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Tagged Highly Degenerate Primer (THDP)-PCR for Community Analysis of Methane- and Ammonia-oxidizing Bacteria Based on Copper-containing Membrane-bound Monooxygenases (CuMMO)

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[Abstract] We describe a two-step PCR strategy using tagged highly degenerate primer (THDP-PCR) targeting copper-containing membrane-bound monooxygenases (CuMMO) genes for community analysis of methane- or ammonia-oxidizing bacteria. This strategy consists of a primary CuMMO gene-specific PCR followed by a secondary PCR with a tag as a single primer. This strategy remarkably increases the divergence of CuMMO gene amplicons while maintaining PCR efficiency without obvious amplification bias. This THDP-PCR strategy can be extended to other functional gene-based community analysis with design of new highly degenerate primer covering target functional gene sequences.

Keywords: Tagged highly degenerate primer, Two-step PCR, CuMMO

[Background] Gene types in CuMMO superfamily are divergent and existent primer sets can only cover some CuMMO types (Tuomivirta *et al*., 2009). To cover the divergent types of genes in the CuMMO superfamily, highly degenerate primers are inevitable, but previous strategies using highly degenerate primers have limitations when applied to environmental samples, like low PCR efficiency or non-specific amplification (Ledeker and De Long, 2013). We recently used a two-step PCR strategy with tagged highly degenerate primers, designated THDP-PCR, to amplify a wide range of genes in the CuMMO family with satisfactory PCR efficiency and no obvious amplification bias (Wang *et al*., 2017).

Materials and Reagents

1. Pipette tips (20 µl, 200 µl, 1,000 µl) (Thermo Fisher Scientific, Thermo Scientific™, catalog numbers: 3521-HR, 3551-HR, 3101-05-HR)
2. 1.5 ml Eppendorf tubes (Eppendorf, catalog number: T9661-1000EA)
3. Strip PCR tubes and caps (Roche Diagnostics, catalog number: 1667009001)
4. PowerSoil™ DNA Isolation Kit (Mo Bio Laboratories, catalog number: 12888-100)
5. Bovine serum albumin (BSA) (Sigma-Aldrich, catalog number: A7906)
6. Premix *Taq* (Takara Bio, catalog number: R004A)
7. dNTP mixture
8. Tris-HCl (pH 8.3)

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9. Potassium chloride (KCl)
10. Magnesium chloride ($MgCl_2$)
11. AxyPrep™ PCR Cleanup Kit (Corning, Axygen®, catalog number: AP-PCR-250)
12. Primers

Primer name	Sequence (5'-3')	Degeneracy
A189-tag	5'-GCCGGAGCTCTGCAGATATCGGNGACTGGGACTTCTGG-3'	4
HD616-tag	5'-GCCGGAGCTCTGCAGATATCAYCWKVCKNAYRTAYTCVGG-3' [a]	4,608
Tag-barcode	5'-XXXXXXXXGCCGGAGCTCTGCAGATATC-3' [b]	1

[a]*Degenerate: K, G/T; R, A/G; Y, C/T; W, A/T; V, G/A/C; N, A/T/C/G.*

[b]*XXXXXXXX indicates the presence of the 8 nt barcode to differentiate samples in the mixture during pyrosequencing.*

Equipment

1. Pipettes (0-10 µl, 10-100 µl, 100-1,000 µl) (Eppendorf, catalog numbers: k03030, k03031, k03032)
2. Vortex-genie™ 2 (Mo Bio Laboratories, model: Vortex-Genie® 2)
3. PICO 17 centrifuge (Thermo Fisher Scientific, Thermo Scientific™, model: Heraeus™ Pico™ 17)
4. Automated thermal cycler TP600 (Takara Bio, model: TP600)

Software

1. Mothur (www.mothur.org)
2. BLAST+ (ftp://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/LATEST/)
3. Megan5 (http://ab.inf.uni-tuebingen.de/software/megan5/)

Procedure

1. Sample collection and nucleic acid extraction

   Extract genomic DNA from soil or sediment samples (0.25 g wet weight) using a PowerSoil™ DNA Isolation Kit (Mo Bio Laboratories, USA) according to the manufacturer's instructions.
2. THDP-PCR method (Figure 1)

