	Temperature	Time	Cycles
Initial denaturation	95 C	3 min	1
Denaturation	95 C	30 s	8
Annealing	55 C	30 s	8

Step	Temperature	Time	Cycles
Extension	72 C	30 s	8
Final extension	72 C	5 min	1
Hold	4 C	Hold	–

4. Clean the indexed PCR product with AMPure XP beads (0.8x ratio) as in Section 6.3. Elute in 27.5 uL.

7.5. Library Quantification and QC

1. Quantify each library using the Qubit dsDNA HS Assay ([SOP-L700](#), Section 6.4).
2. Verify library size by running 1 uL on a 1.5% agarose gel. The expected size is the amplicon plus approximately 120 bp of adapter and index sequence.
3. Libraries with concentrations below 1 nM may not sequence well. Re-amplify or exclude them.

7.6. Library Pooling and Submission

1. Normalize each library to an equimolar concentration (typically 4 nM) by diluting with nuclease-free water.
2. Pool equal volumes of all normalized libraries into a single tube.
3. Quantify the pooled library by Qubit.
4. Prepare the sample sheet (Illumina Experiment Manager format or the sequencing facility's template) listing every sample with its index combination.
5. Submit the pooled library and sample sheet to the sequencing facility per their submission guidelines.
6. Record the submission date, facility, run type (e.g., MiSeq 2x250 or 2x300), and expected turnaround in the lab notebook.

8. Quality Control

- Every library prep run must include: positive control (mock community, if available), NTC, and extraction blank carried through both PCR1 and PCR2
- NTC and extraction blanks must show no visible band on the verification gel and no detectable concentration on the Qubit. If blanks are positive, the entire batch is suspect (Salter et al., 2014).
- Verify amplicon size on gel after PCR1; verify library size (amplicon – adapters) on gel after PCR2
- Record the number of PCR1 cycles and the index assignments for every sample; these are required for data analysis and troubleshooting

9. Waste Disposal

Waste Type	Container	Disposal Method
Used SPRI beads	Standard trash	Non-hazardous
Ethanol wash waste	Chemical waste or evaporate in fume hood	Small volumes can evaporate
Used PCR tubes, plates, tips	Standard trash	Non-hazardous
Gel waste	Per SOP-L703	Depends on stain used

10. References

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11. Related SOPs

SOP	Title	Relationship
L700	DNA Extraction	Upstream: provides template DNA
L703	PCR and Gel Electrophoresis	Amplicon verification
L705	Bioinformatics Pipeline	Downstream: sequence processing
L706	eDNA Metabarcoding	eDNA-specific workflow
F701	Soil Microbial Sampling	Field collection

12. Training Record

Before preparing amplicon libraries independently, personnel must:

1. Read this SOP, [SOP-L703](#), and [SOP-L700](#) in full
2. Demonstrate SPRI bead cleanup with correct bead ratios and wash steps
3. Prepare a supervised library from positive control DNA through pooling, with clean controls and correct library size on gel
4. Complete the sequencing facility's sample submission training
5. Be signed off by the PI or lab manager



Environmental Analysis Program

Pomona College
Biogeochemistry Laboratory

SOP-L705

Bioinformatics Pipeline (QIIME 2 / DADA2)

Standard Operating Procedure

SOP Number	SOP-L705
Revision	0.1
Author(s)	Marc Los Huertos
Date	2026-06-26
Organization	EA Program, Pomona College

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1. Purpose

This SOP describes the bioinformatics workflow for processing amplicon sequencing data (16S rRNA, ITS, 18S rRNA) from raw demultiplexed FASTQ files through quality filtering, denoising, taxonomic classification, and generation of analysis-ready feature tables. The pipeline uses QIIME 2 as the primary framework with DADA2 as the denoising algorithm (Bolyen et al., 2019; Callahan et al., 2016). DADA2 resolves amplicon sequence variants (ASVs) at single-nucleotide resolution, replacing the older OTU-clustering approach with a more reproducible and sensitive method (Callahan et al., 2017).

