Applied and Environmental Microbiology, Nov. 2009, p. 7253–7260 Vol. 75, No. 22
0099-2240/09/$12.00 doi:10.1128/AEM.00796-09

Accurate Quantification of Microorganisms in PCR-Inhibiting Environmental DNA Extracts by a Novel Internal Amplification Control Approach Using Biotrove OpenArrays▽†‡

R. van Doorn,[1,2] M. M. Klerks,[1,3]* M. P. E. van Gent-Pelzer,[1] A. G. C. L. Speksnijder,[4] G. A. Kowalchuk,[2,5] and C. D. Schoen[1]

Plant Research International B.V., Droevendaalsesteeg 1, 6708 PB Wageningen, The Netherlands[1]; NIOO-Centre for Terrestrial Ecology, P.O. Box 40, 6666 ZG Heteren, The Netherlands[2]; Innosieve Diagnostics B.V., Celsiuslaan 5, 5251 ZB Vlijmen, The Netherlands[3]; Public Health Laboratory, Cluster Infectious Diseases, Health Service Amsterdam, Nieuwe Achtergracht 100, 1018 WT Amsterdam, The Netherlands[4]; and Free University of Amsterdam, Institute of Ecological Science, de Boelelaan 1085, 1081 HV Amsterdam, The Netherlands[5]

Received 8 April 2009/Accepted 22 September 2009

PCR-based detection assays are prone to inhibition by substances present in environmental samples, thereby potentially leading to inaccurate target quantification or false-negative results. Internal amplification controls (IACs) have been developed to help alleviate this problem but are generally applied in a single concentration, thereby yielding less-than-optimal results across the wide range of microbial gene target concentrations possible in environmental samples (J. Hoorfar, B. Malorny, A. Abdulmawjood, N. Cook, M. Wagner, and P. Fach, J. Clin. Microbiol. 42:1863–1868, 2004). Increasing the number of IACs for each quantitative PCR (qPCR) sample individually, however, typically reduces sensitivity and, more importantly, the reliability of quantification. Fortunately, current advances in high-throughput qPCR platforms offer the possibility of multiple reactions for a single sample simultaneously, thereby allowing the implementation of more than one IAC concentration per sample. Here, we describe the development of a novel IAC approach that is specifically designed for the state-of-the-art Biotrove OpenArray platform. Different IAC targets were applied at a range of concentrations, yielding a calibration IAC curve for each individual DNA sample. The developed IACs were optimized, tested, and validated by using more than 5,000 unique qPCR amplifications, allowing accurate quantification of microorganisms when applied to soil DNA extracts containing various levels of PCR-inhibiting compounds. To our knowledge, this is the first study using a suite of IACs at different target concentrations to monitor PCR inhibition across a wide target range, thereby allowing reliable and accurate quantification of microorganisms in PCR-inhibiting DNA extracts. The developed IAC is ideally suited for high-throughput screenings of, for example, ecological and agricultural samples on next-generation qPCR platforms.

Real-time PCR-based nucleic acid amplification is currently the most commonly used strategy for the quantification of microorganisms and specific gene expression in environmental samples. Such PCR-based nucleic acid amplification is sensitive, accurate, and relatively fast and allows the detection, cultivation-independent identification, and quantification of microorganisms.

Despite the advantages of PCR-based assays, one major drawback is potential inhibition of the amplification reaction by compounds that are often coextracted with nucleic acids from the sample matrix (9, 14, 26, 30). Therefore, much research has been directed toward the development of optimized DNA extraction protocols for difficult environmental samples (1, 2, 8, 18, 21, 23, 24, 27, 31, 32). Nevertheless, coextraction of PCR-inhibiting compounds often cannot be completely prevented, thereby potentially leading to false-negative results (4, 5, 7, 13). Moreover, the occurrence of partial PCR inhibition can lead to inaccurate target quantification, thereby underestimating the true number of assayed targets present in the sample (26).

A straightforward approach to detect PCR inhibition is the inclusion of an internal amplification control (IAC) (6, 7, 16, 28). An IAC is a nontarget DNA sequence that is coamplified with the target under the same reaction conditions and in the same reaction tube. Most currently used IACs can be divided into two distinct groups: competitive and noncompetitive IACs (7). In competitive IACs, the target and IAC are amplified with the same primer set. In noncompetitive IACs, both the target and IAC are amplified with different primer sets (7). In such strategies, however, competition between the IAC and the target DNA for primers (competitive IAC), nucleotides, and polymerase enzymes (competitive and noncompetitive IAC) can occur (7, 11, 12). Although the absence or presence of a target (qualitative detection) can usually be determined unambiguously after proper optimization via both IAC strategies, the competition for reaction components makes accurate quantification problematic. Therefore, to allow accurate adjustments to quantitative data in the case of partial PCR inhi-

* Corresponding author. Mailing address: Innosieve Diagnostics B.V., Celsiuslaan 5, 5251 ZB Vlijmen, The Netherlands. Phone: 31-646717500. Fax: 31-317418094. E-mail: info@innosieve.com.

† Supplemental material for this article may be found at http://aem.asm.org/.

‡ Publication 4574 of The Netherlands Institute of Ecology (NIOO-KNAW).

▽ Published ahead of print on 2 October 2009.

