Eliminating false positives in a qPCR assay for the detection of the *uidA* gene in *Escherichia coli*

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ABSTRACT

Due to contaminant *Escherichia coli* DNA present in recombinant *Taq* polymerase reagents, it is not possible to reliably detect low levels of *E. coli* in samples using the quantitative polymerase chain reaction (qPCR) assay. Native *Taq* polymerase was successfully used in this study to detect five *uidA* gene copies (5 fg of genomic DNA) of the *uidA* gene.

Key words | contaminant DNA, detection limit, *E. coli*, native *Taq* polymerase, qPCR, *uidA* gene

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SHORT COMMUNICATION

During the development of a rapid 2-step quantitative polymerase chain reaction (qPCR) assay to detect and quantify *Escherichia coli* (*E. coli*) using the *uidA* gene, positive reactions were consistently being observed in the non-template control (NTC) samples. Investigation into the cause of the false-positive reactions determined that there was contaminant *E. coli* DNA in the recombinant polymerase reagents being used. This contamination meant that it was not possible to reliably detect low levels of *E. coli* in the sludge and wastewater samples being tested. It was previously reported that many commercial qPCR reagents contained contaminant genomic DNA from bacterial sources including *E. coli* (Tseng *et al.* 2003; Shannon *et al.* 2007); however, this may not be well known in the research community. *E. coli* is the most commonly used indicator bacteria for the assessment of water, wastewater and sludge treatments, and the presence of contaminant genomic DNA can particularly be an issue when PCR-based methods are used due to false positives. To increase the sensitivity and confidence in the qPCR assay for detecting low levels of *E. coli*, it was necessary to eliminate the contaminant DNA from the PCR reagents.

Efforts were taken to clean up the recombinant polymerase reagents. Two types of methods have been proposed in the literature to clean up commercial reagents of the contaminant bacterial DNA: (1) ultrafiltration; and (2) DNase I enzyme treatment. Ultrafiltration methods (proposed by Mohammadi *et al.* (2003) and Yang *et al.* (2002)) were not tried due to the unknown potential reduction of components in the reagents and the effect this may have on the qPCR assay. Ultrafiltration was also shown to decrease PCR sensitivity in one account (Mohammadi *et al.* 2003), but not in another (Yang *et al.* 2002). The second method, using DNase I enzyme to treat the polymerase reagents, was tested. Both a commercial DNase I kit (AMPD1 from Sigma) and a published DNase I cleanup method (Tondeur *et al.* 2004; Silkie *et al.* 2008) were tested.

In this study, the DNase I treatment showed complete PCR inhibition with the Silkie *et al.* (2008) cleanup method and false positives still present with the commercial kit from Sigma (Figure 1(a–b). and Table 1). The DNase I enzyme that was used by Silkie *et al.* (2008) in the DNase I cleanup method was no longer available and another source was tested. This alternative DNase I reagent may be the cause of the difference in results found. Refined DNase I treatment methods have also been published, however, these refined methods are more difficult to use since they must first assess the quantity of contaminant DNA

