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Quantitative Polymerase Chain Reaction Assay to Study the Role of Genetic Elements Involved in Regulating Plasmid Copy Number

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We have created a quantitative polymerase chain reaction (qPCR) assay that can measure relative plasmid copy number. The assay uses a calibrator plasmid, pCHCS, containing both β-lactamase (*bla*) and d-1-deoxyxylulose 5-phosphate synthase (*dxs*). *bla* is a single-copy plasmid-based target gene and *dxs* is a single-copy reference gene encoded in the *Escherichia coli* genome. We generated standard curves from the *bla* and *dxs* qPCR amplification of various dilutions of pCHCS. These standard curves were used to relate gene copy numbers of *bla* and *dxs* to qPCR C_q values. We demonstrated that our assay responds in a dose-dependent manner. Using this assay we attempted to evaluate the copy number of several ColE1- derived plasmids bearing mutations in genetic elements involved in regulating plasmid copy number. The results followed the trend of increasing plasmid copy numbers in *E. coli* DH5α cells harboring plasmids pBR322, pBART or pUC19. We discuss the utility and some of the remaining challenges associated with this assay for measuring plasmid copy number.

pBR322 and pUC19 are two ColE1-type plasmids commonly used to introduce foreign DNA into cells via transformation experiments (1). Plasmids must control their replication to coexist within their host with a minimal metabolic burden (2). There are three main types of control mechanisms for plasmid copy number. One mechanism involves sequences, known as iterons, which bind to replication initiator proteins (2). The second mechanism involves antisense RNA binding to proteins, and the third mechanism involves antisense RNA binding to a complementary RNA primer (2). The inhibition or activation of these control mechanisms dictate the plasmid copy number of different plasmids within cells.

Certain genetic elements can facilitate or interfere with the plasmid copy number control mechanisms within a cell. For example, Rop, a protein encoded by some plasmids, interferes with plasmid replication by stabilizing the interaction between antisense RNA and the RNA primer required for plasmid replication (1). The antisense RNA is known as RNA I, and the RNA primer is known as RNA II. Rop-enabled hybridization therefore slows down plasmid replication. On the other hand, some plasmids destabilize the RNA hybrid, therefore increasing plasmid replication. For example, plasmids can encode a G to A point mutation in RNA II and this decreases the ability for RNA I to hybridize to RNA II (1). Plasmid size is an additional plasmid characteristic that is hypothesized to interfere with plasmid replication control mechanisms (3). It is thought that since a larger plasmid will place a higher metabolic burden on the cell, larger plasmids will have lower copy numbers (3).

Understanding the effect of each of these genetic elements on plasmid copy number can enable a better understanding of plasmid maintenance. Previously it has been observed that when pUC19 and pBR322 are transformed into the same cell only pUC19 is maintained. We sought to elucidate the role of each of the replication control mechanisms during pUC19 co-transformation with pBR322. is excluded in co-transformations of these plasmids into *E. coli* cells (3). pUC19 does not express the Rop protein, does encode the G to A point mutation in RNA II, and has a smaller size (Table 1) (3). On the other hand, pBR322 does express Rop protein, does not encode the G to A point mutation in RNA II, and has a large plasmid size (Table 1) (3). These characteristics explain the fact that pBR322 will have a lower plasmid copy number than pUC19 in single transformation experiments (3).

To understand the contribution of each of these characteristics on plasmid copy number, each characteristic must be studied independently. Previous groups have created plasmids that can enable this (Table 1). pANPT was created by Al-Shaibani *et al*. (2014), and is a pBR322-derived plasmid that has its tetracycline resistance gene deleted (Table 1) (5). pBART was created by Chang *et al.* (2014), and is a pUC19-derived plasmid with the insertion of the tetracycline resistance gene and the *rop* gene (Table 1) (6). pCAWK is a pBR322-derived plasmid with insertional inactivation of the *rop* gene, created by Airo *et al.* (2012) (Table 1) (7). We must quantify the plasmid copy numbers of cells transformed with each of these plasmids to determine the contribution of each characteristic on the differing plasmid copy numbers.