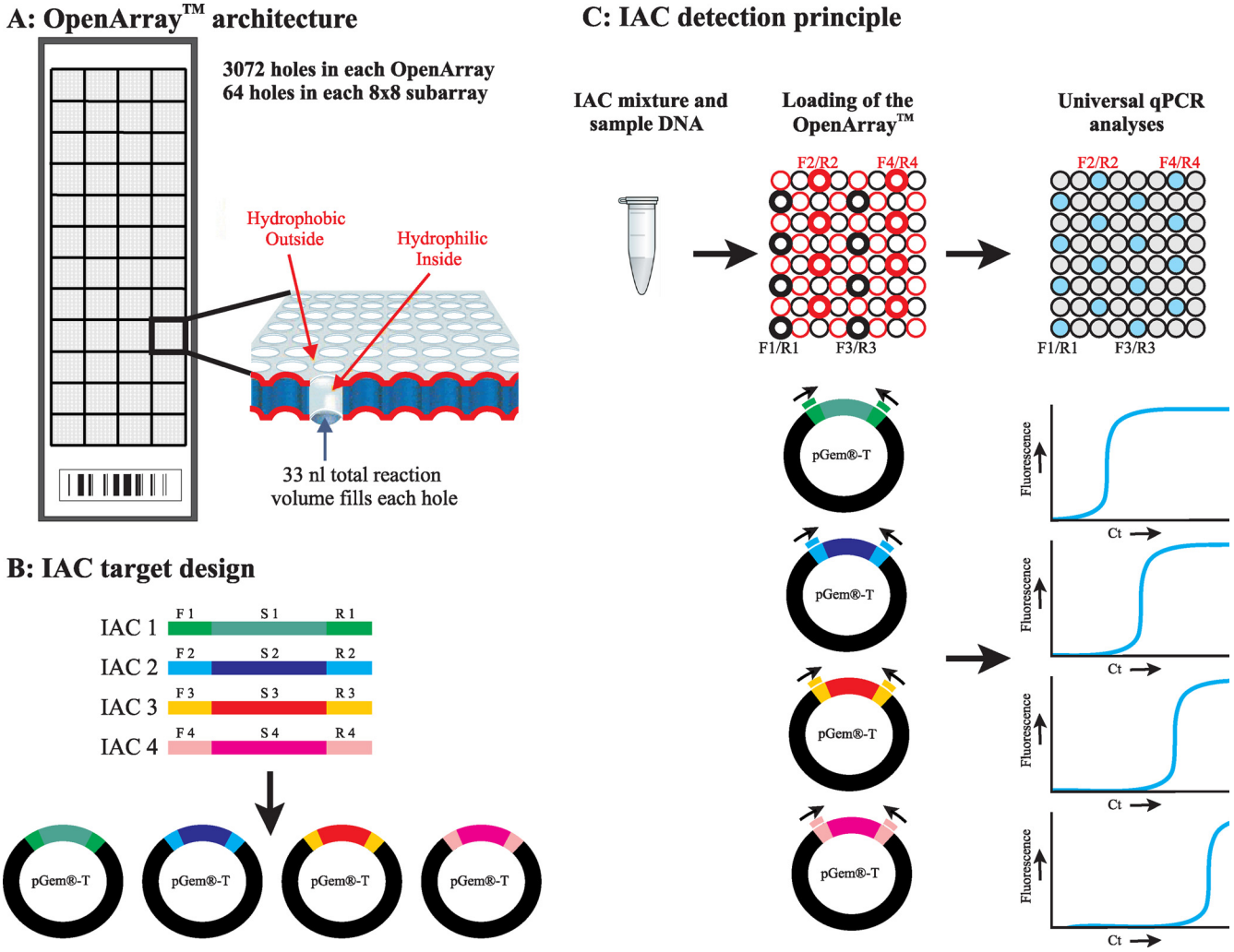

FIG. 1. Schematic overview of the internal amplification control (IAC) on the Biotrove OpenArray system. (A) OpenArray architecture. The OpenArray has 48 subarrays, each containing 64 microscopic 33-nl through holes. The primers are preloaded into the holes. The sample combined with the reaction mixture is autoloaded by the surface tension of the hydrophilically coated holes and the hydrophobic surface of the OpenArray. (B) IAC target design. Each IAC target consists of a 60-nucleotide-long spacer DNA fragment (S1, S2, S3, S4) flanked by IAC-unique primer sequences (F1/R1, F2/R2, F3/R3, F4/R4). The sequence order of the spacer DNA fragments is randomized for each IAC, but all of the IACs are equal in nucleotide composition. The IAC-unique primer pairs ensure IAC-specific amplification in a real-time PCR. IAC targets were cloned into pGem-T vectors. (C) IAC detection principle. A mixture containing a range of concentrations of the four IAC targets, the DNA sample, and real-time PCR reagents is loaded onto a subarray. The IAC targets are independently amplified with the IAC-unique primers which are spotted into selected through holes. Amplification is monitored with SYBR green dye, and potential PCR inhibition is assessed based on the C_T numbers of the IAC target mixture.

bition, separate reactions for each target and (noncompetitive) IAC should be performed. A drawback of this approach is the resulting dramatic increase in the number of reactions that have to be performed, which is of particular concern when large-scale screening of samples is required or in cases where only a small amount of template DNA is available.

A solution to this problem was offered by the recent development of next-generation quantitative PCR (qPCR) platforms like the Biotrove OpenArray system. This novel qPCR platform provides high-density and low-volume qPCR microarrays that are capable of accommodating 3,072 reactions per array (OpenArray; BioTrove Inc., Woburn, MA) (15, 25, 29). The OpenArray contains 48 subarrays, each consisting of 64 microscopic through holes with a volume of 33 nl (Fig. 1A) into which primer pairs are preloaded as specified by the user. Depending on the assay layout, a single OpenArray allows parallel testing of up to 144 samples against a maximum of 3,072 targets.

To date, most IACs have been applied in a single concentration. It has, however, been shown that IACs used at high concentrations may fail to detect weak PCR inhibition and that inhibition of target amplification may be target concentration dependent (7, 22). Here, we hypothesized that accurate quantification by real-time PCR requires an IAC with a wider concentration range. Fortunately, new-generation qPCR platforms facilitated the development of such a new type of amplification control without increased labor or cost.

In this report, we describe a newly developed IAC approach

TABLE 1. Performance and reproducibility of different IAC template strategies for qPCR

Target	C_T (SD)[a]		
	Single-stranded PCR product	Double-stranded PCR product	Plasmid
IAC1	21.10 (2.81)	19.13 (0.33)	17.85 (0.11)
IAC2	20.60 (0.39)	18.68 (0.42)	17.49 (0.06)
IAC3	21.00 (1.47)	18.07 (0.08)	17.84 (0.23)
IAC4	20.45 (0.85)	17.32 (0.05)	18.79 (0.11)

[a] Data represent averages of three qPCR replicates.