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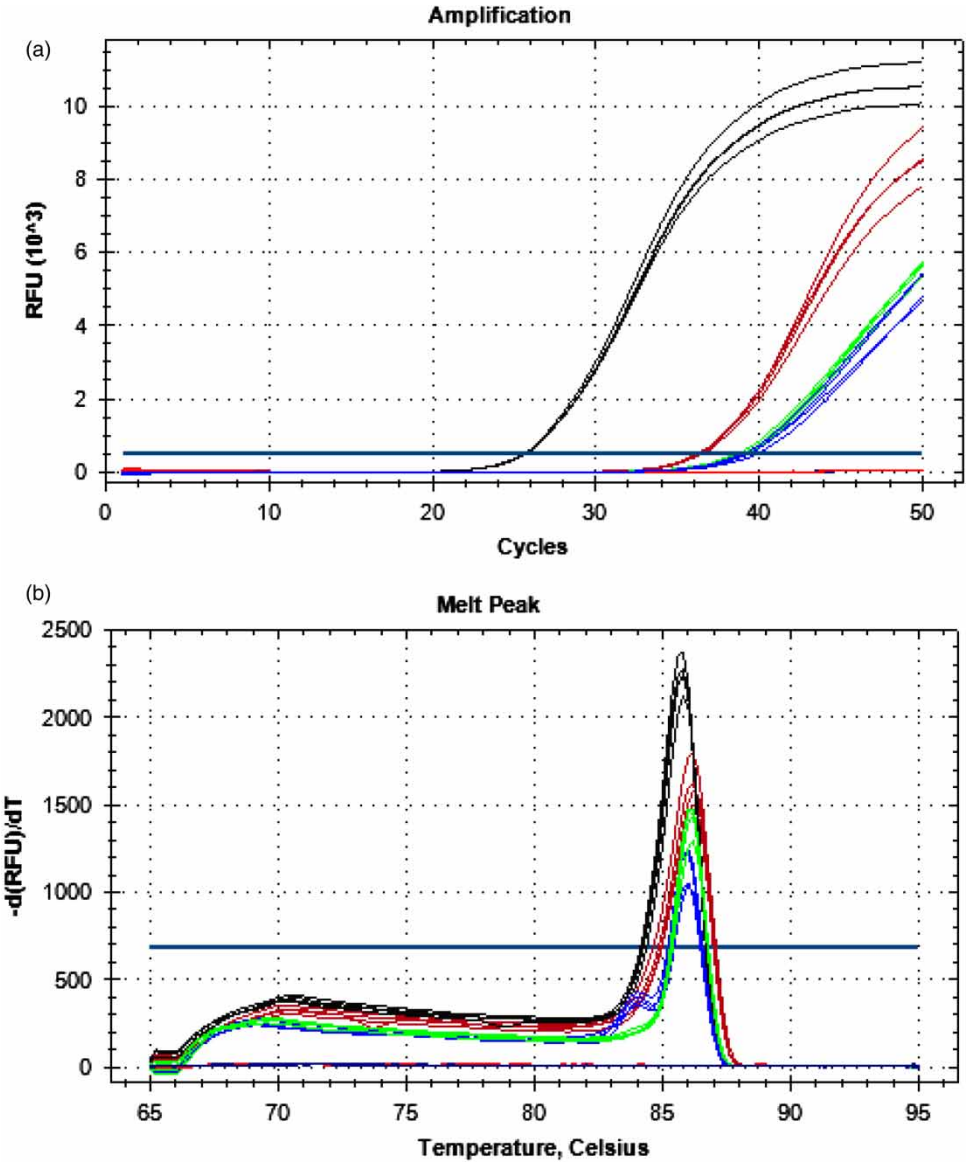

Figure 1 | (a) Amplification for the comparison of DNase I treatment methods showing the mean C_T values of 6 series of reactions, from left to right: C_T of 25.76 (black) represents the positive control, *E. coli* K12 template of 1.167×10^4 *uidA* gene copy number-untreated; C_T of 36.41 (dark red) represents the non-template control (NTC)-untreated; the next 2 sets are lumped together at $\sim C_T$ 40, these represent the NTC (green) and test – *E. coli* K12 template of 1.167×10^4 *uidA* gene copy number (blue) – commercial method from Sigma. The Silkie *et al.* (2008) method completely inhibited the qPCR reaction as shown by the flat line (red/navy blue) at 0 relative fluorescence units (RFU) (10^3). Refer to Table 1 for complete data. (b) Melt peak analysis for the comparison of DNase I treatment methods showing consistent melting temperatures for all positive signal reactions confirming amplification specificity to the *uidA* gene 166 bp amplicon. The black lines represent the positive controls, *E. coli* K12 template of 1.167×10^4 *uidA* gene copy number, dark red lines represent the NTC untreated; green lines represent the NTC and blue lines for the test – commercial method from Sigma. The Silkie *et al.* (2008) method completely inhibited the qPCR reaction as shown by the red/navy blue line at 0 – d(RFU)/dT. Please refer to the online version of this paper to see this figure in colour: http://www.iwaponline.com/jwh/toc.htm.

per reagent and then a customized decontamination protocol must be developed (Corless *et al.* 2000; Heininger *et al.* 2003). It was decided not to try the refined methods due to the additional costs, procedural steps and time, and expertise required.