Past techniques to measure plasmid copy number have involved DNA hybridization protocols, which are very laborious and often do not provide accurate results due to the short lifespan of radioactively labeled probes (4). Lee *et al.* (2006) have reported a real-time qPCR assay that yields fast, reliable measures of plasmid copy number (4). Lee *et al*. were able to calculate the copy number of pBR322 by using ratios of *bla* gene presence to *dxs* gene presence measured through qPCR experiments (4). *bla* is a single-copy gene for β-lactamase found on the pBR322 plasmid (but is not found in *E. coli* genome) (4). *dxs* is a single-copy gene for d-1-deoxyxylulose 5-phosphate synthase found on *E. coli* genome (but is not found in plasmids) (4). *bla* can therefore be referred to as the target

TABLE 1. Characteristics of plasmids derived from pUC19 and pBR322.

Plasmid	Original Plasmid	Special Features	Size (bp)	Rop[1]	G to A mutation[2]	Plasmid Source
pUC19	pUC19	N/A	2686	No	Yes	-
pBART	pUC19	Insertion of *tet*^{R} and *rop*	4989	Yes	Yes	Chang *et al.* (2014)
pBR322	pBR322	N/A	4361	Yes	No	-
pCAWK	pBR322	Insertional inactivation of *rop*	4395	No	No	Airo *et al.* (2012)

1 Rop is a protein that stabilizes the dimerization of RNA I with RNA II, resulting in decreased replication efficiency.
2 The G to A point mutation is in RNA II, which decreases the affinity of RNA I for RNA II, resulting in increased replication efficiency.

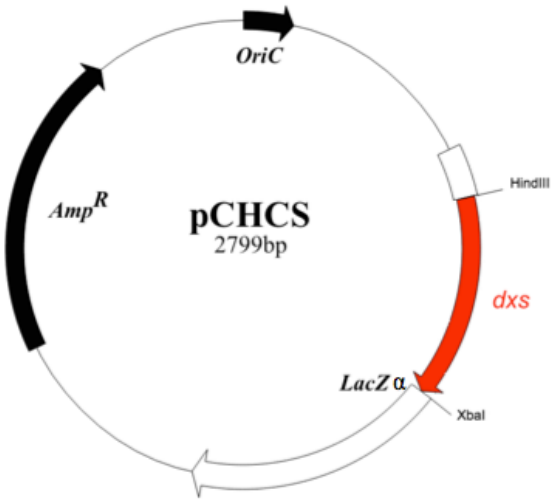

FIG 1. Plasmid map of pCHCS plasmid.

gene, which will vary with plasmid copy number and *dxs* as the reference gene, which is represented at one copy per cell. Lee *et al.* (2006) created a vector containing both *dxs* and *bla* in order establish a standard curve for target gene amplification during qPCR versus gene copy number (4). This standard curve was used to calculate plasmid copy number (4).

We sought to apply the qPCR approach outlined by Lee *et al.* (2006) to create an assay to measure plasmid copy number. We describe the construction of pCHCS, a pUC19-derived plasmid containing a single copy of the *bla* gene and the *dxs* gene (Fig 1) (4). We demonstrate that this assay is dose responsive by titrating the concentration of input plasmid. This assay can be used to understand the role of genetic elements that have been manipulated in plasmid variants such as pANPT, pBART and pCAWK. By comparing the respective plasmid copy numbers of different plasmids, we can determine the effect of each factor hypothesized to affect plasmid copy number. This can enable an understanding of the characteristics of plasmids that allow their maintenance versus exclusion.

Bacterial strains, plasmids, growth media. *E. coli* strain DH5α, as well as *E. coli* DH5α harboring the plasmids pUC19, pBR322, pANPT, and pBART were obtained from the culture collection of the Microbiology and Immunology Department at the University of British Columbia. Bacteria were cultured in Luria-Bertani (LB) liquid media made from 10 g/L of tryptone, 5 g/L of yeast extract and 10 g/L of NaCl. Overnight cultures were prepared by inoculating 5 mL of LB broth incubated at 37°C on a shaking platform set to 190 rpm for 15-20 hours. Ampicillin (Sigma-Aldrich) was at a final concentration of 25μM.

