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Sensitive detection of sample interference in environmental qPCR

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ABSTRACT

Sample interference in environmental applications of quantitative PCR (qPCR) can prevent accurate estimations of molecular markers in the environment. We developed a spike-and-recovery approach using a mutant strain of *Escherichia coli* that contains a chromosomal insertion of a mutant GFP gene. The method was tested in water samples by separately reducing extraction efficiency or adding humic acids and ethanol, compounds that often contaminate environmental DNA extracts, and analyzing qPCR amplification of the spiked *E. coli* control and human fecal *Bacteroides* markers (HF183 and HF134). This approach, coupled with previously developed kinetic outlier detection (KOD) methods, allowed sensitive detection of PCR inhibition at much lower inhibitor concentrations than alternative approaches using Cq values or amplification efficiencies. Although HF183 was more sensitive to the effects of qPCR inhibitors than the *E. coli* control assay, KOD methods correctly identified inhibition of both control and HF183 assays in samples containing as little as 0.1 ng humic acids per reaction or 5% ethanol. Because sigmoidal modeling methods allow distinction of qPCR inhibition from poor DNA recovery, we were able to simultaneously identify qPCR-inhibited reactions and estimate recovery of nucleic acids in environmental samples using a single control assay. Since qPCR is currently used to estimate important water quality parameters that have serious economic and human health outcomes, these results are timely. While we demonstrate the methods in the context of water quality regulation, they will be useful in all areas of environmental research that use qPCR.

1. Introduction

Both identification of a water body's contaminant sources and assessment of human health risks posed to recreational users are shifting to a reliance on quantitative PCR (qPCR). The tool has received increased use because of its ability to rapidly quantify bacterial and viral markers from the environment, identify contaminant sources (Newton et al., 2011; Peed et al., 2011; Sauer et al., 2011), and estimate the human health risk associated with contaminated water bodies (Wade et al., 2006, 2008, 2010). Thus, proper management to reduce future contamination events and minimize human exposure to dangerous pathogens is becoming increasingly dependent on the precision and accuracy of qPCR. In October 2012, the US Environmental Protection Agency will issue revised water quality criteria including recommendations on the use of molecular methods, PCR and qPCR, to estimate water quality (http://water.epa.gov/scitech/swguidance/standards/criteria/

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health/recreation/update.cfm). Given the adoption of molecular methods into management strategies, a high degree of accuracy and precision is needed to maintain their efficacy and credibility. Specifically, in order to set criteria that will be used for a variety of sample types, a method of comparing samples is needed.

The complexity and composition of environmental samples can challenge accurate quantification by qPCR. Suspended organics and sediment can interfere with DNA extraction and reduce recovery of target molecular markers. Complex biomolecules, such as humic acid, can inhibit polymerase activity (Tsai and Olson, 1992; Schriewer et al., 2011) and sequester DNA templates from the amplification reaction (Baar et al., 2011). Furthermore, reagents used in DNA purification protocols, such as ethanol, may not be removed completely and can also inhibit amplification.

Several approaches have attempted to alleviate this "sample interference" in marker quantification. Nucleic acid extraction methods that increase DNA yield while removing most qPCR inhibitors have been developed specifically for troublesome water samples (e.g., MoBio PowerWater DNA Extraction Kit). Likewise, the incorporation of mutant *Taq* polymerase (Baar et al., 2011) and the use of additives (Kreader, 1996; Braid et al., 2003; Abolmaaty et al., 2007; Schriewer et al., 2011) have helped mitigate the effects of qPCR inhibition. Despite these developments, sample interference is found in many environmental sample sets (e.g., Boehm et al., 2009; Peed et al., 2011) and methods for its detection are still needed to prevent serious misinterpretation of results.

