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A new cell-direct quantitative PCR based method to monitor viable genetically modified *Escherichia coli*

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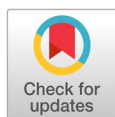
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Abstract

The development and commercialization of industrial genetically modified (GM) organisms is actively progressing worldwide, highlighting an increased need for improved safety management protocols. We sought to establish an environmental monitoring method, using real-time polymerase chain reaction (PCR) and propidium monoazide (PMA) treatment to develop a quantitative detection protocol for living GM microorganisms. We developed a duplex TaqMan quantitative PCR (qPCR) assay to simultaneously detect the selectable antibiotic gene, ampicillin (*AmpR*), and the single-copy *Escherichia coli* taxon-specific gene, D-1-deoxyxylulose 5-phosphate synthase (*dxs*), using a direct cell suspension culture. We identified viable engineered *E. coli* cells by performing qPCR on PMA-treated cells. The theoretical cell density (true copy numbers) calculated from mean quantification cycle (Cq) values of PMA-qPCR showed a bias of 7.71% from the colony-forming unit (CFU), which was within ±25% of the acceptance criteria of the European Network of GMO Laboratories (ENGL). PMA-qPCR to detect *AmpR* and *dxs* was highly sensitive and was able to detect target genes from a 10,000-fold (10^{-4}) diluted cell suspension, with a limit of detection at 95% confidence ($LOD_{95\%}$) of 134 viable *E. coli* cells. Compared to DNA-based qPCR methods, the cell suspension direct PMA-qPCR analysis provides reliable results and is a quick and accurate method to monitor living GM *E. coli* cells that can potentially be released into the environment.

Introduction

Genetic modification technology was developed to overcome the limitations of traditional breeding techniques, and it involves purpose-specific manipulation of genes using the latest molecular biology tools. Organisms developed through genetic modification are commonly referred to as genetically modified organisms (GMOs) or living modified organisms (LMOs) in legal terms (Lee and Suh, 2011). GMO technology has played a vital role in industrial development in the 21st century, and has been applied in several bioindustries including medicine, chemistry, environment, food, energy,

agriculture, and fisheries (Choi, 2020).

GMO technology has been most successfully utilized in the development of GM crops. As of 2019, GM crops were grown on 190.4 million hectares in 29 countries. Furthermore, since the beginning of commercialization of these crops in 1994, their accumulated cultivation area has reached 2.7 billion hectares (ISAAA, 2019). Although GM crop cultivation is not currently allowed in Korea, GM crops are imported and used for food and feed purposes, and their domestic import totaled 11.45 million tons and amounted to approximately $3.4 billion as of 2021 (KBCH, 2022a).

Industrial GMOs are another widely used commercial application of GMO technology, and are defined as "Genetically modified organisms used in industries such as the textile industry, the machine industry, chemical industry, electronics industry, the energy industry, and the resource industry excluding genetically modified organisms for testing, research, agriculture, livestock, health care, environmental purification, or marine and fisheries." by Article 1-2 Subparagraph 5 of the Act on the Movement of Genetically Modified Organisms (LMO Act). A research report from the Korean Society for Biotechnology and Bioengineering (KSBB, 2020) described active research underway worldwide on industrial GM microorganisms, for example, in the production of 1,3-propanediol, polylactic acid, a biodegradable polymer, succinic acid and 1,4-butanediol.

The industrialization of GM microorganisms in Korea is being developed and while it is lower than other leading nations, research and development are underway in several biotechnology companies and universities to generate commercially available host strains (KSBB, 2020). For example, an energy company is using GM microorganisms to develop a commercialized production process for 2,3-butanediol. Moreover, a food company has developed lactic acid-producing GM yeast strains and operates commercial production of lysine and glutamic acid using *Corynebacterium glutamicum* as host cells. Additionally, research is actively underway in several universities to establish GM microorganisms such as GM yeast, which generates 2,3-butanediol, and GM *Escherichia coli* which yields retinol, a wrinkle-improving bio-material. While no GM crops have been approved for commercialization, six industrial GMOs have been commercialized, including five microorganisms and one plant cell line (KBCH, 2022b). In addition, seven GM microorganisms were approved and commercialized for food products, including *C. glutamicum*, which produces the enzyme, D-fructose-4-isotropic.

Recently, as the worldwide production and industrial use of GMOs has gained momentum with an increase in the number of reported LMO research facilities, the demand for safety and safety management for using and developing GMOs has also increased. In Korea, the Ministry of Trade, Industry, and Energy is in charge of industrial GMO safety management according to Article 2, No. 5 of the LMO Act. The safety management is carried out by dividing GMOs into two groups: GMOs that are released into the natural environment (open system) and GMOs that are used in production processes (closed system) (KBCH, 2022b). Since GM plants, animals, and microorganisms are produced in manufacturing facilities, preventing their leakage into the external environment is one of the most critical issues in safety management. It is crucial to monitor possible external outflows that may occur during production, transportation, disposal, and wastewater treatment, as GM microorganisms are difficult to detect by visual inspection.