2. Scope

- **Applies to:** Processing of Illumina paired-end amplicon sequencing data (MiSeq, NextSeq) for 16S, ITS, and 18S marker genes generated from EA Program environmental samples
- **Does not apply to:** Shotgun metagenomics; long-read sequencing (PacBio, Nanopore); transcriptomics (RNA-seq); ddPCR data analysis (see [SOP-L702](#))
- **Location:** EA Program lab computers or personal workstations with QIIME 2 installed; high-performance computing cluster if available

3. Responsibilities

Role	Responsibility
Lab PI	Approve pipeline parameters and taxonomy database
Trained Personnel	Run the pipeline per this SOP; document all parameters
New Students	Complete training before independent analysis

4. Definitions

- **ASV (Amplicon Sequence Variant):** An exact DNA sequence resolved from amplicon sequencing data by denoising algorithms (DADA2, Deblur); a higher-resolution alternative to OTUs that retains single-nucleotide differences between sequences.
- **DADA2:** A denoising algorithm that models sequencing error rates to distinguish true biological sequences from sequencing errors; produces ASVs directly from paired-end reads without clustering.
- **Demultiplexing:** The computational step of assigning reads to their source sample based on index sequences; produces per-sample FASTQ files from a multiplexed sequencing run.
- **Feature table (OTU/ASV table):** A matrix of counts where rows are samples, columns are ASVs or OTUs, and each cell contains the number of reads from that sample assigned to that sequence variant; the primary data object in QIIME 2.
- **FASTQ:** A text-based file format storing DNA sequences and their per-base quality scores (Phred scores); the standard output format from Illumina sequencers; paired-end data produces R1 and R2 FASTQ files per sample.
- **OTU (Operational Taxonomic Unit):** A cluster of sequences grouped by a similarity threshold (typically 97% identity); a coarser representation of diversity than ASVs; largely superseded by ASV-based approaches.
- **Phred quality score (Q score):** A logarithmic measure of base-call accuracy: $Q = -10 \log_{10}(P_{error})$; Q20 = 99% accuracy; Q30 = 99.9% accuracy; used to set quality-trimming thresholds in DADA2.
- **QIIME 2:** An open-source bioinformatics platform for microbiome analysis; manages data as typed artifacts (.qza) with embedded provenance records; wraps DADA2, taxonomic classifiers, diversity analyses, and visualization tools.
- **Rarefaction:** Subsampling each sample to the same number of reads to correct for unequal sequencing depth before diversity comparisons; samples below the rarefaction depth are excluded.

- **Taxonomic classifier:** A trained machine-learning model (e.g., naive Bayes classifier trained on SILVA or UNITE) that assigns taxonomy to ASV sequences by comparing them to a reference database.

5. Software Requirements

Software	Version (minimum)	Notes
QIIME 2	2024.2 or later	Install via conda/mamba; see https://docs.qiime2.org
DADA2 (QIIME 2 plugin)	Bundled with QIIME 2	R package also available for standalone use
R and RStudio	R 4.3+	For downstream statistical analysis; see SOP-L801
phyloseq (R package)	1.44+	For importing QIIME 2 artifacts into R (McMurdie & Holmes, 2013)
FastQC	0.12+	Optional; for initial read quality inspection

Install QIIME 2 in a dedicated conda environment to avoid dependency conflicts:

```
# Example installation (check docs.qiime2.org for current commands)
conda env create -n qiime2-2024.2 \
  --file https://data.qiime2.org/distro/amplicon/qiime2-amplicon-2024.2-py39-linux-conda.yml
conda activate qiime2-2024.2
```

6. Procedure

6.1. Data Organization

1. Create a project directory with the following structure:

```
project_name/
  raw_data/      # Demultiplexed FASTQ files from sequencing facility
  qiime2/        # QIIME 2 artifacts and visualizations
  results/       # Exported tables, trees, taxonomy
  scripts/       # Shell and R scripts
  metadata/      # Sample metadata TSV file
```

2. Prepare a sample metadata file (`metadata.tsv`) in tab-separated format. The first column must be `sample-id` matching the FASTQ filenames. Include all experimental variables (site, treatment, date, depth, replicate). Validate the file at <https://keemei.qiime2.org/> (Rideout et al., 2014).

6.2. Import Raw Reads

Import demultiplexed paired-end FASTQ files into a QIIME 2 artifact:

```
qiime tools import \  
  --type 'SampleData[PairedEndSequencesWithQuality]' \  
  --input-path raw_data/ \  
  --input-format CasavaOneEightSingleLanePerSampleDirFmt \  
  --output-path qiime2/demux.qza
```

Visualize read quality:

```
qiime demux summarize \  
  --i-data qiime2/demux.qza \  
  --o-visualization qiime2/demux-summary.qzv
```

Open demux-summary.qzv at <https://view.qiime2.org> to inspect quality score profiles. Use these plots to determine truncation parameters for DADA2 (Section 5.3).

