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Short communication

Comparison of droplet digital PCR and quantitative real-time PCR in *mcrA*-based methanogen community analysis

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ABSTRACT

Two different quantitative PCR platforms, droplet digital PCR (dd-PCR) and quantitative real-time PCR (qPCR), were compared in a *mcrA*-based methanogen community assay that quantifies ten methanogen sub-groups. Both technologies exhibited similar PCR efficiencies over at least four orders of magnitude and the same lower limits of detection (8 copies μL-DNA extract^{-1}). The *mcrA*-based methanogen communities in three full-scale anaerobic digesters were examined using the two technologies. dd-PCR detected seven groups from the digesters, while qPCR did five groups, indicating that dd-PCR is more sensitive for DNA quantification. Linear regression showed quantitative agreements between both of the technologies (R^2 = 0.59–0.98) in the five groups that were concurrently detected. Principal component analysis from the two datasets consistently indicated a substantial difference in the community composition among the digesters and revealed similar levels of differentiation among the communities. The combined results suggest that dd-PCR is more promising for examining methanogenic archaeal communities in biotechnological processes.

Biological production of methane as a renewable energy has received extensive attention in the field of biotechnology [1]. For instance, anaerobic digestion is a typical biotechnological process for reduction of waste biomass along with production of methane-containing biogas. Methanogens (methane-producing archaea) are strictly anaerobic and slowly growing, and require different growth conditions [2]. Therefore, it is very difficult to scrutinize the methanogens present in these biotechnological processes using culture-dependent techniques. Technical advances in molecular microbial ecology have enabled rapid and complete examination of methanogen communities in anaerobic digestion systems without cultivation [10,14,17]. For instance, Steinberg and Regan [14] developed a methanogen community assay, based on the alpha-subunit of the methyl coenzyme M reductase (*mcrA*) as a phylogenetic marker. The basis of the assay is to quantify ten different groups within the methanogen community using quantitative real-time PCR (qPCR).

The nature of qPCR is to extrapolate the initial concentration of target DNA with an external DNA calibrator [5]. For the *mcrA*-based assay, ten different external DNA calibrators must be prepared, which is an expensive, laborious, and time-consuming process, because they are not readily available [9]. Recently, droplet digital PCR (dd-PCR) has been developed as a new platform for DNA quantification [6]. The most important advantage of dd-PCR over qPCR is to enable the absolute quantification of DNA concentrations without external calibrators [6,13]. In addition, dd-PCR is less susceptible to PCR inhibitors present in the DNA extracts than qPCR [12]. Earlier studies have demonstrated the accuracy and precision of dd-PCR in the quantitative detection of bacteria and viruses in clinical samples [4,7,15]. The primary objective of this study was to compare dd-PCR and qPCR in the *mcrA*-based community assay. Each group was quantified from three full-scale anaerobic digesters using both technologies, and the two community datasets were compared.

Three wastewater treatment facilities are located in Seoul, South Korea. An anaerobic digester was selected from each of the facilities. They are all cylindrical and continuously stirred tank reactors, receiving municipal sewage sludge. They were designated as A (an operational temperature of 38 °C and a HRT of 19 days), B (38 °C and 43 days) and C (52.5 °C and 40 days). Sludge was collected in sterile polyethylene bottles from the recirculation loop of each digester. DNA was extracted using a NucleoSpin Soil kit (Macherey-Nagel GmbH, Düren, Germany) according to the manufacturer's recommendations. DNA was eluted in 100 μL of the elution buffer. There were three replicates per digester.

The *mcrA*-based community assay consists of a single forward/reverse primer set and 10 different hydrolysis probes targeting Methanobacteriaceae *mcrA* (*mbac*), Methanobacteriaceae *mrtA*

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(*mrtA*), Methanocorpusculaceae (*mcp*), Methanospirillaceae (*msp*), *Methanosarcina* (*msar*), Methanosaetaceae (*msa*), uncultured *mcr-7* group (*mcr-7*), uncultured *mcr-2a* group (*mcr-2a*), uncultured *mcr-2b* group (*mcr-2b*), and uncultured Fen cluster (*Fen*) [14]. dd-PCR was performed using a QX100™ droplet digital PCR system (Bio-Rad, Pleasanton, USA) according to the manufacturer's recommendations. The reaction mixture (20 μL) contained 1× dd-PCR master mix (Bio-Rad), 0.9 μM each primer, 1 μM probe and 1 μL template DNA. PCR amplification was carried out on a 2700 GeneAmp® PCR system (Applied Biosystems, Foster, USA). PCR was initiated at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 90 s, and 1 cycle at 98 °C for 10 min. Data were obtained and analyzed using the QX100™ droplet reader (Bio-Rad) and QuantaSoft software (Bio-Rad). The QuantaSoft program generates absolute quantities per microliter-reaction mixture (a total of 20 μL-reaction volume) from given numbers of positive droplets and negative droplets. The obtained values were multiplied by 20 to calculate quantities in microliter-DNA extracts. qPCR was performed using an Applied Biosystems 7300 system as previously described [9]. dd-PCR was used in order to determine the concentrations of the external DNA calibrators with multiple probe sites [9] for qPCR because it accurately provides absolute quantification of target DNA [3,4,6]. The 25-μL reaction mixture contained 1× PCR buffer, 0.2 μL Ace-*Taq* (Genenmed, Seoul, Korea), 0.3 mM dNTPs mix, 0.25 μM each primer, 0.15 μM probe, 1× ROX (Invitrogen, Carlsbad, USA), 1× SYBR green I (Invitrogen) and 1 μL template DNA. PCR was initiated at 95 °C for 3 min, followed by 40 cycles at 95 °C for 15 s and 55 °C for 90 s.