Qualitative polymerase chain reaction (PCR) methods are generally used to detect GM microorganisms, but real-time quantitative PCR methods are used for quantitative analysis (Ali et al., 2012; Dreo et al., 2014; Kim et al., 2016). Moreover, direct PCR can be used to directly quantify microorganisms in a high-throughput manner by acquiring template DNA directly from the organism, thus reducing the cost and time by bypassing the DNA extraction step (Park et al., 2021). Direct PCR analysis methods have been developed and reported in various species, including bacteria (Lee et al., 2009b; Ben-Amar et al., 2017), algae (Chen et al., 2019), fungi (Walch et al., 2016), and viruses (Choo and Kim, 2006). However, direct PCR

assays can result in false positives due to DNA amplification from dead cells or ejected DNA in addition to living cells (Rudi et al., 2005). Noke et al. developed a method to circumvent this issue by treating samples with propidium monoazide (PMA) dyes (Nocker et al., 2006), which penetrate membrane-damaged or dead cells, but not living cells. Thus, PMA only binds to ejected DNA or DNA templates from membrane-damaged or dead cells and inhibits their PCR amplification. As a result, PMA treatment enables selective amplification of DNA templates from living cells during direct PCR (Nocker et al., 2006; Zhao et al., 2013).

Relatively few studies have reported the monitoring of environmental emissions of GM microorganisms, which have been described for crops such as corn (Lee et al., 2009a; Han et al., 2014; 2015), soybeans (Lee et al., 2009a), canola (Lee et al., 2007; Lee, 2020), and grass (Lee et al., 2014). Therefore, this study aimed to establish safety management protocols for monitoring the environmental emissions of GM microorganisms. Here, we describe a new method for the qualitative and quantitative monitoring of *E. coli* in environmental emissions by employing direct PCR combined with PMA treatment.

Materials and Methods

Escherichia coli culture and cell suspension preparation

A modified pGAL-HIR525 (6,593 bp) plasmid harboring an ampicillin-resistance gene (*AmpR*), was transformed into *E. coli* DH5α cells. A single transformed colony was inoculated into 10 mL Luria-Bertani (LB) broth (BD Difco™, Franklin Lakes, NJ, USA) supplemented with 50 µg·mL⁻¹ of ampicillin and grown overnight (18 h) at 37℃ with 150 rpm of agitation. The optical density at 600 nm (OD_{600}) was determined in triplicate samples, and 1 mL of O/N (18 h) cell culture was used for serial cell dilutions. A 10^{-1} diluted cell suspension was prepared by adding 100 µL of cell culture to 900 µL of LB broth. Further diluted cell suspensions of 10^{-2} to 10^{-8} were prepared using the same method. All dilutions were performed on ice.

Primer design for PCR and TaqMan real-time PCR (qPCR) assay

Two primer sets specific for the ampicillin resistance gene (*AmpR*) and the *E. coli* taxon-specific endogenous D-1-deoxyxylulose 5-phosphate synthase gene (*dxs*, GenBank accession number AF035440; Lee et al., 2006) were used. For dual-plex qPCR, dual-labeled hydrolysis probes of 5′-FAM/3′-BHQ1 for *AmpR* and 5′-HEX/3′-BHQ1 for *dxs* were designed as shown in Table 1.

Table 1. Primers and probes targeting the ampicillin resistance gene, *AmpR,* and the taxon-specific, single copy gene, D-1-deoxyxylulose 5-phosphate synthase gene, *dxs,* in *E. coli*.

Target gene	Direction	Primer and probe sequence	Product size
AmpR	Forward	5′-GGTTTCTTAGACGTCAGGTGGC-3′	181 bp
	Reverse	5′-GGGAATAAGGGCGACACGG-3′	
	Probe	5′-FAM-CGGGGAAATGTGCGCGGAACCCC-BHQ1-3′	
dxs	Forward	5′-AAGGCATTGTGAAGCGTCGT-3′	160 bp
	Reverse	5′-CTGGCGGCCATTTCCAGAAT-3′	
	Probe	5′-Hex-CGCTGAACGCCACGCTGGTCG-BHQ1-3′	

DNA standard curve production

E. coli genomic DNA and pGAL-HIR525 plasmid DNA were extracted using Wizard® Genomic DNA purification Kit (Promega, Madison, WI, USA) and Wizard® Plus Minipreps DNA purification system (Promega, Madison, WI, USA), respectively, following the kit instructions. DNA quality and concentration were confirmed through DNA electrophoresis using 1.5% agarose gels and a NanoDrop spectrophotometer (MicroDigital Co., Ltd., Seongnam, Korea), respectively.

