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Note

'Ready Mixed', improved nucleic acid amplification assays for the detection of *Escherichia coli* DNA and RNA

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ABSTRACT

The selective amplification of *E. coli* nucleic acid sequences could be used for the early warning of faecal contamination in environmental samples. Modified assays for *E. coli* DNA and RNA markers are presented with improved integrity and performance over existing methods, and demonstrated using 'ready mixed', preserved reagent mixtures.

The presence of *Escherichia coli* (*E. coli*) in the environment is considered globally as probable evidence of faecal contamination, and therefore it is subject to statutory surveillance for various water and food safety assurance practices (Edberg et al., 2000). *E. coli* detection methods are generally culture based, and the provision of timely results is limited by the bacterial growth rate; typically they take in excess of 18 h (SCA, 2016). This increases public health risk, particularly during short-lived, stochastic contamination events. Nucleic acid amplification is a culture-independent technique, used to detect microorganisms by *in vitro* replication (amplification) of their DNA or RNA sequences. These methods can be employed to detect and enumerate *E. coli* with a better selectivity than cell culture (Walker et al., 2017), and in a few hours or less. The detection of *E. coli* nucleic acids could complement existing culture-based methods, particularly as rapid, 'early warning' risk indicators.

In this study, two 'improved' nucleic acid amplification assays are reported for the specific detection of *E. coli* DNA or RNA sequence markers. Each assay has been demonstrated using a convenient 'ready mixed' format whereby complete and dry-preserved reagent mixtures were prepared in advance, and then activated by simply rehydrating the mixtures with a water sample containing *E. coli* nucleic acids. In one assay, the detection of *E. coli* DNA was achieved using a quantitative real time PCR (qPCR) method targeting a fragment of the *ybbW* gene, based upon a highly selective primer set originally described by Walker et al. (2017). A prior limitation of this method was the use of SYBR Green DNA binding dye for real-time fluorescence detection during qPCR. SYBR Green will bind to any DNA sequence which can lead to false-positive amplification from the presence of non-specific amplification products or primer duplex formation ('primer-dimers') (Zipper et al., 2004). Here, a *ybbW*-specific fluorometric hydrolysis probe was designed and tested in combination with the existing PCR primers to reduce background fluorescence signals and the likelihood of false positive amplification. A second assay targets *E. coli* mRNA by amplifying a fragment of the *clpB* gene transcript sequence. The same mRNA target has already been employed for the specific detection of *E. coli* in water samples using isothermal Nucleic Acid Sequence Based Amplification or NASBA (Min and Baeumner, 2002; Malek et al., 1994; Heijnen and Medema, 2009). The mRNA encodes a heat-shock response molecular chaperone protein and is induced by gently heating the bacteria prior to RNA isolation, enabling sub-single-cell sensitivity (Heijnen and Medema, 2009). However, NASBA, which was recently compared to qPCR methods (Walker et al., 2017), displays significantly higher run-to-run variability than PCR and is unreliable at low template concentration. The reagents are also comparatively expensive and not widely available (Honsvall and Robertson, 2017). Accordingly, here the method was adapted to work using reverse transcription qPCR (RT-qPCR) for indirect mRNA amplification with hydrolysis probe-based real time fluorescence detection. The oligonucleotides used in this study were designed using Geneious R10 software (Biomatters Ltd., New Zealand) and synthesised by Integrated DNA Technologies (IDT Ltd., UK). Hydrolysis probes contained a covalently linked Fluorescein (FAM) at the 5′ end, and an Iowa Black (IABk) quencher at the 3′ end, and a secondary, internal ZEN quencher, 6 nucleotides from the 5′ terminus. The sequences are shown in Table 1.

Reaction mixtures were prepared in sterile, nuclease-free PCR tubes (LightCycler 8-tube strips, Roche Molecular Systems Inc.). The qPCR reactions were prepared to contain 20 mM Tris-HCl (pH 8.3), 100 mM KCl, 1.5 mM $MgCl_2$, 2 mM each dNTPs, 80 nM each primer, 40 nM hydrolysis probe, 0.2% (*w/v*) Sucrose, 0.2% (w/v) Trehalose and 2 U of GoTaq G2 DNA polymerase (Promega, UK); the final volume was 100 µL. The RT-qPCR reactions were prepared using the GoTaq Probe 1-Step RT-qPCR System (Promega, UK) following the manufacturers

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Table 1
Oligonucleotides used in this study.