TABLE 2. Independence of IAC template amplification in PCR[a]

IAC composition	C_T (SD)			
	IAC1[b]	IAC2[c]	IAC3[d]	IAC4[e]
Single target	14.53† (0.06)	17.90‡ (0.09)	20.51¶ (0.22)	24.82§ (1.31)
Target mixture	14.58† (0.11)	17.91‡ (0.15)	20.65¶ (0.20)	24.86§ (1.12)

[a] The indicated IAC concentrations (IAC target/μl PCR master mix) were amplified on the Biotrove OpenArray platform. Different symbols indicate statistically significant differences between C_T values. Student's t test ($P < 0.05$, $n = 20$).
[b] Concentration, 1,670 fg/μl.
[c] Concentration, 167 fg/μl.
[d] Concentration, 16.7 fg/μl.
[e] Concentration, 1.67 fg/μl.

in which different IAC targets are applied at a range of concentrations, thereby providing a calibration IAC curve that enables more accurate target quantification. Primers for the different IACs are spotted along with target-specific primer pairs in separate through holes per subarray, while the IAC target mixture is spiked into the environmental DNA extracts (Fig. 1B and C). The DNA-IAC mixture is then loaded onto the OpenArray subarray, and all targets are amplified and monitored individually in real time (Fig. 1C).

We describe the development, testing, and application of a novel IAC approach for high-throughput screening of environmental samples on next-generation qPCR platforms. Soil DNA extracts varying in their degrees of PCR inhibition were used to demonstrate that this IAC strategy can accurately compensate for partial PCR inhibition during the detection of microbial targets in complex environmental samples. The benefits of our novel IAC approach are discussed with respect its application to various complex matrices where accurate quantification of targets is desired.

MATERIALS AND METHODS

IAC oligonucleotide design. Four single-stranded internal amplification control (IAC) oligonucleotides were designed. Each IAC oligonucleotide consisted of a 60-nucleotide-long DNA fragment based on a part of the DNA sequence of the green fluorescent protein-encoding gene (20) flanked by 20-nucleotide-long IAC-specific primer sequences (see Appendix S1 in the supplemental material). The sequence order of the 60-nucleotide-long fragment was randomized for each IAC, thereby preventing IAC cross-hybridization during qPCR while guaranteeing equal nucleotide compositions for all four IAC targets. The IAC-specific primer pairs have equal melting temperatures (T_ms) to allow universal SYBR green-based detection by real-time PCR. The primer pairs were chosen from the GeneFlex TagArray set (Affymetrix Inc., Santa Clara, CA) so as to minimize IAC secondary structures and optimize primer T_m, specificity, and amplification efficiency. Potential for secondary structures, primer T_m, and primer specificity were examined with the Visual OMP 6.0 software (DNA Software Inc.). The prediction parameters were set to match PCR conditions ([monovalent ions] = 0.075 M, $[Mg^{2+}]$ = 0.005 M, T = 60°C). When necessary, IAC sequences were adjusted slightly to avoid secondary structures that might interfere with efficient qPCR.

The IAC oligonucleotides and primers used (see Appendix S1 in the supplemental material) were synthesized by Eurogentec SA (Seraing, Belgium).

IAC plasmid generation. A double-stranded PCR amplicon of each IAC oligonucleotide target was generated with the IAC-specific primer pairs by PCR. For each PCR, an initial 2-min incubation at 95°C was followed by 35 cycles consisting of 30 s at 95°C, 30 s at 60°C, and 72°C for 60 s. After the final cycle, samples were incubated at 72°C for 10 min and cooled to 4°C afterwards. PCR products were run on a 2% (wt/vol) agarose gel and visualized after electrophoresis by staining with ethidium bromide, followed by UV transillumination. Each IAC amplicon yielded a 100-bp product. The IAC amplicons were excised from the agarose gel, purified with the QIAquick gel extraction kit (QIAgen GmbH, Hilden, Germany), and cloned into the pGEM-T easy vector (QIAgen); this was followed by transformation into competent *Escherichia coli* cells by a heat shock protocol according to the manufacturer's instructions. Plasmid DNA was isolated from positive transformants with the QIA-prep spin minikit (QIAgen), and the presence of the correct inserts was confirmed by sequencing. Finally, the amount of plasmid DNA was quantified by comparison to a standardized DNA marker after gel electrophoresis and used as an IAC.

Testing of IAC strategies by real-time PCR. An IAC mixture composed of single-stranded IAC oligonucleotides, purified double-stranded IAC PCR amplicons, or plasmids containing the IAC inserts was tested for amplification reproducibility. Testing of these IAC target strategies (Table 1) was performed with a 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA). The target inputs were 10 zmol, 50 fg, and 1 pg for the IAC oligonucleotide, PCR amplicon, and plasmid targets, respectively. SYBR green-based qPCR was performed with 1× SYBR green PCR Master Mix (Applied Biosystems), 10 ng sonicated salmon sperm DNA, the IAC template, and 300 nM of each primer. The reaction mixtures were initially incubated at 50°C for 2 min, followed by 10 min of denaturation at 95°C and 40 cycles of 15 s at 95°C and 1 min at 60°C.

Biotrove OpenArray real-time PCR. Amplification of the IAC mixture and environmental samples was followed in real time with an OpenArray NT Cycler (BioTrove Inc., Woburn, MA). Samples were loaded into OpenArray plates with the OpenArray NT Autoloader according to the manufacturer's protocols. Each subarray was loaded with 5.0 μl of master mix containing IAC targets (amounts depending on the experiment) and reagents in a mixture (final concentrations) of 1× LightCycler FastStart DNA Master SYBR green I mix (Roche Diagnostics GmbH, Mannheim, Germany), 0.2% Pluronic F-68 (Gibco, Carlsbad, CA), 1:4,000 SYBR green I (Sigma-Aldrich, St. Louis, MO), 0.5% (vol/vol) glycerol (Sigma-Aldrich), and 8% (vol/vol) deionized formamide (Sigma-Aldrich). The final assay concentration for all of the primer pairs was 300 nM.

The PCR OpenArray thermal cycling protocol consisted of 90°C for 10 min, followed by cycles of 28 s at 95°C, 1 min at 55°C, and 70 s at 72°C (imaging step). The maximum number of PCR cycles was set to 33. Due to the smaller reaction volume in the OpenArray plates, additives in the PCR master mix and different surface properties, the annealing temperature of the Biotrove OpenArray system had to be adjusted to mimic the PCR conditions used in the 7500 Real-Time PCR system. Simulation of Biotrove PCR conditions in Visual OMP 6.0 software (DNA Software Inc.) indicated that an annealing temperature of 55°C in the Biotrove OpenArray corresponded to a 60°C annealing temperature in the 7500 Real-Time PCR system.