Standard curves of *AmpR* and *dxs* were constructed using a 10-fold serial dilution series ranging from 1×10^3 to 1×10^8 copies·μL^{-1} of genomic (4,534,037 bp) and plasmid DNA (6,593 bp), respectively. Copy numbers·μL^{-1} were calculated from genomic and plasmid DNA concentrations using the ratio of (6.02×10^{23} [copy·mol^{-1}] × DNA amount [g]) to (DNA length [bp] × 660 [g·mol^{-1}·bp^{-1}]) as described by Whelan et al. (2003). For the *AmpR* gene, single-plex qPCR was performed using serially diluted plasmid DNA to produce *AmpR* standard curve. However, the standard curve for *dxs* was produced by dual-plex qPCR using serial diluted genomic DNA. A total of two intra-runs with three repeats of each qPCR run were performed and mean quantification cycle (Cq) values of each diluted DNA were used for producing standard curves for *AmpR* and *dxs*. The Cq values were plotted against the logarithm of their initial template copy numbers. Each standard curve was generated using linear regression of the plotted points. PCR amplification efficiency (E) was calculated from the slope of each standard curve following the equation $E = 10^{-1/slope} - 1$ (Rasmussen, 2001). Relative standard deviation (RSD%) and bias% were calculated to evaluate qPCR precision and trueness of both *AmpR* and *dxs*. Bias% was calculated using the following formula: bias% = (mean value - true value)/true value*100.

PCR and dual-plex qPCR analysis

PCR was performed using SapphireAmp® fast PCR Master mix (Takara Bio, San Jose, CA, USA) in a total volume of 25 μL, which contained 1 μL diluted cell suspension (on ice), 10 pmol each of the forward and reverse primers, and 12.5 μL master mix. Nuclease-free water (SDW) was used as the non-template control. The following amplification conditions were used: pre-denaturation at 95℃ for 5 min and 30 cycles of 95℃ for 15 s, 60℃ for 10 s, and 72℃ for 15 s. PCR products were confirmed on a 1.5% agarose gel.

Dual-plex qPCR was performed on a StepOne™ Real-time PCR System (Applied Biosystems, Foster City, CA, USA). Reactions were carried out in a final reaction volume of 20 μL and consisted of 10 μL of TOPreal™ qPCR 2X PreMIX (TaqMan Probe for multiplex, Enzynomics, Daejeon, Korea), 10 pmol of two sets of primers/probes, and 1 μL cell suspension (on ice) or 1 μL DNA. SDW and LB broth were used as negative controls. qPCR was performed under the following conditions: 95℃ for 10 min and 40 cycles of 95℃ for 30 s and 60℃ for 40 s. Target DNA abundance was calculated using Cq values, which correspond to the number of cycles required to reach a systematic default threshold value. A total of 10 colonies were cultured, and 10 qPCR intra-runs with three repeats of each qPCR run were used for qPCR assays. Relative standard deviation (RSD%) was calculated to evaluate qPCR precision of both *AmpR* and *dxs*.

PMA treatment for viable cell quantification

Each 450 μL of serially diluted *E. coli* cell suspension was prepared and treated with 1 μL of 20 mM PMA (PMAxx™ Dye, Biotium Inc., Fremont, CA, USA) and was incubated for 5 min in the dark (with shaking). The samples were exposed on PMA-Lite™ LED Photolysis Device (Biotium Inc., Fremont, CA, USA) for 15 min following manufacturer's instructions. Each 1 μL PMA-treated cell suspension was directly used for qPCR assay. PMA-qPCR assays were performed in five intra-

runs with one qPCR run consisting of two repeats of PMA-treated dilutions and one repeat of non-PMA treated control dilution. For sensitivity determinations, four PMA-qPCR runs with three repeats of each run were additionally performed using diluted cell suspensions from 10^{-3} to 10^{-6} dilution. The limit of detection ($LOD_{95\%}$) was determined using the Quodata web application (Grohmann et al., 2016; https://quodata.de/content/validation-qualitative-pcr-methods-single-laboratory).

Determination of colony forming unit (CFU) using qPCR and plate counting

Cell count·μL^{-1} of *E. coli* was estimated using qPCR mean Cq and PMA-qPCR mean PMA-Cq values of each dilution, based on the DNA standard curves of both targets. CFU per microliter of *E. coli* was determined using plate counting on *AmpR* LB plates. Each 100 μL of 10^{-6}, 10^{-7}, and 10^{-8} diluted cell suspension was spread individually on the plates, and CFU per microliter was calculated as follows: CFU·μL^{-1} = count number $\times$ 1/dilution rate $\times$ 1/inoculum /1,000. A total of 10 replications were performed (same samples were used for qPCR assays) and CFU per microliter was determined from the mean value of 10 repeats.

Statistical analysis

Statistical analysis were performed using Microsoft Excel to determine the average values (Mean) and standard deviation (SD) of Cq, PMA-Cq and plate counting for CFU·μL^{-1}. Also, regression analysis was used to construct standard curves, and to determine dynamic ranges of diluted cell suspensions and PMA-treated diluted cell suspension.

