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A re-evaluation of dilution for eliminating PCR inhibition in soil DNA samples

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ABSTRACT

Quantitative real-time PCR (qPCR) analysis for accurate quantification of targeted microbial genes is compromised by the presence of co-extracted inhibitors from soil samples. Dilution of DNA extracts is a commonly-used method to reduce levels of inhibition. However, the applications of dilution method are mostly empirical, and need to be further elaborated. Here, we propose a dilution model to re-evaluate dilution as a method to eliminate qPCR inhibition. We found that DNA extracts without dilution or with a minor dilution (e.g., 10-fold) resulted in qPCR inhibition for most of studied soils. However, excessive dilution (e.g., 200- or 400-fold) caused an overestimation of the quantified gene copy numbers. Only under a moderate dilution range could qPCR inhibition be efficiently eliminated, which has been well captured by our proposed dilution model. The pre-testing of qPCR inhibition for determining the appropriate dilution range for extracted DNA samples aids accurate quantification of nucleic acids in soils.

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1. Introduction

Quantitative real-time PCR (qPCR) enables quantification of taxonomic or functional abundance for specific organisms or organism groups (in particular for those with functional similarity, e.g., denitrifying bacteria). For environmental samples, such as poultry manure, digester material, wastewater, or soil, when associated with co-extracted qPCR-inhibitors during DNA extraction process, the concerns about assay sensitivity of qPCR have not been adequately addressed (Albers et al., 2013; Lebuhn et al., 2004). Inhibitory components in qPCR reaction cause complete or partial inhibition of the amplification of target DNA fragments. The consequences of this may include decreased detection accuracy (when estimating copy numbers of target DNA fragments) or increased numbers of false negatives (when estimating presence/absence) (McKee et al., 2015; Opel et al., 2010). Even a slight inhibition during qPCR has been demonstrated to cause a substantial shift in quantified gene copy numbers in environmental samples (Bustin et al., 2009).

During DNA extraction of soil samples, inhibitory components such as humic substances are co-extracted along with nucleic acids. To overcome the inhibitory effect on qPCR reaction, strategies including qPCR-reaction modifications and pre-qPCR treatments have been established. Modifications of qPCR-reaction involve alterations of the qPCR recipe, including the addition of performance-enhancing additives, e.g. bovine serum albumin (BSA), and the selective use of high-fidelity thermostable DNA polymerases (Kreader, 1996). Pre-qPCR treatments, such as purification and dilution of DNA extracts have also been adopted for alleviating or eliminating qPCR inhibition (LaMontagne et al., 2002; McKee et al., 2015).

Dilution of DNA extracts can lower the concentration of co-extracted inhibitory components in soils, and thus improve qPCR amplification (Schneider et al., 2009). Certain researchers normally seem to have diluted DNA extracts in a relatively empirical way. For example, 10-fold dilution is commonly used. Other researchers spiked exogenous standards into DNA extracts to estimate the effects of dilution. These exogenous standards are chosen so as not to interfere with any naturally occurring DNA fragments in the samples (Daniell et al., 2012; Lindberg et al., 2007; Moran et al., 2013;

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Schneider et al., 2009). A simple protocol for quantifying inhibitory effects on qPCR involves a serial dilution of the DNA extracts, and then a fixed amount of the exogenous standard is spiked into these diluted DNA extracts individually (Schneider et al., 2009). The aim is to find a proper dilution point where qPCR inhibition could be minimized. In these tests, only inhibitory substances in DNA extracts were assumed to be diluted. However, one concern is that the target DNA fragments and inhibitory components are always simultaneously altered when diluted (Fig. 1, comparing A to C). That is, accompanied by the dilution of co-extracted inhibitory components, the target DNA fragments were also diluted, which increases quantification uncertainties. However, to our knowledge this "co-dilution" issue has never been explored. Moreover, inhibition levels are highly dependent on the concentration of the target gene in the DNA extracts. Stronger inhibition may occur when quantifying genes with low copy numbers compared to those with high copy numbers (Lindberg et al., 2007). For genes with low copy numbers, dilution may further lead to increases in gene susceptibility to inhibitors (Albers et al., 2013; Lindberg et al., 2007; McKee et al., 2015). The combined influence of co-diluted inhibitors and target genes on qPCR inhibition is currently unknown.

In the present study, using the co-dilution scenario, we spiked an artificial target gene into our soil DNA extracts, and then evaluated the inhibitory effect of co-extracted inhibitors on the quantification of this target gene. The soil samples were collected covering a large area of China, and varied with soil type and organic matter content. Therefore, we had the opportunity to evaluate dilution effects on soils that differed widely in organic matter content. The artificial gene was spiked to each soil sample at two distinct concentrations (*i.e.*, 3.64×10^5 and 3.64×10^3 gene copies per qPCR reaction, respectively) in order to find out whether there are big differences in inhibition levels when genes with distinct copy numbers were quantified. After spiking, DNA extracts were diluted stepwise to 400 times. C_t values for target gene along dilution ranges were quantified using qPCR. By comparing these C_t values to controls, we can provide a proper evaluation of the dilution method to explore the following four aspects (Fig. 2): (***i***) to judge whether dilution is necessary and whether it is an efficient approach in reducing or eliminating qPCR inhibition; (***ii***) to find out whether genes with low copy numbers are more susceptible to inhibitors when diluted than with high copy numbers; (***iii***) when dilution is feasible, to determine the appropriate dilution ranges in which qPCR inhibition could be minimized; (***iv***) to test the applicability of this dilution method.