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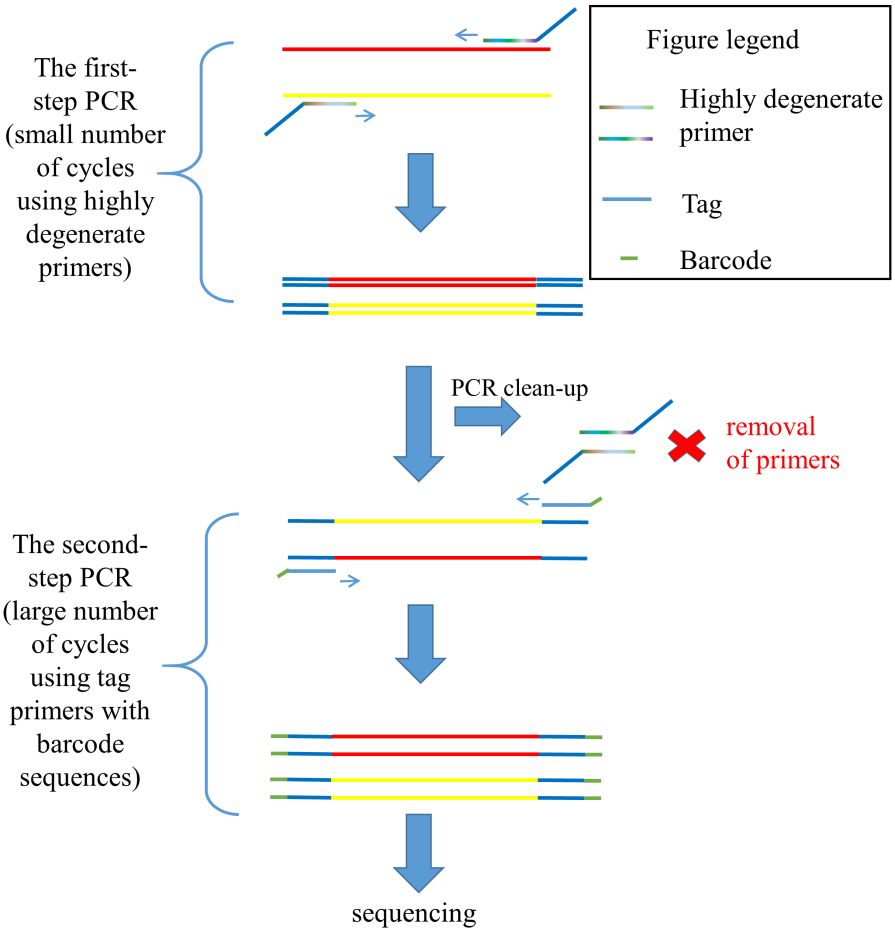

Figure 1. Principle of THDP-PCR

a. First step of THDP-PCR

i. PCR reaction recipe. Final volume: 50 µl

H_2O	18 µl
A189-tag (10 µM)	2 µl
HD616-tag (10 µM)	2 µl
BSA (10 mg/ml)	2 µl
Sample DNA	1 µl

Note: Approximately 2 to 20 ng per sample, H_2O for negative control.

Premix *Taq* (see Table 1)	25 µl

Table 1. Composition of Premix *Taq*™ solution

TaKaRa *Taq*	1.25 U/25 µl
dNTP mixture	each 0.4 mM
Tris-HCl (pH 8.3)	20 mM
KCl	100 mM
$MgCl_2$	3 mM

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Note: Reverse primer HD616-tag was designed based on the CuMMO sequences downloaded from FunGene Pipeline. We aligned the sequences and identified the conserved regions, then introduced degeneracies to cover different types of CuMMO gene sequences, developing the reverse primer HD616. A 20-nt-long tag was added to the 5' end of both primers, A189 and HD616, to generate the tagged primers A189-tag and HD616-tag, respectively, for THDP-PCR amplification. Tag sequence was from virus genome (Canine astrovirus strain HUN/2012/115, GenBank: KX599351.1) and will not bind to bacterial genomes and thus avoids non-specific PCR amplification. Because tag is used as a single primer in the second step of the THDP-PCR, its length is set to 20 nt, a standard length for primers.