Results

PCR analysis of serially diluted *E. coli* cell suspensions

Serially diluted *E. coli* cell suspensions were used as templates in place of DNA for direct PCR amplification of *AmpR* and *dxs*. Fig. 1 shows the amplification specificity of *AmpR* and *dxs* primer sets. We detected one specific fragment of the expected size corresponding to 181 and 160 bp for *AmpR* and *dxs*, respectively. No non-specific products were detected using SDW, indicating high primer specificity for both target genes. Serial dilution PCR revealed that the detection limits of *AmpR* and *dxs* were 10^{-4} and 10^{-3} diluted samples, respectively.
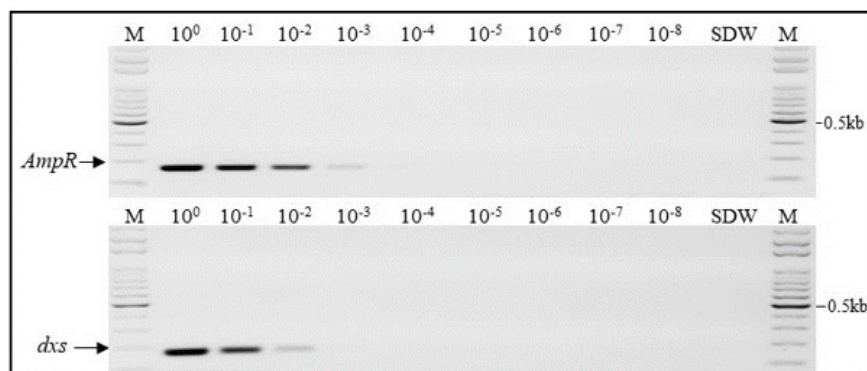

Fig. 1. Serially diluted direct cell suspension PCR analysis for *E. coli AmpR* and *dxs* genes. 10^{0} indicates cell culture with a mean CFU of 1.31 $\times$ 10^{6} per microliter (μL); SDW indicates nuclease-free water as the negative control.

qPCR parameters of DNA

qPCR performance including efficiency, slope, and correlation coefficient were determined using standard curves obtained from genomic and plasmid DNA isolated from *E. coli*. As shown in Fig. 2, both *AmpR* and *dxs* assays showed good performances in the range between 1×10^3 and 1×10^8 DNA copies, corresponding to plasmid DNA of 7.23 - 723 pg and genomic DNA of 5 - 500 ng, respectively. The standard curve efficiencies were 102.0% and 96.5% with slopes of -3.2727 and -3.4094, and linear correlation coefficients of both standard curves were 0.9983 and 0.9987, for *AmpR* and *dxs*, respectively. qPCR assay precision, evaluated using relative standard deviation (RSD%) of the Cq values obtained in six qPCR assay varied from 0.24 to 2.57% for *AmpR* and 0.31 to 1.38% for *dxs*. Trueness expressed in terms of bias (bias%) ranged from 4.95 to 20.55% for *AmpR* and -3.52 to 20.34% for *dxs* (Table 2). qPCR assays for both targets showed an acceptable level of precision and trueness, which were within the limit of $\pm 25\%$ as detailed in European Network of GMO Laboratories (ENGL) guidelines.
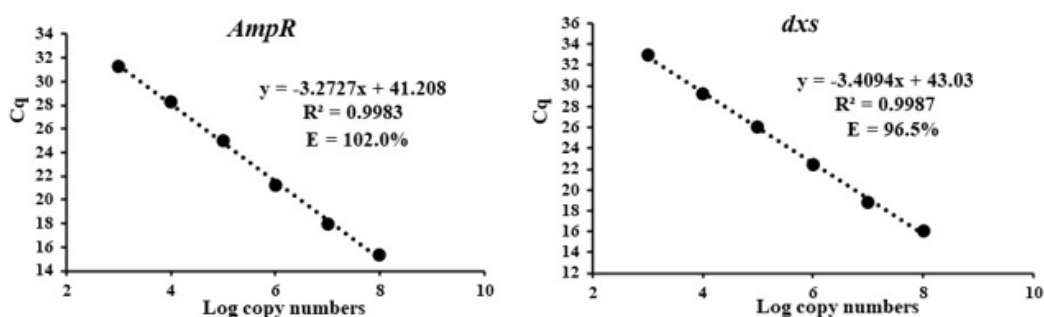

Fig. 2. Standard curves of *AmpR* and *dxs* produced via qPCR assays using serial dilutions of plasmid and genomic DNA extracted from *E. coli*, respectively. Cq, quantification cycle; E, qPCR efficiency; R^2, linear correlation coefficient.

Table 2. Quantitative real-time PCR (qPCR) repeatability for two genes using serial dilutions of plasmid and genomic DNA extracted from *E. coli*.

Target gene	DNA copie	Mean Cq[y]	Mean copy number	SD	RSD (%)	Bias[z] (%)	Total DNA (ng)
AmpR	100,000,000	15.32	79,931,128	0.08	0.49	-20.07	0.723
	10,000,000	18.01	12,055,171	0.44	2.43	20.55	0.0723
	1,000,000	21.29	1,204,398	0.55	2.57	20.44	0.00723
	100,000	25.05	85,925	0.59	2.35	-14.08	0.000723
	10,000	28.26	9,050	0.67	2.38	-9.50	0.0000723
	1,000	31.33	1,049	0.08	0.24	4.95	0.00000723
dxs	100,000,000	16.09	79,816,182	0.18	1.11	-20.18	500
	10,000,000	18.89	12,034,407	0.26	1.38	20.34	50
	1,000,000	22.47	1,074,084	0.13	0.56	7.41	5
	100,000	26.04	96,480	0.08	0.31	-3.52	0.5
	10,000	29.30	10,648	0.38	1.31	6.48	0.05
	1,000	32.96	902	0.26	0.79	-9.85	0.005

SD, standard deviation; RSD, relative standard deviation.