Colony PCR to amplify the *dxs* gene from *E. coli* DH5α cells. Modified *dxs* primers were ordered from Integrated DNA Technologies (IDT). These primers were designed with a HindIII restriction site added on to the 5'- end of the forward primer and an XbaI restriction site added on to the 5' - end of the reverse primer, as shown in Table 2. PCR was performed using Platinum® *Pfx* DNA Polymerase (Invitrogen™, cat #11708-013). The colony PCR was performed as per the manufacturer's instruction. Touchdown PCR was performed using the Whatman Biometra T-gradient thermocycler with the following cycling conditions: complete denaturation for 5 minutes at 94°C, 35 amplification cycles with 94°C for 15 seconds, 50°C - 55°C with -0.15°C increments for 30 seconds, 68°C for 1 minute, and final elongation for 5 minutes at 68°C. Positive controls for PCR were set up using primers for *E. coli yidC* gene. The *yidC* primers were obtained from the UBC Microbiology and Immunology department (8). PCR products were purified using PureLink® PCR Purification Kit (Invitrogen, cat#K3100-01) following the method described in manufacturer's manual. The concentration of the purified product was determined and the purity of the sample was confirmed using a Thermoscientific NanoDrop 2000c Spectrophotometer.

Gel electrophoresis of PCR product. 5 μL of 10X loading buffer (Invitrogen™) was added to 5 μL 100 bp DNA ladder (Invitrogen™, cat #15628-019), and 2 μL of loading buffer added to each PCR reaction. 20 μL of each PCR reaction (with loading buffer) was then loaded into a 2.0% (w/v) agarose DNA gel (2.0 g agarose, 100 mL SYBR® safe DNA gel stain in 0.5X TBE (Invitrogen™)). 5 μL of the DNA ladder (with loading buffer) was loaded into two wells. The gel was run in a 1X TBE buffer solution diluted from a 5X stock solution of TBE buffer (54 g/L Tris, 27.5 g/L boric acid, 3.75 g/L Na_2EDTA, distilled water) at 100 volts for 75 minutes. The gel was then imaged using an Alpha Imager (Multimage™ Light Cabinet).

pUC19 plasmid isolation. A colony of pUC19-bearing *E. coli* DH5α cells was used to inoculate 5 mL of LB broth with a 100 μg/mL concentration of ampicillin (Sigma- Aldrich). Plasmid DNA was isolated using the Invitrogen PureLink® Quick Plasmid Miniprep kit (cat#K2100-11) according to the manufacturer's instructions. The concentration and purity of the isolated plasmid

TABLE 2. Target and amplicon information for primers used in conventional PCR and qPCR.

Target	Primer Sequence (5' to 3')		Expected amplicon size (bp)
*dxs**	Fwd	CATTCTAGACGAGAAACTG GCGATCCTTA	113
	Rev	CATAAGCTTCTTCATCAAGC GGTTTCACA	
dxs	Fwd	CGAGAAACTGGCGATCCTT A	113
	Rev	CTTCATCAAGCGGTTTCACA	
bla	Fwd	CTACGATACGGGAGGGCTT A	81
	Rev	ATAAATCTGGAGCCGGTGA G	

*This primer set includes a HindIII restriction site on Fwd primer and an XbaI restriction site on the Rev primer.

were determined using the Thermoscientific Nanodrop 2000c Spectrophotometer.

Restriction digest of pUC19 and *dxs* PCR product. Restriction enzyme kits for XbaI and HindIII were obtained from Invitrogen™ (cat#15226-012 ,cat#15207-012). Isolated pUC19 plasmid DNA was double digested with XbaI and HindIII. We followed the manufacturer's protocol, using 10 µL of DNA, and 1 µL each of XbaI and HindIII. A similar double digest was performed with *dxs* PCR product but with 15 µL of DNA sample added. Digests were incubated at 37°C for 1 hour to induce enzyme activity and then 65°C for 20 minutes to inactivate the enzymes.

Ligation of *dxs* into pUC19 to create pCHCS. Digested *dxs* was ligated into digested pUC19 using Invitrogen's Rapid ligation protocol for plasmid cloning of DNA fragments. 1 unit of T4 DNA ligase, 2.74 µL of digested pUC19 (19.4 ng/µL) and 0.23 µL of digested *dxs* (28.9 ng/µL) were used in the reaction, obtaining a 3:1 plasmid to vector ratio. A negative control sample was set up in parallel, but with the addition of water instead of *dxs* insert DNA. Ligation reaction tubes were incubated overnight for 21 hours.