The Biotrove OpenArray NT Cycler System software (version 1.0.2) uses a proprietary calling algorithm that estimates the quality of each individual threshold cycle (C_T) value by calculating a C_T confidence value for the amplification reaction. In our assay, C_T values with C_T confidence values below 800 (average C_T confidence of the nontarget amplification reactions plus 3 standard deviations) were considered background signals. Higher C_T confidence levels were considered positive and were analyzed further for amplicon specificity by studying the individual melting curves.

Independence of IAC amplifications. Because the IAC template mixture is spiked in the PCR master mix, all IAC templates are present in each of the 33-nl through holes. To evaluate the specificity of the developed IAC templates, each IAC template was PCR amplified with its corresponding primer pair as a single template and as part of the IAC mixture. Reaction mixtures containing a single IAC target (IAC1, 1.67 pg/μl PCR mixture; IAC2 167 fg/μl PCR mixture; IAC3, 16.7 fg/μl PCR mixture; IAC4, 1.67 fg/μl PCR mixture) and reaction mixtures containing all four IAC targets were PCR amplified and analyzed on the Biotrove OpenArray platform as described above. Sonicated salmon sperm DNA was added as background DNA to each PCR mixture at a final concentration of 2 ng/μl PCR mixture. The five different IAC combinations were analyzed by using 20 qPCR replicates for each data point (Table 2). Statistical data analysis was performed with the Student t test ($P < 0.05$, $n = 20$).

IAC calibration curve construction. IAC calibration curves were constructed with the Biotrove OpenArray system. Each IAC template was added to the

TABLE 3. Characteristics of the tested fields

Field	Soil type	Crop(s)	Lutum (%)	pH KCl	Organic matter (%)
1	Marine clay	Beetroot	12.3	7.4	1.9
2	Marine clay	Grass-clover	24.3	7.4	2.7
3	Marine clay	Parsnip, pumpkin	16.3	7.0	9.4
4	Marine clay	Grass-clover	15.5	7.4	4.0
5	Sandy soil	Pumpkin	2.5	4.9	1.7
6	Sandy soil	Wheat	1.5	5.0	2.6

qPCR mixture at a different concentration, resembling a 10-fold dilution series (IAC1, 1.67 pg/μl PCR mixture; IAC2, 167 fg/μl PCR mixture; IAC3, 16.7 fg/μl PCR mixture; IAC4, 1.67 fg/μl PCR mixture). The four IAC targets were analyzed by using 60 qPCR replicates for each data point of the IAC calibration curve. Finally, regression analysis was performed on the positive C_T values per IAC target.

Effect of PCR inhibition on IAC calibration curve performance. To assess the effect of PCR inhibition on IAC regression curve performance in the Biotrove OpenArray system, two typical PCR inhibitors related to soil sample DNA extracts were tested. Ethanol (used for the final washing step in many DNA isolation protocols) and humic acids (the major PCR-inhibiting compound in soil DNA extracts) were added to the PCR mixtures at different concentrations. IAC-PCR mixtures (see above) with final concentrations of 5%, 3%, 1.5%, 1%, 0.5%, and 0.1% ethanol or 13 ng/μl, 10 ng/μl, 7 ng/μl, 4 ng/μl, and 1 ng/μl humic acids (Sigma-Aldrich) were PCR amplified and analyzed on the Biotrove OpenArray platform as described above. Sonicated salmon sperm DNA was added as background DNA to each PCR mixture at a final concentration of 2 ng/μl PCR mixture. Each IAC target was PCR amplified with its corresponding primer pair by using 60 repetitions for each reaction condition in the Biotrove OpenArray platform. Finally, regression analyses were performed on the positive IAC C_T values per sample.

Soil sample analyses. Six arable fields having different biotic and abiotic properties (Table 3) were sampled in August 2005 (19). DNA was extracted from 0.6 g (wet weight) of each soil sample with the Mobio Ultra Clean soil DNA isolation kit (BIOzymTC, Landgraaf, The Netherlands). An additional 50 mg of glass beads (<106 μm) was added to each microtube. Cells were lysed by bead beating twice for 30 s each time in a cell disrupter (Hybaid Ribolyser; Thermo Fisher Scientific, Waltham, MA). After the bead-beating step, DNA was extracted according to the manufacturer's instructions. Next, DNA concentrations were estimated by comparison to a standardized DNA marker after gel electrophoresis.

Next, IAC PCR mixtures (see above) were supplemented with 1 μl of undiluted, 20-fold-diluted, or 40-fold-diluted environmental soil sample DNA. Subsequently, the mixtures were analyzed on the Biotrove OpenArray platform for the density of 16S rRNA genes belonging to several bacterial groups (3) (Table 4) by using 60 PCR replicates for each data point. Regression analysis was performed by using all of the positive IAC C_T values per sample. For each analyzed soil sample, the intercept and slope of the IAC sample regression formula (sample IAC) were analyzed for statistically significant differences with the IAC calibration regression formula (calibration IAC) by linear regression analysis with GraphPad prism 5.0 (www.graphpad.com/demos). When a statistically significant difference was observed between the sample IAC and the calibration IAC regression line (see IAC calibration curve construction), the C_T values resulting from the analyzed targets were corrected for partial qPCR inhibition by using both the sample IAC and calibration IAC regression formulas. Using the C_T value from the analyzed target as the $y_{\text{observed } CT \text{ value sample}}$ value in the sample IAC regression formula, the calculated x_{sample} value was used in the calibration IAC regression formula, finally resulting in a $y_{\text{corrected } CT \text{ value sample}}$ value, representing the C_T value of the analyzed target corrected for qPCR inhibition. The sample IAC regression formula is $Y_{\text{observed } CT \text{ value sample}} = a_{\text{slope IAC sample}} x_{\text{sample}} + b_{\text{intercept IAC sample}}$. The calibration IAC regression formula is $Y_{\text{corrected } CT \text{ value sample}} = a_{\text{slope IAC calibration}} x_{\text{sample}} + b_{\text{intercept IAC calibration}}$.

RESULTS

Testing of IAC strategies by real-time PCR. Accurate calculation of target quantities requires highly reproducible PCR amplification of all of the four IAC templates. To obtain the most reliable IAC template amplification, three different IAC template strategies were tested. As shown in Table 1, the highest variation in C_T values was observed when single-stranded IAC oligonucleotides were used as PCR templates, while PCR products and plasmids showed equally low variation in C_T values for the applied IAC concentrations. This indicates that the latter two template strategies are most suitable for reliable IAC design. The advantages of cloned IAC DNA over a purified PCR product, however, are that it allows simple storage within bacterial cells and it is more stable due to its circularity, guaranteeing the continuous availability and quality of the IAC targets (7). The plasmid strategy was therefore adopted for IAC development in all subsequent experiments.