6.3. Denoising with DADA2

DADA2 performs quality filtering, chimera removal, and denoising in a single step, producing an ASV feature table and representative sequences (Callahan et al., 2016).

```
qiime dada2 denoise-paired \  
  --i-demultiplexed-seqs qiime2/demux.qza \  
  --p-trunc-len-f 240 \  
  --p-trunc-len-r 200 \  
  --p-trim-left-f 0 \  
  --p-trim-left-r 0 \  
  --p-n-threads 4 \  
  --o-table qiime2/table.qza \  
  --o-representative-sequences qiime2/rep-seqs.qza \  
  --o-denoising-stats qiime2/dada2-stats.qza
```

! Setting Truncation Parameters

Choose `--p-trunc-len-f` and `--p-trunc-len-r` based on the quality plots from Section 5.2. Truncate where the median quality score drops below 25–30. The forward and reverse reads must still overlap by at least 20 bp after truncation for successful merging. For 16S V4 (515F/806R, ~290 bp amplicon) with 2x250 reads, typical values are 240 (forward) and 200 (reverse).

Review denoising statistics:

```
qiime metadata tabulate \  
  --m-input-file qiime2/dada2-stats.qza \  
  --o-visualization qiime2/dada2-stats.qzv
```

Check the percentage of reads passing each filter step (input, filtered, denoised, merged, non-chimeric). A healthy run retains > 60% of input reads through the full pipeline.

6.4. Taxonomic Classification

Assign taxonomy to ASVs using a pre-trained Naive Bayes classifier. Download the appropriate classifier from the QIIME 2 Data Resources page (<https://docs.qiime2.org/2024.2/data-resources/>).

For 16S (SILVA 138.1):

```
qiime feature-classifier classify-sklearn \  
  --i-classifier silva-138.1-ssu-nr99-515f-806r-classifier.qza \  
  --i-reads qiime2/rep-seqs.qza \  
  --o-classification qiime2/taxonomy.qza
```

For ITS (UNITE):

```
qiime feature-classifier classify-sklearn \  
  --i-classifier unite-ver9-dynamic-all-29.11.2022-classifier.qza \  
  --i-reads qiime2/rep-seqs.qza \  
  --o-classification qiime2/taxonomy.qza
```

Visualize taxonomy:

```
qiime taxa barplot \  
  --i-table qiime2/table.qza \  
  --i-taxonomy qiime2/taxonomy.qza \  
  --m-metadata-file metadata/metadata.tsv \  
  --o-visualization qiime2/taxa-barplot.qzv
```

6.5. Phylogenetic Tree Construction

Build a rooted phylogenetic tree for diversity analyses:

```
qiime phylogeny align-to-tree-mafft-fasttree \  
  --i-sequences qiime2/rep-seqs.qza \  
  --o-alignment qiime2/aligned-rep-seqs.qza \  
  --o-masked-alignment qiime2/masked-aligned-rep-seqs.qza \  
  --o-tree qiime2/unrooted-tree.qza \  
  --o-rooted-tree qiime2/rooted-tree.qza
```

6.6. Filtering

Remove contaminant ASVs identified in negative controls, mitochondrial and chloroplast sequences, and low-frequency ASVs:

```
# Remove mitochondria and chloroplast
qiime taxa filter-table \
  --i-table qiime2/table.qza \
  --i-taxonomy qiime2/taxonomy.qza \
  --p-exclude mitochondria,chloroplast \
  --o-filtered-table qiime2/table-filtered.qza

# Remove low-frequency ASVs (e.g., singletons)
qiime feature-table filter-features \
  --i-table qiime2/table-filtered.qza \
  --p-min-frequency 2 \
  --o-filtered-table qiime2/table-final.qza
```

For decontamination using negative control data, export the feature table to R and use the **decontam** package (Davis et al., 2018).

6.7. Export for R Analysis

Export QIIME 2 artifacts for downstream analysis in R with phyloseq (McMurdie & Holmes, 2013):

```
qiime tools export \
  --input-path qiime2/table-final.qza \
  --output-path results/

qiime tools export \
  --input-path qiime2/taxonomy.qza \
  --output-path results/

qiime tools export \
  --input-path qiime2/rooted-tree.qza \
  --output-path results/
```

Alternatively, import QIIME 2 artifacts directly into R using the **qiime2R** package.

7. Quality Control

- Inspect the DADA2 denoising stats for every run. Flag samples with fewer than 1,000 reads after denoising.
- Verify that negative controls (NTC, extraction blank) contain fewer than 100 reads. If controls contain substantial reads, use **decontam** to identify and remove contaminant ASVs (Davis et al., 2018; Salter et al., 2014).