Preparation of competent *E. coli* DH5α cells. Competent cells were prepared following the Hancock Lab $CaCl_2$ *E. coli* transformation protocol (9). Competent cells were then suspended in 0.1 M $CaCl_2$ with 15% v/v glycerol. 50 µL aliquots were made and stored in the -80°C freezer.

pCHCS transformation into competent *E. coli*. A heat-shock transformation protocol, based on the Hancock Lab $CaCl_2$ *E. coli* transformation protocol, was used to transform competent *E. coli* cells with pCHCS (9). Dilutions of transformed cells were spread-plated onto LB agar plates containing 25 µM ampicillin supplemented with 40 µL of 0.1mM isopropyl β-D-1-thiogalactopyranoside (IPTG; Invitrogen™, cat#15529-019), and 40 µL of 20 M 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; Fermentas, cat #R0404) added to facilitate blue/white screening. Cells were incubated at 37°C overnight and colony counts were obtained.

Screening pCHCS plasmid. A white colony from the pCHCS-transformed *E. coli* DH5α cells was used to inoculate 5 mL of LB broth with a 100 µg/mL concentration of ampicillin (Sigma-Aldrich). Plasmid DNA was isolated using the PureLink® Quick Plasmid Miniprep kit (Invitrogen™, cat#K2100-11) according to the manufacturer's instructions. The concentration and purity of the isolated plasmid were determined using the Thermoscientific Nanodrop 2000c Spectrophotometer. *bla* and *dxs* were amplified from the isolated pCHCS plasmid using the primers outlined in Table 2. The touchdown PCR protocol was performed using the Whatman Biometra T-gradient thermocycler with the following cycling conditions: complete denaturation for 5 minutes at 94°C, 35 amplification cycles with 94°C for 15 seconds, 50°C -55°C with -0.15°C increments for 30 seconds, 68°C for 1 minute, and final elongation for 5 minutes at 68°C. The PCR reactions were set up according to the manufacturer's manual with 5 µL of 10X *Pfx* Amplification Buffer. pCHCS was also submitted for Sanger sequencing at the Michael Smith Laboratories to further confirm the presence of *dxs* within the original pUC19 plasmid.

Preparation of template DNA for qPCR. Colonies of *E. coli* DH5α cells harboring pBR322, pUC19, pBART or pCAWK were used to inoculate 5 mL of LB broth containing 100 µg/mL of ampicillin (Sigma- Aldrich). The cultures were grown to exponential phase. The growth phase was determined by monitoring the optical density (OD) at 600 nm of the cultures using the Ultrospec 3000 UV/visible Spectrophotometer. DNA was extracted from the samples using the Invitrogen Purelink® Genomic DNA Mini Kit, following the method for Gram-negative bacterial cells described in the manufacturer's manual. The concentration of extracted DNA was measured using a Thermoscientific Nanodrop 2000c Spectrophotometer. The concentration of DNA in each sample was normalized to a concentration of 2 ng/µl using sterile water.

Previously isolated pUC19 plasmid was also added to this template DNA at differing amounts for one of the qPCR experiments.

qPCR using SYBR Green. The BIORAD CFX Connect Real Time System was used for qPCR amplification and analysis. The 20 µl qPCR reaction was prepared as follows: 10 µl of SsoAdvanced™ Universal SYBR® Green Supermix, 1 µl of each 10 µM primer, 2 µl of template DNA and 6 µl of sterile water. The primer sequences for *bla* and *dxs* are shown in Table 2. The cycling protocol was adapted from Lee *et al.* (2006) as follows: initial denaturation for 10 minutes at 95°C followed by 40 cycles of 10 seconds at 95°C, 10 seconds at 62°C and 10 seconds at 72°C (4). The fluorescence signal was measured at the end of each extension step at 72°C. A melting curve analysis was performed at the end of the amplification with a temperature gradient of 0.1°C/s from 70°C to 95°C to confirm the amplification of only one species.

Creation of standard curves to determine plasmid copy number. 10-fold serial dilutions of pCHCS ranging from 1 $x10^5$ to 1 $x10^9$ copies/µl were made to create standard curves for *bla* and *dxs*. A Thermoscientific Nanodrop 2000c Spectrophotometer was used to measure the concentration of the samples and the plasmid length was calculated to be 2799 bp. The following equation was used to calculate the corresponding plasmid copy number (10).

$$\text{DNA (copy)} = \frac{6.02 \times 10^{23}(\text{copy/mol}) \times \text{DNA amount(g)}}{\text{DNA length(dp)} \times 660(\text{g/mol/dp})}$$

C_q values for the amplification of both *bla* and *dxs* of each dilution were obtained in duplicate using qPCR. The average C_q values were plotted against the logarithm of the initial template copy numbers to create the standard curves.