Independence of IAC amplification. To allow accurate quantification, the presence of multiple IAC templates should not influence the PCR amplification, compared to the amplification of each single IAC template separately. Therefore, the specificity of the developed IAC templates was evaluated by testing each IAC template in a PCR with its corresponding primer pair as a single template and as part of the IAC mixture. No statistically significant differences ($P > 0.05$, t test) were observed between the C_T values of the IAC targets when amplified individually and those obtained with the IAC mixture (Table 2). Thus, the presence of multiple IAC templates had no significant influence on the PCR amplification and detection of each individual IAC template, indicating that quantification accuracy should not be compromised by aspecific amplification or interactions during IAC amplification.

IAC calibration curve. Because the IAC template amplifications are truly independent, a calibration IAC curve and a corresponding regression formula could be constructed. As shown in Fig. 2, the correlation between the IAC template concentrations and the observed C_T values is very high ($R^2 = 0.996$). Additionally, the IAC calibration curve was highly reproducible, with standard deviations ranging from 0.12 C_T values at high IAC template concentrations to 0.86 C_T values at low IAC template concentrations close to the detection threshold (Fig. 2).

Effect of PCR inhibition on IAC calibration curve performance. To assess the effect of PCR inhibition on the IAC regression curve performance in the Biotrove OpenArray system, two PCR-inhibiting compounds typically obtained after soil DNA extraction (ethanol and humic acids) were added to the PCR mixture. None of the tested ethanol concentrations (up to a 5% final concentration) had an effect on the IAC regression curve in the Biotrove OpenArray system (data not shown), implying that there was no PCR inhibition. The addition of humic acids, however, resulted in a linear shift of the IAC regression curves (Fig. 3A). In the case of partial PCR inhibition, no difference in C_T value shift was observed between IAC templates at low and high concentrations. Interestingly, at high humic acid concentrations, the IAC templates at lower concentrations appeared to be more sensitive to full PCR inhibition than the IAC templates present at high concentrations (Fig. 3B), indicating that the occurrence of full PCR inhibition in the Biotrove OpenArray system may be target concentration dependent.

Quantification of bacterial groups in environmental soil samples with the IAC calibration curve. The reliability and

TABLE 4. IAC-based adjustments to quantitative data on environmental samples[a]

Field and DNA dilution	IAC sample curve	R^2 value	C_T value[b]							
			Observed				Corrected			
			All bacteria	Alphaproteobacteria	Betaproteobacteria	Actinobacteria	All bacteria	Alphaproteobacteria	Betaproteobacteria	Actinobacteria
1										
0	$y = -3.57x + 29.71$[c]	0.999	14.99 (0.15)	18.25 (0.10)	19.69 (0.13)	17.38 (0.15)	14.10	17.10	18.43	16.30
20×	$y = -3.32x + 28.04$	0.998					13.99 (0.17)	17.45 (0.47)	18.54 (0.44)	16.33 (0.45)
2										
0	$y = -3.46x + 29.44$[c]	0.996	14.98 (0.05)	18.38 (0.12)	19.52 (0.15)	16.84 (0.15)	13.91	17.15	18.23	15.68
20×	$y = -3.23x + 27.66$	0.996					13.79 (0.14)	17.21 (0.40)	18.32 (0.68)	15.53 (0.32)
3										
0	$y = -3.34x + 29.31$[c]	0.987	13.87 (0.06)	17.53 (0.15)	18.93 (0.16)	15.99 (0.17)	12.49	16.08	17.46	14.57
20×	$y = -3.38x + 28.05$	0.991					12.44 (0.10)	15.97 (0.30)	16.79 (0.36)	14.55 (0.19)
4										
0	$y = -3.49x + 29.63$[c]	0.993	14.10 (0.08)	17.67 (0.06)	19.51 (0.16)	16.12 (0.11)	13.03	16.39	17.66	14.93
20×	$y = -3.19x + 27.48$	0.998					12.96 (0.16)	16.16 (0.29)	18.12 (0.32)	14.68 (0.26)
5										
0	$y = -3.53x + 28.48$	0.997	19.17 (0.58)	20.77 (0.73)	21.80 (0.54)	19.41 (0.81)	19.17	20.77	21.80	19.41
NT[d]										
6										
0	$y = -3.75x + 29.76$[c]	0.998	17.15 (0.22)	20.69 (0.56)	26.27 (1.27)	26.62 (1.99)	16.61	19.71	24.60	24.90
40×	$y = -3.86x + 29.99$[c]	0.998	17.20	20.72	ND[e]	ND	16.71	19.70	ND	ND

[a] Data represent averages of 30 PCR replicates.
[b] Observed C_T values for 20- and 40-fold-diluted samples were corrected for the dilution factor by subtracting 4.47 and 5.47 C_T values, respectively. C_T values for the undiluted samples were corrected for the dilution factor only after correction for PCR inhibition.
[c] IAC sample curves are significantly different from IAC calibration curves (linear regression analysis). If no statistically significant difference was found, the C_T values of the assayed targets were not corrected for PCR inhibition and C_T values with only dilution factor correction are given.
[d] NT, not tested.
[e] ND, not detected.