- Confirm that the positive control (mock community) recovers the expected taxa at the expected relative abundances.
- Document all QIIME 2 and DADA2 parameters in the project README and in the analysis scripts. Reproducibility requires recording the exact software versions, classifier version, and truncation lengths.
- Archive raw FASTQ files, the QIIME 2 artifacts, and the analysis scripts in the project GitHub repository per [SOP-L801](#).

8. References

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9. Related SOPs

SOP	Title	Relationship
L700	DNA Extraction	Upstream: sample preparation
L703	PCR and Gel Electrophoresis	Amplicon verification
L704	Amplicon Library Prep	Upstream: library preparation
L706	eDNA Metabarcoding	eDNA-specific application
L801	RStudio and GitHub	Data management and version control
L802	Quality Control and QA	General QA/QC framework

10. Training Record

Before running the bioinformatics pipeline independently, personnel must:

1. Read this SOP in full
2. Complete the QIIME 2 “Moving Pictures” tutorial (<https://docs.qiime2.org/2024.2/tutorials/moving-pictures/>)
3. Process a test dataset under supervision, from import through taxonomy assignment and export to R
4. Demonstrate correct interpretation of DADA2 denoising statistics and quality plots
5. Be signed off by the PI or lab manager



Environmental Analysis Program

Pomona College
Biogeochemistry Laboratory

SOP-L706

eDNA Metabarcoding Lab Workflow

Standard Operating Procedure

SOP Number	SOP-L706
Revision	0.1
Author(s)	Marc Los Huertos
Date	2026-06-26
Organization	EA Program, Pomona College

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1. Purpose

This SOP describes the end-to-end laboratory workflow for environmental DNA (eDNA) metabarcoding, from receipt of field-collected water filter samples through DNA extraction, PCR amplification, library preparation, sequencing, and bioinformatics analysis. eDNA metabarcoding combines

universal or group-specific primers with high-throughput sequencing to detect and characterize aquatic biodiversity from water samples without capturing organisms (Deiner et al., 2017; Taberlet et al., 2012).

This SOP serves as the integrating document that ties together the field collection procedures in [SOP-F700](#) with the molecular and computational SOPs in the L700 series. It does not repeat detailed procedures described in other SOPs but provides the eDNA-specific modifications, QC requirements, and decision points that distinguish eDNA metabarcoding from standard amplicon sequencing of soil or tissue samples.

2. Scope

- **Applies to:** Laboratory processing of eDNA water samples for multi-species detection via amplicon sequencing on Illumina platforms; target taxa include fish (12S), amphibians (16S/12S), macroinvertebrates (COI), and general biodiversity surveys (18S, COI)
- **Does not apply to:** Species-specific eDNA detection by qPCR or ddPCR (single-species assays); soil microbial community analysis (see [SOP-L704](#) and [SOP-L705](#)); field collection procedures (see [SOP-F700](#))
- **Location:** Molecular biology bench, SGM 133

3. Responsibilities

Role	Responsibility
Lab PI	Approve target markers, primers, and sequencing plan
Trained Personnel	Process samples per this SOP; maintain contamination controls
New Students	Complete eDNA-specific contamination training before participating

4. Definitions

- **Amplification blank (no-template control, NTC):** A PCR reaction containing all reagents except sample DNA; run with every PCR plate; any amplification in the NTC indicates contamination and invalidates the entire plate.
- **Blocking primer:** A modified oligonucleotide added to a PCR reaction to selectively suppress amplification of abundant non-target DNA (e.g., host DNA in gut or tissue eDNA samples); allows rare target sequences to be amplified competitively.
- **eDNA metabarcoding:** A method that uses universal or group-specific primers to amplify and sequence a short, taxonomically informative DNA region (barcode) from environmental DNA, yielding a community-level inventory of taxa present in a sample.
- **Environmental DNA (eDNA):** Genetic material shed into the environment by organisms through mucus, feces, skin cells, eggs, and other biological material; collected non-invasively from water, sediment, or air.