Two artificial DNA templates with multiple probe sites were developed as reference DNA templates for qPCR of the 10 groups [9]. The two artificial sequences (509 bp long) contain the target DNA region (amplified by the primer pair), with additional flanking 20-bp DNA regions at the both ends. Plasmids with the artificial DNA templates were used to construct standard curves. They were serially diluted 10-fold. The two technologies did not detect DNA at $<10^{-8}$ dilution (equivalent to 8 copies μL^{-1} as measured by dd-PCR). The 10 standard curves constructed by qPCR over the 10-fold serial dilution series (10^{-5}–10^{-8}) showed a slope value of

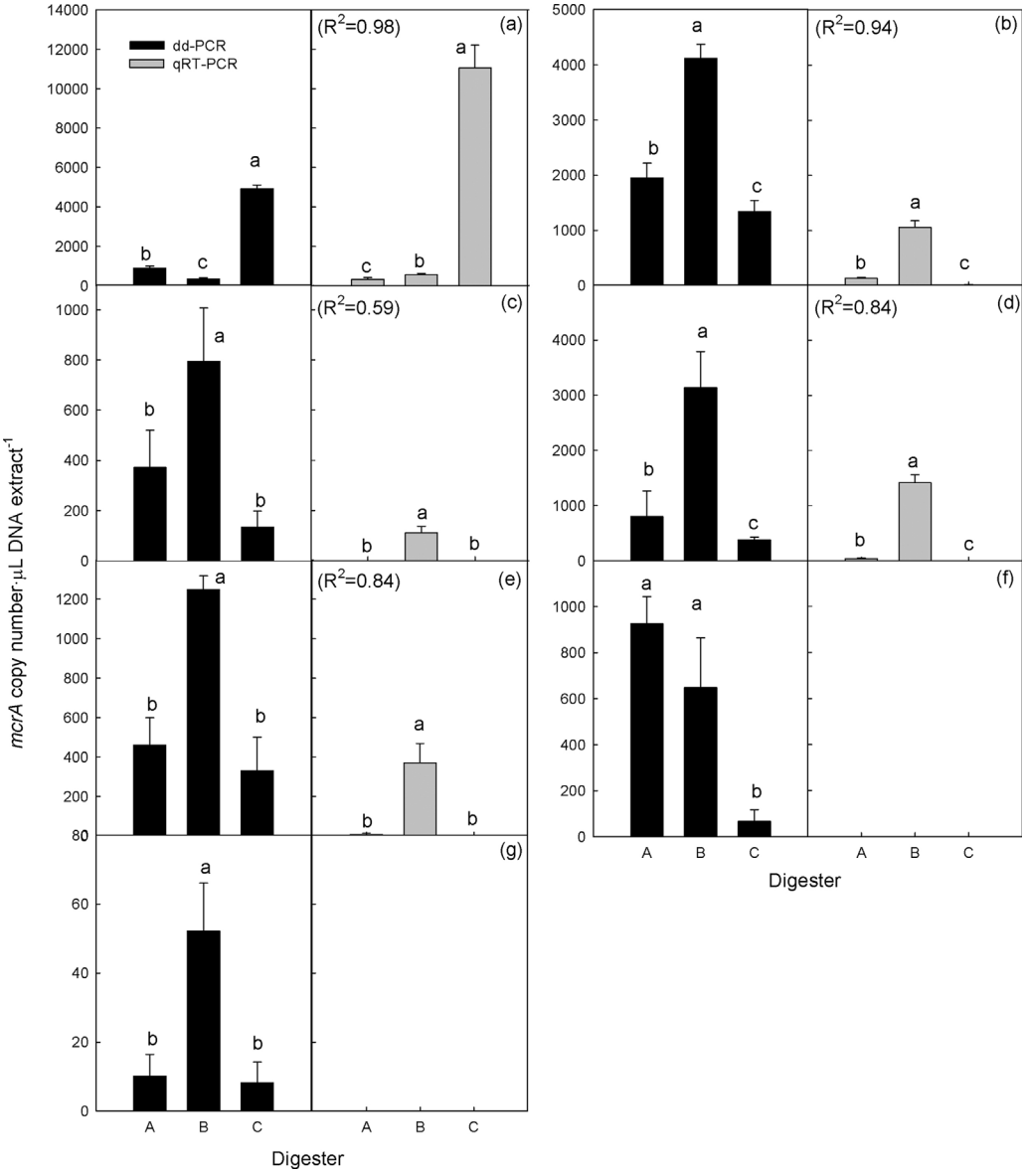

Fig. 1. dd-PCR and qPCR quantification results. (a) *msar*; (b) *msa*; (c) *mcp*; (d) *msp*; (e) *mcr7*; (f) *mcr-2a*; and (g) *mbac*. The three groups (*mrtA*, *mcr-2b*, and *Fen*) were not detected by either of the technologies. Error bars represent ±1 standard deviation of the mean. Different letters (a, b and c) indicate significant difference at $p < 0.05$. Correlation coefficients (R^2) between dd-PCR and qPCR are shown in parentheses.