Currently, there is a wide range of methods to manage sample interference in qPCR. A surrogate approach consists of spiking a known quantity of targets (e.g., cells, genomes, or plasmids), which usually comprise two separate controls, one to estimate extraction recovery and another to test for the presence of inhibitors (Peed et al., 2011). Controls for inhibition can be quite complex, and the methods can be difficult or expensive to reproduce (Pontiroli et al., 2011). Nevertheless, even the most complicated surrogates may not reflect recovery or inhibition of target markers due to differences in cell lysis efficiency or qPCR amplification kinetics (Hugget et al., 2008). Another approach is to dilute the DNA extract before qPCR amplification to the point where inhibitors are too dilute to affect amplification (Cook and Britt, 2007; Rajal et al., 2007; Schriewer et al., 2010). While this approach bypasses assumptions about differential effects of inhibition between assays, it raises the costs of analysis, and can impair the limits of detection (Cook and Britt, 2007). Clearly, alternative methods that are sensitive to the effects of sample interference are needed.

Kinetic outlier detection (KOD) methods that have been used over a wide range of studies to detect inefficient qPCR amplification (reviewed in Bar et al., 2011) may hold promise for environmental qPCR studies. In particular, the amplification compatibility test as proposed by Tichopad and colleagues has been shown to be extremely sensitive to qPCR inhibition by tannins (Tichopad et al., 2010). Instead of deriving a single value, $Cq_{threshold}$ (the fractional cycle number required to reach the quantification threshold) from an amplification curve, the curve is fit to a sigmoidal model, allowing the progression of a qPCR amplification reaction to be described in detail. Derivation of outlier statistics for parameters from each amplification curve allows the detection of inhibited amplification reactions. While these data analysis methods have been shown to be highly sensitive to qPCR inhibitors, they have not been incorporated in absolute quantification methods, nor have they been applied to complex and often challenging environmental samples.

In order to apply these new methods to environmental qPCR, we first developed an internal control that could be used as a surrogate to allow sensitive detection of reduced DNA recovery and qPCR inhibition. Second, we tested amplification compatibility methods in the context of environmental sample analysis. Due to their extreme sensitivity, the methods potentially bypass barriers imposed by differential effects of inhibitors. We used two variations of a KOD method. One could be used with a simple inhibition control to assess the effects of inhibition alone, as previously described (Tichopad et al., 2010). The other could be used with a full process control to assess both inhibition and extraction with a single qPCR assay. To validate control sensitivity, we separately reduced extraction efficiency or added a PCR inhibitor (humic acids or ethanol). Finally, to ensure that conclusions about sample interference drawn from control experiments also apply to assays of interest, we compared the effects of sample interference on control and *Bacteroides* marker recovery and amplification.

2. Methods

2.1. Cell counting

Escherichia coli strain AF504 *gfp* (AF504 attB::bla-rrnBP1::gfpmut3b*; Folkesson et al., 2008) was chosen as a control not only because it grows rapidly and can be quantified with routine methods, but also because it carries a single-copy mutant *gfp* insertion that is not typically found in the environment and can be targeted by qPCR. The *E. coli* control strain was grown in 180 μL LB broth with 100 μg/ml ampicillin and 20 μL mineral oil in 96-well plates (Greiner Bio-One, Monroe, North Carolina, #655098). Optical density readings (595 nm) and aliquots for flow cytometry were taken at roughly 30 min intervals. Cell aliquots were immediately diluted 1:2 in filter-sterilized fixation buffer (0.37% formaldehyde in phosphate buffered saline), vortexed, and stored overnight at room temperature. Cell aliquots were counted using a Guava EasyCyte® flow cytometer (Millipore, Billerica, MA, USA). Aliquots of 10^4 cells/μL were stored in filter-sterilized 15% glycerol solution (pH 7.5) at −20 °C for future use.

2.2. qPCR

We developed a qPCR assay for the *E. coli* control (AF504 *gfp*) using previously published SYBR Green (Invitrogen, Carlsbad, CA) dye chemistry and cycling conditions (Green et al., 2011). Primers used in the AF504 *gfp* qPCR assay (mut3F-5′-CGG TTA TGG TGT TCA ATG CTT TGC GAG ATA CCC, mut3R-5′-ATG GCA CTC TTG AAA AAG TCA TGC CGT TTC) targeted the mutated region of *gfp* mut3, with an annealing temperature of