[y] Cq means quantification cycle value of qPCR assay.

[z] Bias% was calculated as a ratio of (viable cell count of *dxs* - CFU)/CFU × 100.

qPCR and PMA-qPCR performance of *E. coli* cell suspensions

For cell suspension qPCR, 10 repeats of *E. coli* 18 h cultures were prepared and OD_{600} and CFU per milliliter were measured. As shown in Fig. 3A, 18 h cultures had an average OD_{600} of 0.70 $\pm$ 0.04 (range: 0.63 - 0.76), corresponding to an average CFU per milliliter of 1.31 $\times$ 10^9 (range: 1.21 $\times$ 10^9 - 1.42 $\times$ 10^9). The 10 culture replicates showed similar increases in cell density, determined by OD_{600} and $CFU\cdot mL^{-1}$ values, indicating that the growth phase of the 18 h cultures was not stationary and was suitable for qPCR and PMA-treated qPCR analysis. Dual-plex qPCR using culture stocks (10^0) revealed that the Cq values of *AmpR* and *dxs* were 16.17 $\pm$ 0.06 and 22.23 $\pm$ 0.31, respectively (Table 3). Compared to non-PMA treated samples, PMA treatment resulted in higher qPCR Cq values of 18.57 $\pm$ 0.19 and 24.18 $\pm$ 0.26 for both targets, respectively. Variations in Cq and PMA-Cq values were 0.37 and 1.00 RSD% for *AmpR* and 1.06 and 1.41 RSD% for *dxs*, indicating that the qPCR of three inter-run repeats and ten intra-run repeats had good repeatability and reproducibility.

Dual-plex qPCR analysis of serially diluted cell suspensions was performed and the mean Cq values from 10-fold (10^{-1}) to 100,000-fold (10^{-5}) cell dilutions of both target genes are shown in Table 3. *AmpR* and *dxs* qPCR showed good repeatability and reproducibility, corresponding to RSD% ranging from 1.76 to 3.04 and 1.75 to 3.62, respectively. qPCR performance for *AmpR* and *dxs* using five serial cell dilutions showed slopes of -3.285 and -3.134, with linear correlation coefficients of 0.9995 and 0.9988, corresponding to efficiencies of 101.6% and 108.5%, respectively (Fig. 3B and C). These results revealed that the target DNA concentration after qPCR amplification in 10-fold serially diluted cell suspension was identical with the diluted DNA sample, indicating no negative impact of using direct cell suspension on qPCR assays. Furthermore, LB broth and SDW were analyzed as negative controls under the same dual-plex qPCR conditions for both targets to evaluate possible matrix effects. The matrixes of LB broth and SDW had similar Cq values of 35.94 $\pm$ 0.98 and 36.10 $\pm$ 0.68 for *AmpR* and 36.28 $\pm$0.91 and 36.42 $\pm$ 0.59 for *dxs*, respectively, indicating that the combination of primer/probe sets of *AmpR* and *dxs* and the cell culture matrix did not show non-specific cross-reactivity in the qPCR assay. The PMA-treated serially diluted cell suspensions showed qPCR performance slopes of -3.0893 and -3.2302, with linear correlation coefficients of 0.9979 and 0.9976, corresponding to efficiencies of 110.7% and 104.0%, for both targets, respectively (Fig. 3B and C). These results indicated that qPCR amplification using diluted viable cells was not affected by PMA addition to the sample matrix. As a negative control, PMA-treated LB broth showed qPCR Cq values of 35.66 $\pm$ 1.92 and 36.66 $\pm$ 0.53 for both targets, respectively.

Total cell counts and viable cell counts were obtained using qPCR and PMA-qPCR based on the Cq values and constructed DNA standard curves. The mean total cell counts of *AmpR* and *dxs* were calculated to be 126.00 $\pm$ 50.40 ($\times$ 10^6) and 8.13 $\pm$ 6.42 ($\times$ 10^6), respectively, and the mean viable cell counts per microliter were 19.60 $\pm$ 6.63 ($\times$ 10^6) and 1.41 $\pm$ 0.55 ($\times$ 10^6), respectively (Table 3). Considering the copy number of the *dxs* gene is one, the theoretical cell density (true copy number) calculated using PMA-qPCR should be 1.41 $\pm$ 0.55 ($\times$ 10^6), revealing a bias of 7.71% from the plate counting (mean $CFU\cdot\mu L^{-1}$: 1.31 $\times$ 10^6). This bias did not overlap with the limit of 25% as detailed in ENGL guidelines, suggesting acceptable trueness of the PMA-qPCR assay. In addition, the plasmid copy number was calculated using a ratio of 15.50 from the total cell count of *AmpR* to that of *dxs* in the qPCR assay. For PMA-qPCR assay, a ratio of 13.90 was calculated from the viable cell count of *AmpR* to that of *dxs*, implying a plasmid copy number of 14 - 16.