Absolute quantification of plasmid copy number. We used our *bla* and *dxs* standard curves from pCHCS for this quantification (Fig 4). The C_q values from the triplicate qPCR runs of each of our experimental samples were averaged. These average C_q values were used to calculate gene copy numbers for *bla* and *dxs* using the equation derived from the standard curves. The gene copy number for *bla* was divided by the gene copy number for *dxs* to obtain plasmid copy number of each experimental sample.

Characterization of pUC19, pBR322, pANPT, pCAWK and pBART. *E. coli* DH5α transformed with each respective plasmid was used to inoculate 5 mL of LB broth with a 100 µg/mL concentration of ampicillin (Sigma- Aldrich). Plasmid DNA

samples from the overnight cultures were isolated using the Invitrogen PureLink® Quick Plasmid Miniprep kit (cat#K2100-11) according to the manufacturer's instruction. The concentration and purity of the isolated plasmid were determined using the Thermoscientific Nanodrop 2000c Spectrophotometer. Plasmids were digested using HindIII restriction enzyme (Invitrogen™, cat#15207-012) according to protocol described by manufacturer's instructions. 20µL of the digested plasmids, with 5 µL of 10X loading buffer (Invitrogen™) added, and 5 µL of Quick-Load® 2-Log DNA Ladder (0.1-10.0 kb) (New England Biolabs, cat #N0469S) were loaded onto a 1.2% (w/v) agarose gel. The gel was run in 1X TBE buffer and electrophoresed at 100 volts for 1 hour. Bands were visualized and imaged using an Alpha Imager (Multimage™ Light Cabinet).

RESULTS

Construction of pCHCS, a plasmid containing a single copy of *bla* and *dxs*. Our goal was to create a plasmid with both the *bla* and *dxs* genes. To do this we used colony PCR to amplify *dxs* from *E. coli* DH5α cells and purified this gene product. We then used HindIII and XbaI restriction enzymes to digest both the purified PCR product and pUC19 plasmid (containing *bla* gene). The digested plasmid and PCR product were ligated together and transformed into *E. coli* DH5α cells. Ampicillin resistant transformants were screened on agar plates containing X-gal and IPTG. The ligation of *dxs* into the *lacZ*α portion of the multiple cloning site on pUC19 was expected to disrupt *lacZ* coding for β-galactosidase. This eliminates the ability of the transformed bacteria to breakdown X-gal resulting in white colonies of cells transformed with plasmids carrying the *dxs* insertion. Two white colonies were chosen at random and screened for the *dxs* insertion using PCR. PCR of the plasmid isolated from the first white colony resulted in amplification of the *dxs* gene, but not the *bla* gene (Fig 2). The plasmid isolated from the second white colony resulted in amplification of both the *dxs* and the *bla* genes (Fig 2).

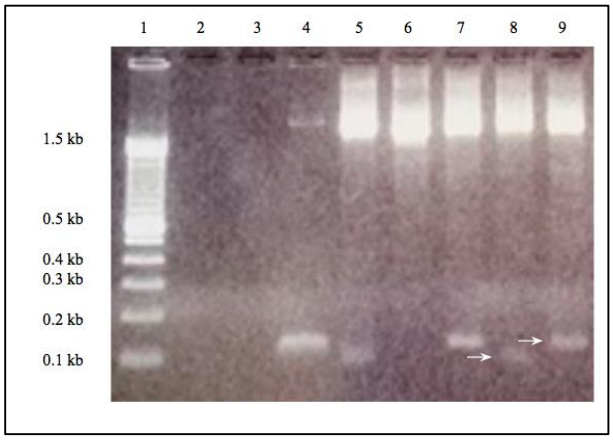

FIG 2. *dxs* and *bla* amplification from pCHCS. Lane 1: 100 kb DNA ladder, lane 2: *dxs* primers (negative control), lane 3: *bla* primers (negative control), lane 4: *dxs* primers and *E. coli* DH5α genomic DNA (positive control), lane 5: *bla* primers and pUC19 (positive control), lane 6: *bla* primers and plasmid DNA isolated from sample colony number one, lane 7: *dxs* primers and plasmid DNA isolated from sample colony number one, lane 8: *bla* primers and plasmid DNA isolated from sample colony number two, lane 9: *dxs* primers and plasmid DNA isolated from sample colony number two.