With the DNase I cleanup methods giving poor results, a 3-step qPCR protocol was developed using a native *Taq* polymerase guaranteed to be free of contaminant *E. coli* DNA. Native *Taq* DNA polymerase is recommended by manufacturers for use in special PCR

Table 1 | C_T values for Figures 1(a) and (b): comparison of DNase treatment methods on the Ssofast Supermix *Taq* polymerase. All reactions were run in quadruplicate

Treatment/ Method	qPCR reaction	Genomic units (*uidA* gene)	Mean C_T	C_T Std. Dev.
Silkie *et al.*	*E. coli* control	1.167×10^4	0.00	–
	NTC		0.00	–
Sigma DNase I	*E. coli* control	1.167×10^4	39.49	0.375
	NTC		39.05	0.229
Untreated	*E. coli* control	1.167×10^4	25.76	0.093
	NTC	~29.5[a]	36.41	0.124

[a]Estimates based on *E. coli* control.

applications where genomic DNA may interfere with amplification specificity or when the target amplicon is within the *E. coli* genome. However, the use of native *Taq* polymerase in qPCR is normally not recommended due to its lower thermal stability eliminating the option of designing a 2-step qPCR assay and increasing the qPCR run time. However, sensitivity preceded over extended qPCR assay run times leading to the development of the method described herein.

This qPCR protocol detects and quantifies a 166 bp segment of the *uidA* gene in *E. coli* (Figure 2). Genomic DNA was isolated from an overnight pure culture of *E. coli* K12 (ATCC 23631) using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Inc., CA, USA). The quantity and quality of the *E. coli* genomic DNA was determined using the Varian CARY-100 BIO UV-VIS spectrophotometer by measuring the absorbance at 260 nm with the purity determined by analyzing the 260:280 nm ratio. A set of 20-mer primers, UAL-1939 (5′-TATGGAATTTCGCCGATTTT-3′) and UAR-2105 (5′-TGTTTGCCTCCCTGCTGCGG-3′), was used to amplify a 166 bp region of the *uidA* gene of *E. coli* (Bej *et al.* 1991). Instead of the rapid 2-step qPCR with an initial denaturation of 2 minutes at 98.0 °C followed by 49 cycles of 2 seconds at 98.0 °C, and an annealing/elongation temperature of 57.2 °C, a 3-step program was used. The 3-step qPCR protocol consisted of an initial 3 minute denaturation at 95.0 °C followed by 49 cycles of denaturation at 95.0 °C for 10 seconds, annealing at 59.2 °C for 20 seconds and an elongation at 72.0 °C for 15 seconds. Each

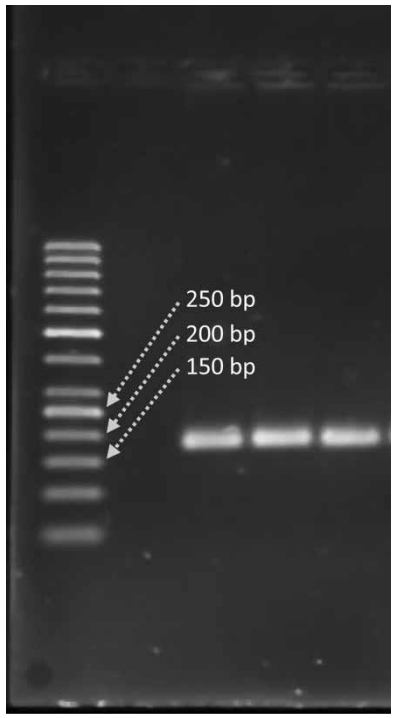

Figure 2 | 0.8% Agarose gel of the 166 bp amplicon (Bej *et al.* 1991) in triplicate Lane 1: 50 bp ladder; Lane 2 was intentionally left empty; Lane 3–5: Genomic DNA isolated from *E. coli* ATCC 23631.