Detection limit of viable cells using PMA-qPCR

The detection limit of dual-plex PMA-qPCR assay is the lowest viable *E. coli* cell numbers that can be detected with 95% confidence. The cell stock with an average CFU of $1.31 \times 10^6 \cdot \mu L^{-1}$ was serially diluted from 10-fold (10^{-1}) to 1,000,000-fold (10^{-6}), while *E. coli* cell numbers were estimated to be 1.31×10^5 to 1.31 copies·μL^{-1}, after PMA treatment. For all treated samples, qPCR consisted of a total of ten repeats containing two inter-run repeats and five intra-run repeats. To improve detection accuracy, we performed several replicates for a total of 22 repeat measurements for the 10^{-3} - 10^{-6}-fold diluted samples. Both assays of *AmpR* and *dxs* detected an average of 131 viable *E. coli* cells from 10,000-fold (10^4) diluted cell suspension (Table 4). Statistical analysis revealed that the $LOD_{95\%}$ on *AmpR* and *dxs* was 134 live cells with a 95% confidence interval of 83 - 216 cells detectable using dual-plex PMA-qPCR assays.
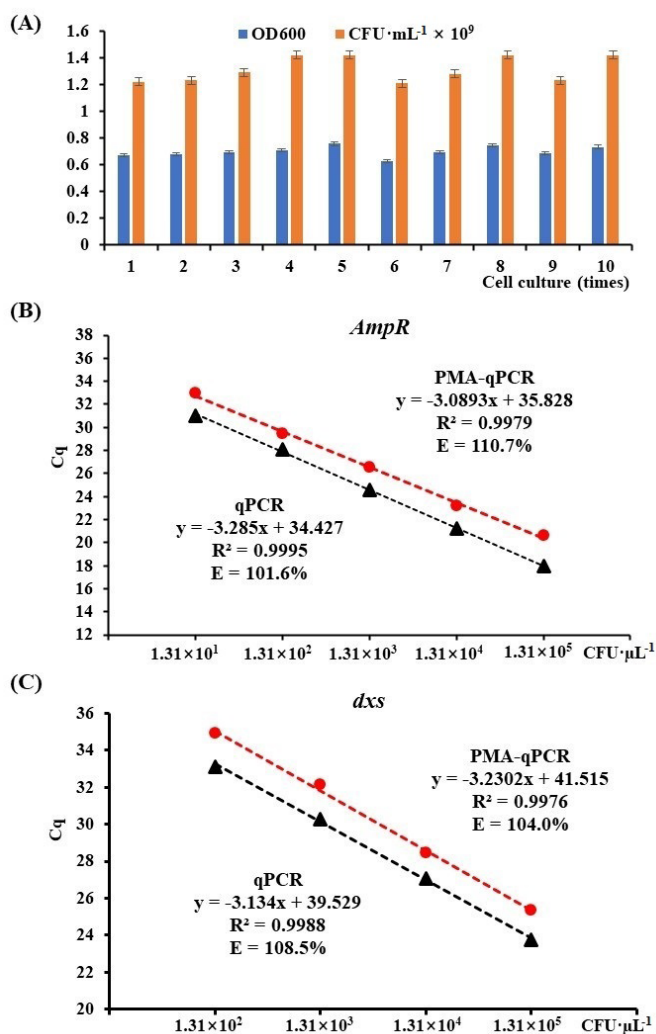

Fig. 3. Dual-plex quantitative real-time PCR (qPCR)d and propidium monoazide–quantitative real-time PCR (PMA-qPCR) assays targeting *AmpR* and *dxs* using serially diluted cell suspensions. (A) OD_{600} and CFU·μL^{-1} of 10 replicate qPCR cell culture suspensions. (B) Dynamic ranges of qPCR and PMA-qPCR for *AmpR*. (C) Dynamic ranges of qPCR and PMA-qPCR for *dxs*. Black triangles and red circles denote Cq of qPCR and PMA-qPCR at each serial cell dilution, respectively. Cq, quantification cycle; E, qPCR efficiency; R^2, linear correlation coefficient; OD_{600}, optical density at a wavelength of 600 nm.

Table 3. Quantitative real-time PCR (qPCR) and propidium monoazide–quantitative real-time PCR (PMA-qPCR) performance of the target antibiotic gene, *AmpR* and the taxon-specific gene, *dxs* on serially diluted *E. coli* cell suspensions.