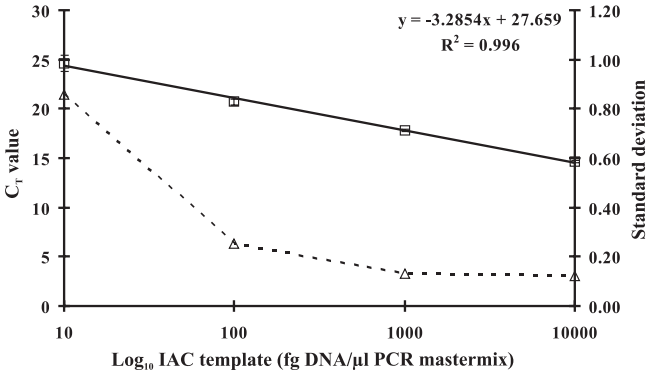

FIG. 2. Reproducibility and precision of the IAC calibration curve in the Biotrove OpenArray. An IAC mixture containing 1,670 fg IAC1, 167 fg IAC2, 16.7 fg IAC3, and 1.67 fg IAC4 per μl PCR master mix was analyzed for PCR amplification reproducibility on the OpenArray platform ($n = 60$). Solid line, IAC calibration curve based on the average of the observed C_T values ($R^2 = 0.996$). Dashed line, standard deviation C_T values.

accuracy of adjustments to quantitative data based on the developed calibration IAC regression formula were tested by using environmental soil samples with different biotic and abiotic properties (Table 3) (19). Undiluted DNA extracts were analyzed by real-time PCR for the density of total bacteria, proteobacteria of the alpha and beta subdivisions, respectively, and actinobacteria (Table 4). In the analyzed soil DNA extract from field 5, the sample IAC regression formula in the undiluted DNA samples was not significantly different from the calibration IAC regression formula, indicating that there was effectively no qPCR inhibition (Table 4). The observed C_T values for this sample therefore did not need to be adjusted. The sample IAC regression formulas for the other five undiluted DNA samples, however, were significantly different from the calibration IAC regression formula, indicating the occurrence of partial qPCR inhibition (Table 4). For these cases, the C_T values resulting from the analyzed bacterial groups were corrected for qPCR inhibition as described above (see Materials and Methods).

Reliability of IAC-based C_T value corrections. To visualize the reliability of IAC-based corrections, the five soil DNA samples that showed PCR inhibition were diluted 20 times (fields 1, 2, 3, and 4) or 40 times (field 6). As shown in Table 4, the sample IAC regression formulas in the diluted DNA samples from fields 1 to 4 were not significantly different from the calibration IAC regression formula. This indicated that a 20-fold dilution was sufficient to completely overcome PCR inhibition, making the correction of the observed C_T values no longer necessary. To enable the comparison between the IAC-corrected C_T values of the assayed targets in the undiluted DNA samples with the C_T values of the assayed targets after 20-fold dilution of DNA samples, a total of 4.47 C_T units (theoretically equal to a 20-fold dilution in qPCR; $2n = 20$, $n = 4.47$, where n is number of PCR cycles) was subtracted from the C_T values of the assayed targets. Next, comparison of the IAC-corrected C_T values of the assayed targets in the undiluted DNA samples with the C_T values in the noninhibiting DNA samples (after 20-times dilution correction) demonstrated that the developed IAC enabled accurate adjustments

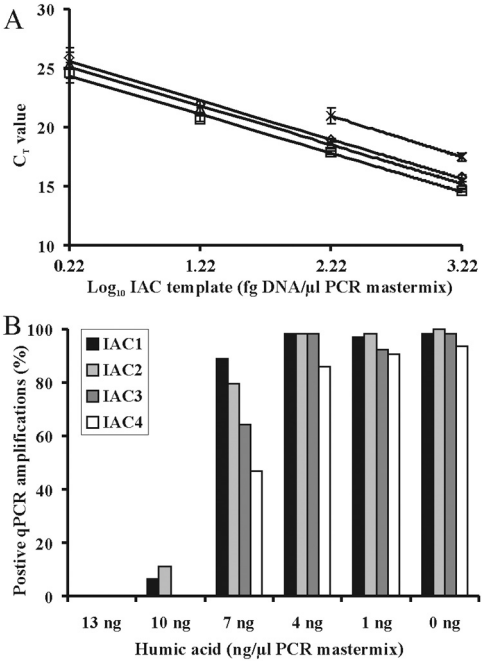

FIG. 3. (A) Effect of artificial PCR inhibition on the IAC curve. Several concentrations of humic acids were added to the IAC PCR mixtures and amplified on the Biotrove OpenArray platform. IAC curves, from top to bottom: ×, 10 ng humic acids per μl PCR mixture; ◇, 7 ng humic acids per μl PCR mixture ($R^2 = 0.996$); Δ, 4 ng humic acids per μl PCR mixture ($R^2 = 0.998$); □, 0 ng humic acids per μl PCR mixture ($R^2 = 0.996$). Data represent averages of 60 PCR replicates ($n = 60$). The error bars represent standard deviations. (B) Effect of artificial PCR inhibition on the number of positive qPCR amplifications. Several concentrations of humic acids were added to the IAC PCR mixtures and amplified on the Biotrove OpenArray platform. Data represent the percentage of positive PCR amplifications out of 60 PCR replicates per IAC target-humic acid concentration combination.

to quantitative data in cases of partial PCR inhibition by environmental samples (Table 4).

The slope of the sample IAC regression formula from the 40-fold-diluted DNA extract from field 6, however, was still significantly different from the slope of the calibration IAC regression formula but equal to the slope of the corresponding undiluted DNA sample IAC regression formula. In this particular case, even a 40-fold dilution was not sufficient to completely eliminate the negative effect of the inhibiting agents in the DNA extract for the sample IAC regression curve, but accurate quantification was still possible with the newly developed IAC system.

DISCUSSION

In this study, we developed a novel internal amplification control (IAC) that allows highly accurate adjustment of quantitative data for partial PCR inhibition. The extremely precise IAC approach described in this study allowed a highly accurate quantification of microorganisms in environmental soil DNA samples that contained substances that partially inhibited PCR amplification.

Environmental soil samples are commonly known to contain variable levels of PCR-inhibiting compounds (30). These com-

pounds are often coextracted with soil DNA during nucleic acid extraction procedures. A commonly applied strategy to reduce inhibition of PCR is to simply dilute soil DNA extracts until PCR inhibition is no longer observed. However, also the targets of interest are diluted, which negatively influences detection sensitivity. Additionally, this commonly used strategy (dilution series preparation) is not well suited for use with the new-generation qPCR platforms (15, 17), which require relatively high concentrations of DNA target input in nanoliter reaction volumes. Indeed, we observed that the reproducibility, accuracy, and precision of quantification in the Biotrove OpenArray PCR all decrease at lower input DNA copy numbers, stressing the need for concentrated DNA samples (Fig. 2).