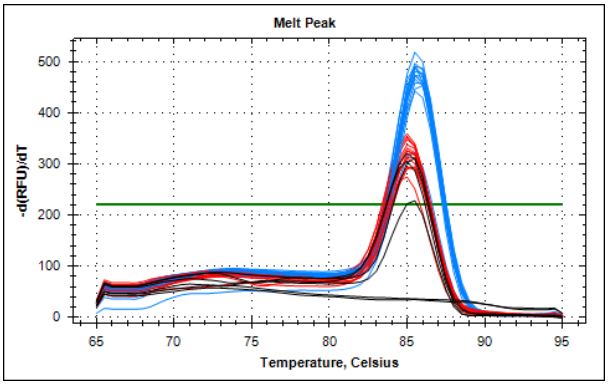

FIG 3. Melt curve analysis for the qPCR amplification of *bla* and *dxs* from diluted samples of pCHCS. The black lines represent melt curves from the amplification of negative controls (template + no primers; primers + no template). The red lines represent melt curves from the amplification of *bla*. The blue lines represent melt curves from the amplification of *dxs*.

The presence of the *dxs* insertion was confirmed by sequencing (Fig S1). Taken together these results show that a plasmid containing both the *dxs* and *bla* gene was created.

qPCR standard curve generation using pCHCS. Standard curves were created so that the gene copy numbers of *bla* and *dxs* in experimental samples could be determined from C_q values. These experimental samples consist of DNA from *E. coli* DH5α cells harboring plasmids. The gene copy numbers obtained from the standard curves can be used to determine plasmid copy numbers of the experimental samples. The curves were created by performing qPCR of multiple dilutions of pCHCS and obtaining C_q values for *dxs* and *bla* genes. The two curves were similar but not identical. The curve for *dxs* amplification had a greater y-intercept value and a lower slope value than the curve for *bla* amplification. The best-fit lines ran through all points of each respective curve indicating that the curves are reliable within the tested range. Melt curve analysis demonstrated the amplification of two separate species meaning that there was no unspecific amplification (Fig 3). Negative controls containing primers (no template) amplified a product past 30 cycles, which is negligible due to the fact that the *dxs* and *bla* species were amplified between 8 and 25 cycles. These results indicate that the qPCR protocol is adequately amplifying the correct genes. The amount of both *dxs* and *bla* gene presence increased with higher concentrations of pCHCS, however the *dxs* gene was consistently amplified at a lower rate.

qPCR measurement of *bla* and *dxs*. The addition of pUC19 plasmid in increasing amounts showed a dose-dependent response in the pCHCS assay. DNA isolated from *E. coli* DH5α cells that were transformed with pUC19 (22.4 ng/µL) was used as a baseline, consistent in all reaction tubes. A dilution of pUC19 was added to the reaction tubes. When pUC19 with a concentration of 12.6 ng/µL was diluted 1/10 and added to the reaction tube, the resulting plasmid copy number was 89.31 (Table 3). With the addition of less pUC19 plasmid (1/100 and 1/1000 dilutions), the resulting plasmid copy number was 14.23 and

TABLE 3. Estimated plasmid copy number by absolute quantification for DNA from pUC19 harboring *E. coli* DH5α cells with added dilutions of pUC19 plasmid.

Dilution of pUC19 added*	Average Cq value		Copies (copies/µl)		Plasmid Copy Number
	bla	*dxs*	*bla*	*dxs*	
1/10	8.52	6.57	3.34 x 10^8	3.74 x 10^6	89.31
1/100	7.54	6.39	3.46 x 10^7	2.43 x 10^6	14.23
1/1000	7.25	6.37	1.79 x 10^7	2.35 x 10^6	7.60

*The undiluted pUC19 concentration was 12.6 ng/ul

TABLE 4. Estimated plasmid copy number of various plasmid-bearing *E. coli* DH5α cells by absolute quantification.