2. Materials and methods

2.1. Soil collection and DNA extraction

The collected soil samples cover a wide range of soil types and have varied water status and organic matter contents according to Web Reference Base (Table S1). Soils belonging to "Histosols" (1#) and "Chernozems" (2#) have the highest organic matter contents (230 and 121 g/kg, respectively). "Vertisols" (3#), "Gleysols" (4#), and "Fluvisols" (5#) are waterlogged soils. "Leptosols" (6#), "Luvisols" (7#) are soils collected in mountain-slope deposits, and "Anthrosols" (8#) are manipulated paddy soils. Other soils, such as "Ferralsols" (9#), "Solonchaks" (10#), and "Arenosols" (11# and 12#) are iron/aluminum-rich or quartz-rich soils, and thus have the lowest organic matter contents (ranging from 19.3 to 4.93 g/kg).

Six cores within A horizon (and O horizon if present) were randomly taken for each soil (Table S1) by using cutting ring samplers (7 cm in diameter and 5 cm in height). These cores were mixed to obtain a homogeneous and representative sample for each soil, and passed through a 2.5-mm sieve to removed large debris. The six aliquots (approx. 0.15–0.30 g of fresh soils) for each soil were stored at −80 °C.

DNA extraction for each aliquot was performed according to the method of Miller et al. (1999). DNA was extracted using SDS-chloroform chemical treatment combined with low-speed bead mill homogenization, and then precipitated by 75% ethanol. After precipitation, DNA extracts were re-suspended in pure water. In this mentioned protocol, only some classic/basic steps were involved. We used pure water rather than TE solution here to avoid any potential inhibition of qPCR by TE. Before extraction, a phosphate-buffered Triton X-100 solution (100 mmol/L Tris, 100 mmol/L Na_4PO_7, 100 mmol/L Na_2EDTA, 1.0% PVP, 100 mmol/L NaCl, 0.05% Triton X-100, pH 10.0) was used to wash soil samples thrice to remove as many humic components as possible. To reduce incomplete cell lysis, we pooled three successive extracts using the protocols suggested by Feinstein et al. (2009), who showed that combination of three supernatants after cell lysis can maximize DNA yields and reduce bias due to the incomplete DNA extraction. For some samples, we further handled them by silica-based purification or using certain commercial kit for DNA extraction.

The concentration of extracted DNA was determined using a spectrophotometer (NanoDrop ND-2000c, Thermo, USA). To calculate the concentration of co-extracted inhibitory components, the absorbance at 230 nm (A_{230}) of the DNA extracts was first measured spectrophotometrically as a proxy for humic-like components (LaMontagne et al., 2002). Thereafter, the A_{230} value for each DNA extract was compared to that of a known-concentration humic acid sodium salt solution (Aldrich, Milwaukee, WI).

2.2. Preparation of the exogenous standard

The exogenous standard used in this study was kindly provided by T. J. Daniell. An artificial DNA fragment was first generated through *in-situ* mutation of an *E. coli* 16S ribosomal gene, in which the three 3′ terminal recognition bases were altered to the requisite complementary bases (Daniell et al., 2012). Such a mutated DNA fragment can be amplified with Mut-F (5′–CCTACGGGAGGCACGTC-3′) and Mut-R (5′-ATTACCGCGGCTGGACC-3′) primers. Thereafter, this DNA fragment was cloned into pGEM-T Easy (Promega, Southampton, UK) and then transformed into *E. coli* DH10B cells. Plasmid from transformants with the correct insert (the mutated DNA fragment) was extracted using AxyPrep™ Plasmid Midiprep Kit (Daniell et al., 2012). After linearization with *NotI* (Promega, Southampton), the linearized mutant plasmid was further purified and then quantified by absorption at 260 nm using NanoDrop ND-2000c.

The initial concentration for this plasmid was 64 ng/μl. After dilution, we spiked it into our DNA extracts as an exogenous standard with a final concentration of 1.11×10^8 gene copies/dry soils (equivalent to 3.64×10^5 gene copies per qPCR reaction) and 1.11×10^6 gene copies/dry soil (equivalent to 3.64×10^3 gene copies per qPCR reaction), respectively. These concentrations (*i.e.*, low and high) were chosen because they are generally within the same order of magnitude as the observed abundance of total microorganism, and as the observed abundance of specific functional groups (e.g., denitrifying bacteria) in soils, respectively (Martins et al., 2011).

To confirm the specificity of Mut-F and Mut-R primer set which was artificially designed to amplify the mutated DNA fragment (which served as the target gene in qPCR reaction), we used this primer set to test each soil sample individually, and found no amplification for any endogenous DNA fragments in extracts. We further confirmed that such a mutated DNA fragment cannot be amplified by the normal primer set used to target the 16S ribosomal gene. Moreover, due to its short length, it would not interfere with

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