Name	Sequence (5′ – 3′)	Reference
ybbW forward primer	TGATTGGCAAAATCTGGCCG	(Walker et al., 2017)
ybbW reverse primer	GAAATCGCCCAAATCGCCAT	
ybbW hydrolysis probe	FAM-CCGCCG[*ZEN*]AAAACGATATAGATGCACGG- IABkFQ*	This study
clpB forward primer	GCGACAATCCGGTCTTCA	This study
clpB reverse primer	AAATCCACATTTCTGACGAGG	(Heijnen and Medema, 2009)
clpB hydrolysis probe	FAM-CTTCCA[*ZEN*]GGCGAATCACTTTACCCGG-IABkFQ*	This study

recommended protocol except that the final 20 µL reactions were mixed with 80 µL of RT-PCR grade water to a final volume of 100 µL. The mixtures were frozen at −80 °C, and then lyophilised using a sterile vacuum chamber in tandem with an a ZL(8 L) Lyophilisation Instrument (SPScientific, UK). The vacuum chamber was decontaminated before use by applying DNAaway (Sigma, UK) and RNaseZap (Thermofisher, UK) chemicals according to the manufacturers recommended method, to remove contaminating DNA and RNase respectively. Lyophilisation took place for precisely 16 h at < 200 µBar, at which point the mixtures had formed a crystalline, white powder. Air for re-pressurisation passed through a 0.2 µm Sterivex filtration unit (Millipore, USA). Decontamination was assessed by no template control reactions showing null amplification. After varying periods of time in storage at room temperature, the lyophilised reaction mixtures were activated by adding a 20 µL solution of *E. coli* DNA or RNA containing a known quantity of the target sequence. The DNA template was prepared from genomic DNA isolated from a type strain of *E. coli* (NCTC 9001), exactly as described by Walker *et al (*Walker et al., 2017*)*. RNA template was prepared by *in vitro*, T7 RNA Polymerase-driven expression of a truncated *clpB* gene sequence fused to a 5′ T7 promoter sequence. Full details of the template preparation are given in the supporting information. The template samples were quantified, and stock solutions were prepared to contain an estimated 10^5 to 10 copies per 20 µL. Each qPCR reaction was carried out using a LightCycler 96 qPCR instrument (Roche, UK) with an initial denaturation of 95 °C for 2 min, followed by 40 cycles of 95 °C for 20 s and 60 °C for 60 s. The RT-qPCR reactions were carried out as described above, except for the inclusion of an initial reverse transcription step of 42 °C for 15 min prior to thermal cycling.

When prepared and performed exactly as described above, the preserved mixtures could be used to amplify between 10 and 10^5 estimated target sequence copies, as shown in Fig. 1. Furthermore, the integrity and reliability of each method was improved when compared to prior versions. For example, the use of a *ybbW*-specific hydrolysis probe (this study) in place of SYBR green (Walker et al., 2017) for real-time detection of the *ybbW* target eliminated background fluorescence and false-positive amplification over a 40 Cycle PCR, as shown in the supporting information Fig. S1. In addition, the detection of *clpB* mRNA by RT-qPCR (this study) in place of NASBA (multiple prior studies; (Walker et al., 2017; Min and Baeumner, 2002; Heijnen and Medema, 2009)) markedly reduced the variability between replicate reactions and different runs, as shown in the supporting information Fig. S2. Each 'improved' assay was able to detect at least 10 estimated copies of the target sequence, but when the template was diluted further only a portion of the replicate reactions generated amplification curves (data not shown), and so the limit of quantification (LOQ) was taken to be ≥10 estimated copies for each assay. The relationship between template sequence copy number and C_t was linear over 5 orders of magnitude, with a typical linear fit (R^2) of 0.9934 (*clpB* RT-qPCR) and 0.9991 (*ybbw* qPCR). The efficiency of the primers was determined using the method of Pfaffl (Pfaffl, 2001) and found to be 1.94 (*ybbW*) and 1.83 (*clpB*), when using freshly prepared mixtures. Storage for up to 4 weeks did not impact the LOQ of the qPCR, albeit the amplification rate was reduced (C_t was increased). However, preservation increased the LOQ for the RT-qPCR to 100 estimated copies. After 4 weeks the amplification efficiency for each oligonucleotide set was 1.78 (*ybbW*) and 1.67 (*clpB*); the linear relationship ($R^2 \geq 0.99$) was unaffected by storage. After 6 weeks in storage there was a significant loss in reagent activity, and only the samples containing the highest tested number of target sequence copies (10^5 per reaction) could be amplified (not shown). Accordingly, the 'shelf-life' of the preserved mixtures used in this study was considered to be up to 4 weeks.