Plasmid within *E. coli* DH5α cells	Average Cq value		Copies (copies/µl)		Plasmid Copy Number
	bla	*dxs*	*bla*	*dxs*	
pUC19	7.48	6.68	3.01 x 10^7	4.81 x 10^6	6.26
pBR322	7.23	7.03	1.71 x 10^7	1.07 x 10^7	1.60
pCAWK	7.23	7.13	1.71 x 10^7	1.34 x 10^7	1.27
pBART	6.97	6.74	9.31 x 10^6	5.47 x 10^6	1.70
Control: pCHCS	8.51	8.15	3.25 x 10^8	1.40 x 10^8	2.31

7.60, respectively (Table 3). This shows a dose-dependent response of the pCHCS assay to the amount of plasmid in the assay.

Plasmid copy number of plasmids containing *bla* were measured. qPCR analysis of *bla* and *dxs* was next used to evaluate copy number of plasmids pBR322, pUC19, pBART, and pCAWK. Genomic and plasmid DNA was isolated from *E. coli* DH5α cells containing one of the four previously stated plasmids. qPCR was used to amplify *bla* and *dxs* genes and their respective C_q values were applied to our standard curves. The copy number for *bla* was divided by the copy number for *dxs*, giving us the measured plasmid copy number. As a control, we performed qPCR analysis of purified pCHCS. We expected to obtain a value of 1 since a plasmid sample should contain a 1:1 ratio of *bla* to *dxs*. The value we obtained was 2.31 (Table 4) indicating that the *bla* gene was copied more efficiently than the *dxs* gene. pBR322 is expected to have a plasmid copy number between 15 and 20 (3). We obtained a plasmid copy number of 1.60 using our assay (Table 4). The plasmid copy number of pUC19 was 6.26 which follows the expected trend of pUC19 having a higher plasmid copy number than pBR322 (1). pBART had a plasmid copy number of 1.70 according to our assay. This result was expected, as pBART is a pUC19-derived plasmid with the insertion of the *rop* gene (5). pCAWK, a pBR322-derived plasmid with insertional inactivation of the *rop* gene, had a plasmid copy number of 1.27, which is below the plasmid copy number for pBR322. This result was unexpected. These comparisons show that pUC19 > pBR322 ~ pBART > pCAWK.

DISCUSSION

We have built a qPCR-based method to measure plasmid copy number based on the assay designed by Lee *et al.* (2006) (3). The assay utilizes single-copy genes from plasmid (*bla*) and chromosome (*dxs*) to determine plasmid copy number after transformation of a plasmid into *E. coli* DH5α cells (3). In this study we attempted to use our qPCR-based assay to understand the role of genetic elements in regulating plasmid copy number.

pCHCS plasmid was created and used in qPCR experiments to establish a pair of standard curves for *bla* and *dxs*. The standard curves that we obtained from the amplification of *bla* and *dxs* from dilutions of pCHCS show a linear relationship, but differ slightly in slope and y-intercept values (Fig 4). By comparison, the standard curves for *bla* and *dxs* established by Lee *et al*. (2006) were nearly identical to each other (3). We repeated the qPCR experiment with pCHCS, which resulted in similar standard curves to the first run, therefore we can consider our standard curves to be repeatable.

Since our standard curves are repeatable we felt confident in using them for calculations of plasmid copy number in future experiments, however the discrepancy between the *bla* and *dxs* curves is potentially problematic. *bla* and *dxs* are both single-copy genes and should therefore be copied in a 1:1 ratio. According to our standard curves in Figure 4, pCHCS is not yielding this expected ratio. The primers that we designed were adapted from the primers used by Lee *et al.* (2006), therefore we can assume that this discrepancy is likely not due to issues with our primers. The discrepancy could be attributed to sample preparation, since we did not have access to the kit that Lee *et al.* (2006) used to isolate both genomic and plasmid DNA. We instead used a genomic isolation kit, with manual modifications so that plasmid was included in the isolation. We hypothesize that there was exponentially more genomic DNA than plasmid DNA in the preparation, which could lead to much less *bla* amplification compared to *dxs* amplification in the qPCR reaction. These deceptive C_q values for both *dxs* and *bla* could result in lower plasmid copy numbers than expected, which is consistent with our results (Table 4).

Another potential factor influencing our standard curves is qPCR reagents and machinery. Although we followed the qPCR protocol outlined by Lee *et al.* (2006), we used different qPCR reagents and a different qPCR thermocycler, which may explain this discrepancy. Further optimization of the protocol with our qPCR reagents and thermocycler could potentially yield comparable standard curves.