In order to test the developed IAC system, we induced PCR inhibition by adding ethanol and humic acids to the IAC samples at a range of concentrations. Addition of ethanol up to even a 5% final concentration did not result in any PCR inhibition in the Biotrove OpenArray system (data not shown). Higher ethanol concentrations were not expected or realistic based on typical DNA extraction protocols and were therefore not tested. PCR inhibition with humic acids, however, did result in significantly different sample IAC regression curve intercepts compared to the intercept of the calibration IAC regression curve. Since the slopes did not appear to be significantly different, artificially induced partial PCR inhibition resulted in a linear C_T shift of all IAC concentrations. Thus, partial PCR inhibition in terms of C_T values was not target concentration dependent in these samples. Analyzing the number of negative PCR amplifications at very high humic acid concentrations, however, indicated that full PCR inhibition in the Biotrove OpenArray system is more likely to occur at low target concentrations. This suggests that, in cases of very strong inhibition at low IAC concentrations, which result in negative PCR amplifications, a DNA sample might still have to be diluted to allow accurate IAC-based quantification. Such strong PCR inhibition was, however, only observed in the artificially inhibited PCR amplifications at very high humic acid concentrations and not in the analyzed DNA extracts from actual environmental samples (see below).

The developed calibration IAC regression formula was tested with DNA extracts from six marine clay and sandy soil environments, which contained various amounts of organic matter (19). The relative abundances of three bacterial groups (the alpha and beta subdivisions of proteobacteria and actinobacteria) and the density of total bacteria in the soils were determined, while the potential PCR inhibition was monitored with the newly developed IAC. As expected, marine clay soils with high organic matter contents showed the highest degree of PCR inhibition, while sandy soils with low organic matter contents generally showed lower PCR inhibition. Contrary to those of the samples where PCR inhibition was induced artificially, the slopes of the sample IAC curves obtained with the PCR-inhibiting environmental samples were often different from the slope of the calibration IAC curve. Thus, partial PCR inhibition in environmental samples can be target concentration dependent, stressing the need for IACs at multiple target concentrations for more precise quantification (see Appendix S2 in the supplemental material). By comparing C_T values obtained from diluted DNA extracts with the IAC-corrected C_T values obtained from the inhibiting DNA extracts, we demonstrated that target-specific C_T values could be accurately adjusted for partial PCR inhibition with the developed IAC. It should be noted that the IAC primer pairs have no relation to the primer pairs of the assayed targets and that each new target primer pair has to be tested for the PCR inhibition correction in combination with the IAC system before reliable IAC-based quantification can be performed with environmental samples.

In the field 6 soil DNA extract, the slope of the IAC sample regression curve remained significantly different from the slope of the calibration IAC regression curve, even after 40-fold dilution of the DNA sample. Interestingly, the undiluted and 40-fold-diluted DNA sample IAC regression formulas were highly similar, implying that 40-fold dilution was not sufficient to neutralize completely the negative effect of the undiluted DNA extract on the sample IAC regression curve (further dilutions were not tested because of too much loss of sensitivity). Nevertheless, this event did not affect the possibility of accurate quantification with this new IAC principle.

Next-generation qPCR platforms, like the Biotrove OpenArray system, allow parallel mass testing of environmental samples against a wide range of microorganisms. Reliable quantification requires amplification controls that can correct for partial PCR inhibition, as is often observed for environmental soil DNA extracts. We demonstrated that artificially induced partial PCR inhibition in the Biotrove OpenArray system is not target concentration dependent, while low target concentrations were shown to be more sensitive to full PCR inhibition. Partial PCR inhibition in environmental samples, however, did appear to be target concentration dependent. The developed IAC system can be easily adapted for incorporation into TaqMan probe-based detection assays, and the bacterial cells containing the IAC plasmids could potentially be used as extraction controls in future experiments (10). In addition, the performance of DNA extraction methods can be evaluated. The described IAC system enables accurate adjustments to obtain quantitative data from environmental DNA extracts that possibly inhibit PCR and can be applied for routine screenings of samples from different environmental systems.

ACKNOWLEDGMENTS

We thank Eelco Franz (RIKILT-Institute of Food Safety) for his assistance with statistical data analysis. We acknowledge Richard van Hoof (Plant Research International) and Julia Rieckmann (Alterra) for their assistance with IAC design and for providing information about the performance of the assayed-target primers, respectively. We also acknowledge Joeke Postma and Mirjam Schilder (Plant Research International) for kindly providing soil DNA samples and data on soil characteristics.

This work was partially funded by the Dutch Ecogenomics Consortium, work package 3.4.

REFERENCES

1. **Arbeli, Z., and C. L. Fuentes.** 2007. Improved purification and PCR amplification of DNA from environmental samples. FEMS Microbiol. Lett. **272:** 269–275.
2. **Dong, D. X., A. Yan, H. M. Liu, X. H. Zhang, and Y. Q. Xu.** 2006. Removal of humic substances from soil DNA using aluminium sulfate. J. Microbiol. Methods **66:**217–222.
3. **Fierer, N., J. A. Jackson, R. Vilgalys, and R. B. Jackson.** 2005. Assessment of soil microbial community structure by use of taxon-specific quantitative PCR assays. Appl. Environ. Microbiol. **71:**4117–4120.
4. **Gonzague, M., C. Plin, L. Bakkali-Kassimi, A. Boutrouille, and C. Cruciere.** 2002. Development of an internal control for the detection of the African swine fever virus by PCR. Mol. Cell. Probes **16:**237–242.