ii. Automated thermal cycler program

1) Denaturation at 94 °C for 5 min
2) 10 cycles at:
   94 °C for 1 min
   59 °C for 1 min
   72 °C for 1 min
3) 72 °C for 10 min

Note: Conventional (one step) PCR efficiency is not satisfactory for highly degenerate primers with degeneracy higher than 48 based on our experiment.

b. PCR clean-up

Purify the entire volume (50 µl) of the first-step PCR product using an AxyPrep™ PCR Cleanup Kit (Axygen, China) according to the manufacturer's instructions to remove unbound degenerate primers.

Note: Unbound long tagged 3'-end unmatched degenerate primers will also bind to templates but not trigger the polymerization and thereby prevent the binding of the single tag primer to the first-step PCR products, so PCR clean-up between the two steps is necessary.

c. Second step of THDP-PCR

i. PCR reaction recipe. Final volume: 50 µl

H_2O	17-19 µl
Tag-barcode (10 µM)	4 µl
PCR clean-up product	2-4 µl

Note: Including the negative-control sample.

Premix *Taq* (see Table 1)	25 µl

ii. Automated thermal cycler program

1) Denaturation at 94 °C for 5 min
2) 35-40 cycles at:
   94 °C for 1 min
   59 °C for 1 min

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72 °C for 1 min

3) 72 °C for 10 min

Note: The used clean-up product volume and cycle numbers in the second step PCR can adjusted to suit different kinds of samples. For some low-abundance samples, try to increase the clean-up product volume to 4 µl and cycle numbers to 40.

3. Pyrosequencing

   Mix together THDP-PCR products from different samples and sequence via Roche_454 pyrosequencing (GS FLX Titanium System) according to the manufacturer's instructions.

Data analysis

1. Trim pyrosequencing reads using the command trim.seqs in mothur with length > 300 and q > 25, and no ambiguous sequences allowed.
2. Translate the trimmed sequences into amino acid sequences using FrameBot tools in Functional Gene Pipeline/Repository (http://fungene.cme.msu.edu/) to remove potential non-specific sequences which cannot be translated into CuMMO-related amino acid sequences.

   Note: The nucleotide sequences which can pass the translation are retrieved using the command get.seqs in mothur from trimmed sequences, based on this step's results. The following analysis steps are based on the retrieved nucleotide sequences, not polypeptides.
3. Identify and remove chimeras using the commands chimera.uchime and remove.seqs in mothur.
4. Split the sequences into different samples by the barcode sequence using the command split.seqs in mothur.
5. Classify the split sequences with BLAST and the lowest common ancestor method in MEGAN using the expanded CuMMO database.

   Note: The original database and parameters used in this step are interpreted by Dumont et al. (2014) in the article and supplementary files. The reference database used in the classification is expanded by Wang et al. (2017) from the original database to suit the broad coverage of the highly degenerate primers, and the detailed information is listed in the article and supplementary files (Wang et al., 2017).

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References

1. Dumont, M. G., Luke, C., Deng, Y. C. and Frenzel, P. (2014). Classification of *pmoA* amplicon pyrosequences using BLAST and the lowest common ancestor method in MEGAN. *Front Microbiol* 5: 34.
2. Ledeker, B. M. and De Long, S. K. (2013). The effect of multiple primer-template mismatches on quantitative PCR accuracy and development of a multi-primer set assay for accurate quantification of *pcrA* gene sequence variants. *J Microbiol Methods* 94(3): 224-231.
3. Tuomivirta, T. T., Yrjala, K. and Fritze, H. (2009). Quantitative PCR of *pmoA* using a novel reverse primer correlates with potential methane oxidation in Finnish fen. *Res Microbiol* 160(10): 751-756.
4. Wang, J. G., Xia, F., Zeleke, J., Zou, B., Rhee, S. K. and Quan, Z. X. (2017). An improved protocol with a highly degenerate primer targeting copper-containing membrane-bound monooxygenase genes for community analysis of methane- and ammonia-oxidizing bacteria. *FEMS Microbiol Ecol* 93(3): fiw244.