



Environmental Analysis Program

Pomona College
Biogeochemistry Laboratory

SOP-L702

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

Standard Operating Procedure

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Table of contents

1 Purpose	1
2 Scope	2
3 Responsibilities	2
4 Definitions	2
5 Why ddPCR for This Application	3
6 The Soil Nitrogen Cycle and Functional Gene Markers	3
7 From Instrument Output to Quality-Controlled Data	4
7.1 Reviewing and Exporting Data	4
7.2 Quality Control and Acceptance Criteria	5
8 Calculating Gene Copy Numbers	5
8.1 From Reaction to Sample	5
8.2 R Implementation	6
9 Interpreting Relative Gene Abundances	7
10 References	7
11 Related SOPs	8
12 Training Record	8
13 Appendix A: DMINQ Checklist	9

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

- Bio-Rad Laboratories. (2018). *Droplet digital PCR applications guide*. Bio-Rad Laboratories, Inc.
- Bio-Rad Laboratories. (2024). *QX200 droplet digital PCR system instruction manual*. <https://www.bio-rad.com/en-us/product/qx200-droplet-digital-pcr-system>
- Braker, G., Zhou, J., Wu, L., Devol, A. H., & Tiedje, J. M. (2000). Nitrite reductase genes (*nirK* and *nirS*) as functional markers to investigate diversity of denitrifying bacteria in Pacific Northwest marine sediment communities. *Applied and Environmental Microbiology*, 66(5), 2096–2104. <https://doi.org/10.1128/AEM.66.5.2096-2104.2000>
- Francis, C. A., Roberts, K. J., Beman, J. M., Santoro, A. E., & Oakley, B. B. (2005). Ubiquity and diversity of ammonia-oxidizing archaea in water columns and sediments of the ocean. *Proceedings of the National Academy of Sciences*, 102(41), 14683–14688. <https://doi.org/10.1073/pnas.0506625102>
- Frosteg aard, Aa., Courtois, S., Ramisse, V., Clerc, S., Bernillon, D., Le Gall, F. ccois, Jeannin, P., Nesme, X., & Simonet, P. (1999). Quantification of bias related to the extraction of DNA directly from soils. *Applied and Environmental Microbiology*, 65(12), 5409–5420. <https://doi.org/10.1128/AEM.65.12.5409-5420.1999>
- Henry, S., Bru, D., Stres, B., Hallet, S., & Philippot, L. (2006). Quantitative detection of the *nosZ* gene, encoding nitrous oxide reductase, and comparison of the abundances of 16S rRNA, *nirG*, *nirK*, and *nosZ* genes in soils. *Applied and Environmental Microbiology*, 72(8), 5181–5189. <https://doi.org/10.1128/AEM.00231-06>

- Hindson, B. J., Ness, K. D., Masquelier, D. A., Belgrader, P., Heredia, N. J., Makarewicz, A. J., Bright, I. J., Lucero, M. Y., Hiddessen, A. L., Legler, T. C., Kitano, T. K., Hodel, M. R., Petersen, J. F., Wyatt, P. W., Steenblock, E. R., Shah, P. H., Bousse, L. J., Troup, C. B., Mellen, J. C., ... Colston, B. W. (2011). High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. *Analytical Chemistry*, 83(22), 8604–8610. <https://doi.org/10.1021/ac202028g>
- Huggett, J. F., Foy, C. A., Benes, V., Emslie, K., Garson, J. A., Haynes, R., Hellemans, J., Kubista, M., Mueller, R. D., Nolan, T., Pfaffl, M. W., Shipley, G. L., Vandesompele, J., Wittwer, C. T., & Bustin, S. A. (2013). Digital PCR as a novel technology and its potential implications for molecular diagnostics. *Clinical Chemistry*, 59(12), 1691–1702. <https://doi.org/10.1373/clinchem.2013.214742>
- Kuypers, M. M. M., Marchant, H. K., & Kartal, B. (2018). The microbial nitrogen-cycling network. *Nature Reviews Microbiology*, 16(5), 263–276. <https://doi.org/10.1038/nrmicro.2018.9>
- Levy-Booth, D. J., Prescott, C. E., & Grayston, S. J. (2014). Microbial functional genes involved in nitrogen fixation, nitrification and denitrification in forest ecosystems. *Soil Biology and Biochemistry*, 75, 11–25. <https://doi.org/10.1016/j.soilbio.2014.03.021>
- Lievens, A., Jacchia, S., Kagkli, D., Savini, C., & Querci, M. (2016). Enhanced analysis of real-time PCR data by using a variable efficiency model: FPK-PCR. *Nucleic Acids Research*, 44(10), e95. <https://doi.org/10.1093/nar/gkw007>
- R Core Team. (2024). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing. <https://www.R-project.org/>
- Rotthauwe, J.-H., Witzel, K.-P., & Liesack, W. (1997). The ammonia monooxygenase structural gene *textitamoA* as a functional marker: Molecular fine-scale analysis of natural ammonia-oxidizing populations. *Applied and Environmental Microbiology*, 63(12), 4704–4712. <https://doi.org/10.1128/aem.63.12.4704-4712.1997>
- The dMIQE Group, & Huggett, J. F. (2020). The digital MIQE guidelines update: Minimum information for publication of quantitative digital PCR experiments for 2020. *Clinical Chemistry*, 66(8), 1012–1029. <https://doi.org/10.1093/clinchem/hvaa125>

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