5. **Hartman, L. J., S. R. Coyne, and D. A. Norwood.** 2005. Development of a novel internal positive control for Taqman based assays. Mol. Cell. Probes **19:**51–59.
6. **Hoorfar, J., N. Cook, B. Malorny, M. Wagner, D. De Medici, A. Abdulmaajood, and P. Fach.** 2003. Making internal amplification control mandatory for diagnostic PCR. J. Clin. Microbiol. **41:**5835.
7. **Hoorfar, J., B. Malorny, A. Abdulmawjood, N. Cook, M. Wagner, and P. Fach.** 2004. Practical considerations in design of internal amplification controls for diagnostic PCR assays. J. Clin. Microbiol. **42:**1863–1868.
8. **Ijzerman, M. M., D. R. Dahling, and G. S. Fout.** 1997. A method to remove environmental inhibitors prior to the detection of waterborne enteric viruses by reverse transcription-polymerase chain reaction. J. Virol. Methods **63:** 145–153.
9. **Khan, G., H. O. Kangro, P. J. Coates, and R. B. Heath.** 1991. Inhibitory effects of urine on the polymerase chain-reaction for cytomegalovirus DNA. J. Clin. Pathol. **44:**360–365.
10. **Klerks, M. M., A. H. C. van Bruggen, C. Zijlstra, and M. Donnikov.** 2006. Comparison of methods of extracting *Salmonella enterica* serovar Enteritidis DNA from environmental substrates and quantification of organisms by using a general internal procedural control. Appl. Environ. Microbiol. **72:** 3879–3886.
11. **Klerks, M. M., C. Zijlstra, and A. H. C. van Bruggen.** 2004. Comparison of real-time PCR methods for detection of *Salmonella enterica* and *Escherichia coli* O157:H7, and introduction of a general internal amplification control. J. Microbiol. Methods **59:**337–349.
12. **Maaroufi, Y., J. M. de Bruyne, V. Duchateau, R. Scheen, and F. Crokaert.** 2006. Development of a multiple internal control for clinical diagnostic real-time amplification assays. FEMS Immunol. Med. Microbiol. **48:**183–191.
13. **Monpoeho, S., M. Coste-Burel, M. Coste-Mattioli, B. Besse, J. J. Chomel, S. Billaudel, and V. Ferre.** 2002. Application of a real-time polymerase chain reaction with internal positive control for detection and quantification of enterovirus in cerebrospinal fluid. Eur. J. Clin. Microbiol. Infect. Dis. **21:** 532–536.
14. **Monteiro, L., D. Bonnemaison, A. Vekris, K. G. Petry, J. Bonnet, R. Vidal, J. Cabrita, and F. Megraud.** 1997. Complex polysaccharides as PCR inhibitors in feces: *Helicobacter pylori* model. J. Clin. Microbiol. **35:**995–998.
15. **Morrison, T., J. Hurley, J. Garcia, K. Yoder, A. Katz, D. Roberts, J. Cho, T. Kanigan, S. E. Ilyin, D. Horowitz, J. M. Dixon, and C. J. H. Brenan.** 2006. Nanoliter high throughput quantitative PCR. Nucleic Acids Res. **34:**e123.
16. **Nolte, F. S.** 2004. Novel internal controls for real-time PCR assays. Clin. Chem. **50:**801–802.
17. **Ottesen, E. A., J. W. Hong, S. R. Quake, and J. R. Leadbetter.** 2006. Microfluidic digital PCR enables multigene analysis of individual environmental bacteria. Science **314:**1464–1467.
18. **Persoh, D., S. Theuerl, F. Buscot, and G. Rambold.** 2008. Towards a universally adaptable method for quantitative extraction of high-purity nucleic acids from soil. J. Microbiol. Methods **75:**19–24.
19. **Postma, J., M. T. Schilder, J. Bloem, and W. K. van Leeumen-Haagsma.** 2008. Soil suppressiveness and functional diversity of the soil microflora in organic farming systems. Soil Biol. Biochem. **40:**2394–2406.
20. **Prasher, D. C., V. K. Eckenrode, W. W. Ward, F. G. Prendergast, and M. J. Cormier.** 1992. Primary structure of the *Aequorea victoria* green-fluorescent protein. Gene **111:**229–233.
21. **Rojas-Herrera, R., J. Narvaez-Zapata, M. Zamudio-Maya, and M. E. Mena-Martinez.** 2008. A simple silica-based method for metagenomic DNA extraction from soil and sediments. Mol. Biotechnol. **40:**13–17.
22. **Rosenstraus, M., Z. Wang, S. Y. Chang, D. DeBonville, and J. P. Spadoro.** 1998. An internal control for routine diagnostic PCR: design, properties, and effect on clinical performance. J. Clin. Microbiol. **36:**191–197.
23. **Sagova-Mareckova, M., L. Cermak, J. Novotna, K. Plhackova, J. Forstova, and J. Kopecky.** 2008. Innovative methods for soil DNA purification tested in soils with widely differing characteristics. Appl. Environ. Microbiol. **74:** 2902–2907.
24. **Sebastianelli, A., T. Sen, and I. J. Bruce.** 2008. Extraction of DNA from soil using nanoparticles by magnetic bioseparation. Lett. Appl. Microbiol. **46:** 488–491.
25. **Stedtfeld, R. D., S. W. Baushke, D. M. Tourlousse, S. M. Miller, T. M. Stedtfeld, E. Gulari, J. M. Tiedje, and S. A. Hashsham.** 2008. Development and experimental validation of a predictive threshold cycle equation for quantification of virulence and marker genes by high-throughput nanoliter-volume PCR on the OpenArray platform. Appl. Environ. Microbiol. **74:** 3831–3838.
26. **Stults, J. R., O. Snoeyenbos-West, B. Methe, D. R. Lovley, and D. P. Chandler.** 2001. Application of the 5′ fluorogenic exonuclease assay (TaqMan) for quantitative ribosomal DNA and rRNA analysis in sediments. Appl. Environ. Microbiol. **67:**2781–2789.
27. **Tsai, Y. L., and B. H. Olson.** 1992. Rapid method for the separation of bacterial DNA from humic substances in sediments for polymerase chain reaction. Appl. Environ. Microbiol. **58:**2292–2295.
28. **van Doorn, R., M. Slawiak, M. Szemes, A. M. Dullemans, P. Bonants, G. A. Kowalchuk, and C. D. Schoen.** 2009. Robust detection and identification of multiple oomycetes and fungi in environmental samples by using a novel cleavable padlock probe-based ligation detection assay. Appl. Environ. Microbiol. **75:**4185–4193.
29. **van Doorn, R., M. Szemes, P. Bonants, G. A. Kowalchuk, J. F. Salles, E. Ortenberg, and C. D. Schoen.** 2007. Quantitative multiplex detection of plant pathogens using a novel ligation probe-based system coupled with universal, high-throughput real-time PCR on OpenArrays. BMC Genomics **8:**276.
30. **Watson, R. J., and B. Blackwell.** 2000. Purification and characterization of a common soil component which inhibits the polymerase chain reaction. Can. J. Microbiol. **46:**633–642.
31. **Whitehouse, C. A., and H. E. Hottel.** 2007. Comparison of five commercial DNA extraction kits for the recovery of Francisella tularensis DNA from spiked soil samples. Mol. Cell. Probes **21:**92–96.
32. **Zhou, J. Z., M. A. Bruns, and J. M. Tiedje.** 1996. DNA recovery from soils of diverse composition. Appl. Environ. Microbiol. **62:**316–322.