The inclusivity and specificity of each oligonucleotide set (primers and probe) was evaluated to determine whether the modifications made to existing methods, as described in this study, had any impact on their selectivity for *E. coli*. This was done according to the method of Walker et al. (2017) by PCR amplification of DNA sequences extracted from a panel of *E. coli* strains, and non-*E. coli* bacterial species, full details of which can be found in the supporting information Table S1. PCRs were carried out as described above, but using freshly prepared (*i.e.*, not preserved) reaction mixtures. Each assay was 100% inclusive of 76 unique *E. coli* strains including 72 strains belonging to the ECOR collection, which is considered to represent genotypic variation in *E. coli* (Ochman and Selander, 1984; Patel et al., 2018). The *ybbW* qPCR was able to exclude 22 non-*E. coli* bacterial strains. The *clpB* assay was mostly specific for *E. coli*, except that it was able to detect DNA extracted from closely related species *Shigella* spp., *Escherichia albertii* and *Escherichia fergosonii*. Overall, our results were in agreement with those reported by Walker et al. (2017), who carried out the same tests on unmodified versions of these assays, indicating that the changes described in this study did not negatively impact the selectivity of each method.

In summary, the methods described here can be used to amplify (detect) *E. coli* DNA and RNA sequence markers at concentrations ranging from ≤10 to 100,000 copies, with a strong linear correlation for quantification. Each method was based on existing, state of the art nucleic acid amplification tests for *E. coli*, but including critical modifications to improve integrity and reliability. The use of the dry-preserved reaction format constitutes a streamlined, one-step testing process, suited to automation, and where the potential for human error and contamination are significantly reduced.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2019.105721.

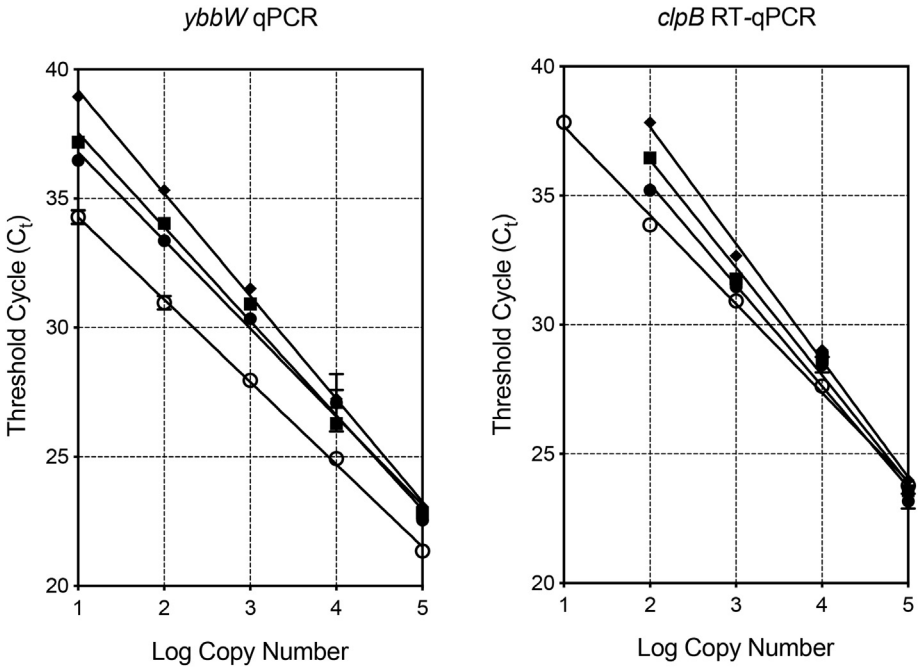

Fig. 1. The oligonucleotides shown in Table 1 were used to amplify *ybbW* DNA sequences by qPCR and *clpB* RNA sequences by RT-qPCR using dry-preserved reaction mixtures which had been stored for up to 4 weeks without refrigeration. After 1 week (●), 2 weeks (■) or 4 weeks (♦) the mixtures were re-hydrated with water containing DNA or RNA template at an estimated concentration of between 10 and 100,000 copies. For comparison, the open circles (○) indicate reactions prepared using fresh reagents which had not been preserved, but did contain an equivalent amount of Trehalose and Sucrose sugars. The results show the mean threshold cycle (C_t) *versus* template copy number from quadruplicate reactions. The error bars, where visible, show the standard error of the mean ($n = 4$). No symbol represents a null amplification.

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