



Environmental Analysis Program

Pomona College
Biogeochemistry Laboratory

SOP-L701

Primer Selection and Design for Nitrogen Cycle Genes – ddPCR

Standard Operating Procedure

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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 μ M. Store at –20 °C.
5. Prepare 10 μ M 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.

9. References

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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