Target gene	Cell dilution	qPCR			PMA-qPCR			CFU·μL^{-1} ($\times 10^6$)	Bias[y] (%)
		Cq[w] (mean ± SD)	RSD (%)	Total cell count·μL^{-1} ($\times 10^6$)[x]	PMA-Cq (mean ± SD)	RSD%	Viable cell count·μL^{-1} ($\times 10^6$)		
AmpR	10^{0z}	16.17 ± 0.06	0.37		18.57 ± 0.19	1.00		1.31 ± 0.01 (1.21 - 1.42)	7.71
	10^{-1}	17.97 ± 0.45	2.53	126.00 ± 50.40	20.61 ± 0.87	4.22	19.60 ± 6.63		
	10^{-2}	21.26 ± 0.47	2.22		23.24 ± 1.04	4.48			
	10^{-3}	24.58 ± 0.43	1.76		27.02 ± 0.95	3.51			
	10^{-4}	28.05 ± 0.51	1.82		30.57 ± 1.10	3.60			
	10^{-5}	31.00 ± 0.94	3.04		33.72 ± 1.38	4.09			
NC (matrix)	LB	35.94 ± 0.98	1.52		35.66 ± 0.68	1.92			
	SDW	36.10 ± 0.68	0.68		-	-			
Dxs	10^{0}	22.23 ± 0.31	1.41		24.18 ± 0.26	1.06			
	10^{-1}	23.76 ± 0.72	3.03	8.13 ± 6.42	25.35 ± 0.60	2.38	1.41 ± 0.55		
	10^{-2}	27.06 ± 0.67	2.47		28.47 ± 0.55	1.95			
	10^{-3}	30.29 ± 0.53	1.75		32.12 ± 0.79	2.45			
	10^{-4}	33.13 ± 1.20	3.62		34.90 ± 0.90	2.57			
	10^{-5}	35.43 ± 0.97	2.74		38.35 ± 1.09	2.85			
NC (matrix)	LB	36.28 ± 0.91	2.50		36.66 ± 0.53	1.44			
	SDW	36.42 ± 0.59	0.59		-	-			
Plasmid copy number[v]				15.50			13.90		

SD, standard deviation; RSD, relative standard deviation; NC, negative control (cell dilution medium); LB, Luria-Bertani; SDW, sterile distilled water.

[v] Plasmid copy number was calculated as a ratio of cell count (viable cell count) of *AmpR* to those of *dxs*.

[w] Cq means quantification cycle value of qPCR assay; Mean ± SD indicate average values and standard deviation.

[x] Total cell counts/μL and viable cell count/μL were calculated from Cq and PMA-Cq according to the DNA standard curves.

[y] Bias was calculated as a ratio of (viable cell count of *dxs* - CFU)/CFU × 100.

[z] 10^{0} refers to cell culture with a mean CFU value of 1.31×10^6 per microliter (μL).

Table 4. Limit of detection (LOD) confirmation using replicate propidium monoazide–quantitative real-time PCR (PMA-qPCR) runs on separate dilution series of suspension cells from *E. coli*.

Targets/cell dilutions	Number of positive reactions/total number of reactions (percentage of positive reactions)							LOD$_{95}$%
	Cell stock	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	
CFU·μL^{-1}	1,310,000	131,000	13,100	1,310	131	13.1	1.31	134 (83 - 216)
dxs	10/10 (100%)	10/10 (100%)	10/10 (100%)	22/22 (100%)	22/22 (100%)	3/22 (13.6%)	0/22 (0%)	
AmpR	10/10 (100%)	10/10 (100%)	10/10 (100%)	22/22 (100%)	22/22 (100%)	19/22 (86.3%)	5/22 (22.7%)	

Discussion

GMOs are commonly utilized in the food and feed industries to produce additives, enzymes, and flavorings. They frequently harbor antimicrobial resistance genes as selection markers, conferring a resistance to different antibiotics including chloramphenicol, ampicillin, kanamycin, bleomycin, and tetracycline (Fraiture et al., 2020). The acquisition of potential antimicrobial resistance genes through horizontal gene transfer from pathogens and other microorganisms is of considerable public health and environmental concern, requiring fast and effective detection methods to monitor living GMOs released into the surrounding environment, such as wastewater, air currents, and facility surfaces from large-scale food and feed processing industries.

Most environmental microbial monitoring experiments use DNA-based real-time PCR detection methods, requiring the filtration, concentration, and culture of the environmental samples using selection media (Heijnen and Medema, 2006). However, these real-time PCR-based methods have limited monitoring efficiency due to technical difficulties in microbial DNA extraction and estimation of the actual number of viable microbial cells present in environmental samples. Moreover, DNA quality and real-time PCR efficiency can be influenced by DNA extraction protocols, which affect DNA recovery rate and integrity, as well as contamination by DNA inhibitors (Piskata et al., 2019). Culture-based methods are currently used to distinguish dead and living microbial cells in environmental sample and to assess the number of living cells. However, real environmental microbial cell numbers may be overestimated due to concentration and culture growth of the target microorganism. Additionally, sample pretreatment with nucleic acid intercalating dyes such as PMA has been proposed to avoid qPCR false-positive results (da Cunha et al., 2020). PMA only binds to DNA from dead or membrane-damaged cells following exposure to bright light, and qPCR amplification of PMA-bound DNA is inhibited (Fittipaldi et al., 2012). However, PMA treatment can reduce DNA yields from treated cells compared to those from untreated living cells (Joo et al., 2019), which can reduce the detection efficiency of DNA-based real-time PCR.