- **Extraction blank:** A tube containing only extraction reagents and no biological material, processed through the full extraction procedure; detects reagent or handling contamination; must yield no target amplification.
- **Field blank:** A filter or collection vessel processed through all field collection steps but with no environmental sample; detects airborne or handling contamination during field work.
- **Metabarcoding locus:** The specific genomic region amplified for community identification; common loci include 12S rRNA (fish), 16S rRNA (bacteria, invertebrates), 18S rRNA (eukaryotes), COI (invertebrates, fish), and ITS2 (fungi, plants).
- **Occupancy model:** A statistical model that accounts for imperfect detection in eDNA surveys by estimating the probability that a species is truly present given detection/non-detection across replicate samples or PCR replicates.
- **PCR replicate:** One of multiple independent PCR reactions run from the same DNA extract; increases detection sensitivity for rare targets and allows estimation of false-negative rates; standard eDNA practice is 3–8 replicates per sample.
- **Sequence read depth (coverage):** The total number of sequencing reads assigned to a sample; directly affects the number of rare taxa detected; EA Program target is a minimum of 10,000 reads per sample after quality filtering.

5. Safety and Hazards

Hazards

- **Chemical:** Lysis buffers (guanidine salts), ethanol, SPRI beads, and DNA stains. See individual SOP safety sections for details.
- **Biological:** eDNA samples may originate from water bodies containing pathogens. Handle with standard precautions.
- **UV radiation:** If using UV transilluminator for gel imaging.

5.1. Required PPE

- ☐ Safety glasses
- ☐ Lab coat
- ☐ Nitrile gloves (changed frequently; mandatory at all steps)

5.2. Emergency Procedures

Refer to [SOP-L003 Hazardous Materials Handling](#).

6. Workflow Overview

The eDNA metabarcoding workflow consists of six stages. Each stage references a detailed SOP.

Stage	Description	SOP Reference
1	Field collection and preservation	F700
2	DNA extraction from filters	L700
3	PCR amplification and verification	L703
4	Library preparation and sequencing	L704
5	Bioinformatics (QIIME 2 / DADA2)	L705
6	Taxonomic interpretation and reporting	This SOP, Section 6.6

7. Procedure: eDNA-Specific Modifications

7.1. Sample Receipt and Logging

1. Upon receipt from the field, verify that all samples match the chain of custody form per [SOP-F008](#).
2. Inspect Sterivex capsules or filter bags for integrity (no leaks, caps intact, preservative present).
3. Log each sample in the lab notebook and [Freezer Inventory Google Sheet](#).
4. Store preserved filters at -20 C (short-term) or -80 C (long-term) until extraction.

7.2. DNA Extraction from Filters

Follow [SOP-L700](#) with the following eDNA-specific modifications:

- **Sterivex capsules:** Open the capsule aseptically using a sterile razor blade or the Sterivex extraction adapter. Transfer the membrane into the PowerBead Pro tube using sterile forceps. If Longmire's buffer was used as preservative, add the buffer to the bead tube as well.
- **Open membrane filters:** Transfer the folded filter directly into the bead tube using sterile forceps.
- **Extraction blanks:** Process one extraction blank per batch. For eDNA, this means running an empty bead tube through the full protocol.
- **Field blanks:** The field blank filter (from [SOP-F700](#)) is extracted alongside samples and serves as the primary contamination control for the entire field-to-lab chain.

7.3. Inhibition Testing

eDNA extracts from natural waters frequently contain PCR inhibitors (humic acids, tannins, metal ions) (McKee et al., 2015). Test for inhibition before committing to full library preparation:

1. Run an internal positive control (IPC) qPCR assay on a subset of extracts (or all extracts, if the batch is small). The IPC uses a synthetic DNA target spiked into the PCR reaction at a known concentration.
2. If the IPC C_t value shifts by more than 2 cycles compared to the uninhibited control, the extract is inhibited.
3. For inhibited samples, dilute the extract 1:5 or 1:10 with nuclease-free water and retest. Alternatively, clean the extract using a OneStep PCR Inhibitor Removal Kit (Zymo Research).

7.4. Primer Selection for eDNA Metabarcoding

Target Taxa	Marker	Primers	Amplicon	Reference
Fish	12S rRNA	MiFish-U-F / MiFish-U-R	~170 bp	Miya et al. (2015)
Amphibians	16S rRNA	16Smam / 16Smam-R (modified)	~250 bp	Vences et al. (2005)
Macroinvertebrates	COI	mlCOIintF / jgHCO2198	~313 bp	Leray et al. (2013)
General eukaryotes	18S rRNA	Euk1391f / EukBr	~130 bp	Amaral-Zettler et al. (2009)
General eukaryotes	COI	fwhF2 / fwhR2n	~205 bp	Vamos et al. (2022)

Note

Primer choice determines which taxa can be detected. Discuss target taxa and primer selection with the PI before beginning library preparation. Multi-marker approaches (e.g., 12S + COI) provide broader taxonomic coverage but require separate library preparations and sequencing runs (Deiner et al., 2017).