25 μL reaction had 2.5 μL 10× PCR buffer, 0.5 μL of 10 mM deoxynucleotide triphosphates (dNTPs), 1.25 μL of 50 mM MgCl, 0.625 μL (0.10 μM) of each primer, 0.5 μL (1 U/μL) native *Taq* polymerase, 1.25 μL of EvaGreen dye, 1 μL of 10 μg/μL bovine serum albumin (BSA), 50 ng of genomic DNA template topped up to 25 μL with DNA free water.

This protocol was free of false-positive reactions in the NTCs in several standard curves and positive control runs, and gave expected threshold cycle (C_T) values when compared with recombinant PCR reagents (Figure 3). All standard curve dilutions and NTCs were done in triplicate and the mean C_T values are shown in Figure 3 and Table 2. The assay with melt curve analysis has increased in time by approximately 30 minutes due to the lower denaturation temperature and the need for separate annealing and elongation steps in the protocol. By eliminating the contaminant *E. coli* DNA from the PCR reagents, this method was able to detect as low as five copies of the *uidA* gene (Figure 3) compared with variable sensitivities of 10–1,000 gene copy numbers

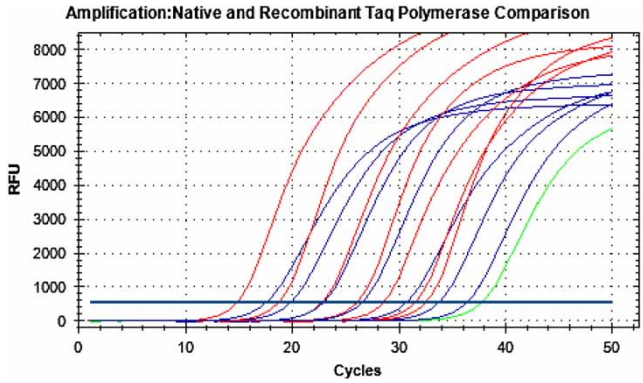

Figure 3 | Comparison of native (red) and recombinant *Taq* polymerase (blue) with the same PCR parameters has shown that both have a similar sensitivity with a detection limit of ~10 genomic units. The NTC (green) signal is due to primer-dimerization and is differentiated in the melt curve analysis (not shown). In an effort to show the direct comparison of both types of polymerase, the protocol designed for the native polymerase was used; this explains the higher C_T shift with the recombinant polymerase. All standard curve dilutions and NTCs were done in triplicate but just the mean data points are shown in the above figure for ease of interpretation. Please refer to the online version of this paper to see this figure in colour: http://www.iwaponline.com/jwh/toc.htm.

Table 2 | Mean C_T values comparing the use of recombinant *Taq* polymerase (R-Taq) and native *Taq* (N-Taq) polymerase (see Figure 3). All reactions were done in triplicate

Taq polymerase	qPCR reaction	Genomic units (*uidA* gene)	Mean C_T	C_T Std. Dev.
R-Taq	Std-01	1.167×10^7	15.32	0.270
	Std-02	1.167×10^6	18.76	0.192
	Std-03	1.167×10^5	22.54	0.455
	Std-04	1.167×10^4	26.18	0.103
	Std-05	1.167×10^3	29.11	0.316
	Std-06	1.167×10^2	31.35	0.517
	Std-07	1.167×10^1	32.26	0.441
	Std-08	1.167×10^0	31.50[a]	1.121
N-Taq	Std-01	1.167×10^7	17.44	0.460
	Std-02	1.167×10^6	19.97	0.133
	Std-03	1.167×10^5	23.06	0.099
	Std-04	1.167×10^4	26.87	0.205
	Std-05	1.167×10^3	30.81	0.463
	Std-06	1.167×10^2	33.80	0.431
	Std-07	1.167×10^1	36.81	0.295
	Std-08	1.167×10^0	34.79[a]	5.073
	NTC		38.06[a]	0.000
	NTC		38.14[a]	0.000
	NTC		37.52[a]	0.000