Here, we describe a PMA-qPCR method to detect viable genetically modified *E. coli* cells harboring the ampicillin resistance gene, *AmpR*, by using direct cell suspensions. To quantify the number of living cells, an *E. coli* single copy endogenous gene, *dxs* (Lee et al., 2006), was used as an internal control, combined with *AmpR* for TaqMan probe based dual-plex qPCR analysis. In addition, the use of the taxon-specific endogenous gene, *dxs,* allows the screening of natural *AmpR*-resistant microorganisms to enhance the assay specificity of the target ampicillin-resistant *E. coli* strains. Cell suspensions were prepared from 18 h cultures, and 10 replicates representing an average OD_{600} of 0.70 and an average CFU of 1.31×10^6 per μL using plate counting were used. As a result, serially diluted cell suspension direct qPCR and PMA-qPCR assays showed good performances on inter- or intra-run replicates, and the use of cell suspensions had little effect on qPCR accuracy and precision, and was suitable for qPCR analysis compared to the use of DNA templates. These results are consistent with similar studies by Sung and Hawkins (2020) on the detection of mycoplasma contamination in mammalian cell lines, and the results indicated that the accumulation of metabolic waste products in cultured cell suspensions had no effect on qPCR precision and robustness.

DNA standard curves generated by plasmid DNA often overestimate the amount of DNA in qPCR due to contamination of different conformations of plasmid DNA, such as supercoiled circular DNA and linear DNA, known for differing DNA amplification efficiencies (Lin et al., 2011). We generated the *AmpR* standard curve using plasmid DNA, and the single copy gene *dxs* standard curve using genomic DNA, with the same dual-plex qPCR assay as the cell suspension, to evaluate the actual cell counts in cell suspensions of qPCR and PMA-qPCR assays. Based on DNA standard curves, we calculated theoretical total cell counts and viable cell counts from the Cq values from qPCR and PMA-qPCR of serially diluted cell suspensions. The 7.71% bias between calculated viable cell counts and CFU by plate counting did not overlap with the baseline $\pm$25% proposed by the ENGL (2015), indicating that monitoring viable *AmpR*-resistant *E. coli* cells using direct cell suspension PMA-qPCR assays was accurate and effective. Moreover, qPCR and PMA-qPCR showed similar cell count ratios of *AmpR* to *dxs*, indicating that the plasmid, pGAL-HIR525, had approximately 14 - 16 copies in *E. coli*.

Zhang and Fang (2006) suggested that reliable standard curves should exhibit slope values between -3.9 and -3.0, corresponding to qPCR efficiency between 80 and 115%, and R^2 values higher than 0.95 when qPCR is applied for microbial quantification in environmental samples. ENGL establishes that the variance of qPCR analysis based on relative standard deviations (RSD%), should be less than 25% for dynamic method ranges. Here, we report optimal DNA standard curves

for *AmpR* and *dxs*, and appropriate linear regression generated by Cq and PMA-Cq values of diluted cell suspensions. Thus, the number of living *AmpR*-resistant *E. coli* cells in environmental samples can be reliably quantified using PMA-qPCR standard curves as shown in Fig. 3B and C, instead of using DNA standard curves. The assay sensitivity confirmed that the detection limit for both targets was at least 134 viable *E. coli* cells with 95% confidence intervals using dual-plex PMA-qPCR. This work describes a novel TaqMan based dual-plex qPCR method to circumvent the technical limitations of DNA extraction, improve detection efficiency, reduce assay time and costs, and accurately detect the number of living *AmpR*-resistant genetically modified *E. coli* cells in environmental samples.

Conclusion

As industrial GM organisms are developed and commercialized, a detection method is needed to quickly and effectively monitor living GMOs emitted to the surrounding environment such as wastewater, air flow, and facility surfaces in large-scale food and feed processing industries. In this study, the PMA-treatment combining dual-plex TaqMan-based qPCR method targeting on *Amp*-resistant gene and taxon-specific gene *dxs* was developed to directly detect viable *Amp*-resistant *E. coli* cells from cell culture. qPCR and PMA-qPCR assays using diluted cell suspensions on both targets were confirmed acceptable levels of precision and trueness, which were within the $\pm 25\%$ limit detailed in ENGL guidelines. In addition, qPCR analysis was not affected by the cell culture matrix. The PMA-qPCR assay allows detection limits to be detected at 95% confidence of 134 viable *Amp*-resistant *E.coli* cells. The method can avoid high technical requirements for DNA extraction and purification, save time and cost, avoid false positive detection in living GM cells, and exclude natural *Amp*-resistant microorganisms. This work provides a feasible monitoring approach for living GMO detection in environmental samples.

Conflict of Interests

No potential conflict of interest relevant to this article was reported.

Acknowledgements

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