7.5. PCR and Library Preparation

Follow [SOP-L703](#) for initial PCR screening and [SOP-L704](#) for library preparation, with these eDNA-specific considerations:

- **Replicates:** Run 3 independent PCR replicates per sample to improve detection probability for rare species (Ficetola et al., 2015). Pool the replicates after PCR1 before cleanup and indexing.
- **Cycle number:** Use 35 cycles for PCR1 (higher than standard 16S amplicon work) because eDNA concentrations are typically very low. Be aware that additional cycles increase the risk of chimera formation and tag-jumping (Schnell et al., 2015).
- **Blocking primers (optional):** If non-target taxa (e.g., human DNA) dominate the PCR product, consider using blocking primers or PNA (peptide nucleic acid) clamps to suppress amplification of non-target sequences.
- **Controls:** In addition to the standard NTC and extraction blank, include the field blank and (if available) a positive control consisting of a mock community of known species at known concentrations.

7.6. Bioinformatics and Taxonomic Assignment

Follow [SOP-L705](#) for denoising and taxonomic classification, with these modifications:

- **Reference database:** Use a curated database matched to the marker and target taxa:
 - 12S fish: MitoFish or MIDORI2

- COI: BOLD / MIDORI2
- 16S/18S: SILVA or GenBank nt
- **Species-level assignment:** For eDNA, species-level identification is the primary goal (unlike microbiome studies where genus-level is often sufficient). Use BLAST-based classification (`classify-consensus-blast` in QIIME 2) in addition to or instead of the Naive Bayes classifier for improved species-level resolution (Goldberg et al., 2016).
- **Decontamination:** Use the `decontam` R package to identify and remove contaminant ASVs using the field blank and extraction blank data (Davis et al., 2018).
- **Detection criteria:** An ASV is considered a positive detection for a species only if:
 1. It matches a reference sequence at greater than or equal to 97–99% identity (marker-dependent)
 2. It is absent from all negative controls (field blank, extraction blank, NTC), or its abundance in the sample exceeds 10x the abundance in the highest-read control
 3. It is detected in at least 2 of 3 PCR replicates (if replicates were sequenced independently) (Ficetola et al., 2015)

7.7. Reporting

eDNA metabarcoding results are reported as a species detection list per site with the following metadata:

- ASV sequence and percent identity to reference
- Number of reads per ASV per sample
- Detection across replicates (e.g., detected in 2/3 replicates)
- Negative control results
- Any taxa flagged as potential contaminants

! Important

eDNA metabarcoding provides presence/detection data. Quantitative interpretation of read counts as species abundance is not recommended without careful validation, because PCR amplification bias, primer affinity, and DNA shedding rates vary among taxa (Kelly et al., 2019).

8. Quality Control

- **Field blanks:** Must be negative for all target taxa. Any target taxon detected in a field blank compromises the detection claim for that taxon across all samples in the same sampling event.
- **Extraction blanks:** Must be negative. Positive extraction blanks indicate lab contamination; the entire extraction batch is suspect.
- **NTC:** Must be negative. Positive NTCs indicate PCR contamination.
- **Positive control (mock community):** Must recover all expected species. Failure to detect a known species in the mock community indicates primer bias or PCR failure.

- **Replication:** A minimum of 3 PCR replicates per sample is recommended for occupancy-based detection analyses.
- Document all control results in the project data file and report alongside species detections.

9. Waste Disposal

Refer to waste disposal tables in [SOP-L700](#), [SOP-L703](#), and [SOP-L704](#).

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11. Related SOPs

SOP	Title	Relationship
F700	eDNA Field Sampling	Upstream: field collection
F008	Sample Labeling and COC	Chain of custody
L700	DNA Extraction	Stage 2: extraction
L703	PCR and Gel Electrophoresis	Stage 3: amplification
L704	Amplicon Library Prep	Stage 4: library prep
L705	Bioinformatics Pipeline	Stage 5: sequence analysis
L801	RStudio and GitHub	Data management

12. Training Record

Before processing eDNA samples independently, personnel must:

1. Read this SOP and all related SOPs (F700, L700, L703, L704, L705)
2. Demonstrate aseptic filter handling and extraction under supervision
3. Complete an eDNA-specific contamination awareness session covering pre-PCR/post-PCR separation, filter handling, and control interpretation
4. Process a supervised batch including field blank, extraction blank, and positive control with clean results
5. Be signed off by the PI or lab manager