[a]Primer dimer signal.

reported in the literature (Silkie *et al.* 2008; Hospodsky *et al.* 2010; Blainey & Quake 2010) and seen in our experiments prior to the new protocol.

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REFERENCES

Bej, A. K., DiCesare, J. L., Haff, L. & Atlas, R. M. 1991 Detection of *Escherichia coli* and *Shigella* spp. in water by using the polymerase chain reaction and gene probes for uid. *Appl. Environ. Microbiol.* **57**, 1013–1017.

Blainey, P. C. & Quake, S. R. 2010 Digital MDA for enumeration of total nucleic acid contamination. *Nucleic Acids Res.* **39**, 1–9.

Corless, C. E., Guiver, M., Borrow, R., Edwards-Jones, V., Kaczmarski, E. B. & Fox, A. J. 2000 Contamination and sensitivity issues with a real-time universal 16S rRNA PCR. *J. Clin. Microbiol.* **38**, 1747–1752.

Heininger, A., Binder, M., Ellinger, A., Botzenhart, K., Unertl, K. & Doring, G. 2003 DNase pretreatment of master mix reagents improves the validity of universal 16S rRNA gene PCR results. *J. Clin. Microbiol.* **41**, 1763–1765.

Hospodsky, D., Yamamoto, M. & Peccia, J. 2010 Accuracy, precision, and method detection limits of quantitative PCR for airborne bacteria and fungi. *Appl. Environ. Microbiol.* **76**, 7004–7012.

Mohammadi, T., Reesink, H. W., Vandenbroucke-Grauls, C. & Savelkoul, P. H. M. 2003 Optimization of real-time PCR assay for rapid and sensitive detection of eubacterial 16S ribosomal DNA in platelet concentrates. *J. Clin. Microbiol.* **41**, 4796–4798.

Shannon, K. E., Lee, D.-Y., Trevors, J. T. & Beaudette, L. A. 2007 Application of real-time quantitative PCR for the detection of selected bacterial pathogens during municipal wastewater treatment. *Sci. Total Environ.* **382**, 121–129.

Silkie, S. S., Tolcher, M. P. & Nelson, K. L. 2008 Reagent decontamination to eliminate false-positives in *Escherichia coli* qPCR. *J. Microbiol. Methods* **72**, 275–282.

Tondeur, S., Agbulut, O., Menot, M. L., Larghero, J., Paulin, D., Menasche, P., Samuel, J. L., Chomienne, C. & Cassinat, B. 2004 Overcoming bacterial DNA contamination in real-time PCR and RT-PCR reactions for LacZ detection in cell therapy monitoring. *Mol. Cell. Probes* **18**, 437–441.

Tseng, C. P., Cheng, J. C., Tseng, C. C., Wang, C. Y., Chen, Y. L., Chiu, D. T., Liao, H. C. & Chang, S. S. 2003 Broad-range ribosomal RNA real-time PCR after removal of DNA from reagents: melting profiles for clinically important bacteria. *Clin. Chem.* **49**, 306–309.

Yang, S., Lin, S., Kelen, G. D., Quinn, T. C., Dick, J. D., Gaydos, C. A. & Rothman, R. E. 2002 Quantitative multiprobe PCR assay for simultaneous detection and identification to species level of bacterial pathogens. *J. Clin. Microbiol.* **40**, 3449–3454.

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