In order to test whether or not our qPCR assay can detect differences in *bla* gene copy number, we

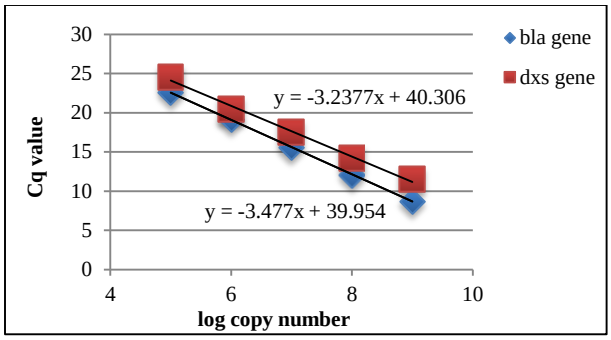

FIG 4. Standard curves for *bla* and *dxs* qPCR amplification of pCHCS dilutions.

performed an experiment where differing amounts of pUC19 plasmid were added to otherwise identical reaction tubes. Each tube contained equal amounts of *E. coli* DH5α cells harboring pUC19, and then different amounts of pUC19 plasmid dilutions was added to each tube. Since the tubes already contained pUC19 before additional plasmid was added, we were not expecting plasmid copy numbers to be proportional based on the amount of plasmid that was added to the tubes (Table 3). For example, the plasmid copy number of the 1/100 dilution of pUC19 (14.23) is not ten times higher than the plasmid copy number of the 1/1000 dilution (7.60) (Table 3). This experiment was performed simply to demonstrate that our assay is able to respond in a dose-dependent manner to the amount of plasmid present. This justifies the use of our assay in future qPCR experiments with student-made plasmids.

Upon testing the validity of our qPCR assay with our control, pCHCS, it yielded a plasmid copy number of 2.31, which is greater than the expected value of 1.0. This result was not anticipated since a sample of pCHCS should have a 1:1 ratio of *bla* to *dxs*, due to the fact that both *bla* and *dxs* are single-copy genes. Further optimization of our qPCR protocol could refine our standard curves of pCHCS in order to obtain a plasmid copy number of 1.0 for pCHCS control samples in the future. As stated above, this error could be due to the discrepancy with our standard curves and could be refined with improved sample preparation, and optimization of the qPCR with our reagents and equipment. The unexpected plasmid copy number for pCHCS could also be due to defects in the pCHCS plasmid itself. It is possible that *dxs* is being copied less efficiently in pCHCS than in its natural cell environment, resulting in the observed higher C_q value for *dxs* relative to *bla*. Since *dxs* was ligated into the pUC19 plasmid to create pCHCS, this decreased efficiency in *dxs* copy number could be the result of improper ligation. Lee *et al.* (2006) used a different calibrator plasmid in their experiments, therefore this is a plausible factor in the observed plasmid copy number for pCHCS.

Taking this into account, a correction factor could be applied to our standard curves to correct for the different efficiencies associated with copying *bla* and *dxs* in pCHCS. A correction factor can be applied to a set of data where there is error, in order to allow for comparison to a second set of data. If a correction factor were applied to our pCHCS standard curves so that the *dxs* and *bla* curves were identical to each other, the plasmid copy number for pCHCS would be 1.0. This would result in standard curves that could more reliably measure plasmid copy number for other plasmids. A correction factor was not needed for Lee *et al.* (2006), since their standard curves for *dxs* and *bla* were identical.

All of our plasmid copy numbers for our experimental samples were much lower than anticipated. The plasmid copy number that we obtained for pBR322 should have been the lowest of all of our experimental samples at a value of 15-20 (3). However, we obtained a much lower value of 1.60 for the plasmid copy number of pBR322. This error could once again be attributed to our DNA isolation method. Due to the isolation kits available, it is hypothesized that plasmid DNA was not being isolated as efficiently as genomic DNA. Consequently *bla* would have been underestimated in the qPCR, thus yielding lower than expected values for plasmid copy number. Additionally, this discrepancy could be caused by the C_q values of the sample plasmids being below the linear range of our standard curves. We do not know the characteristics of the standard curves below or above the range depicted in Figure 4, therefore it is possible that the curves are non-linear outside of this range. Since the C_q values are below the range this may be the cause of the observed low plasmid copy numbers.

Our data do not align with values reported in the literature for plasmid copy numbers, however it is consistent with our hypothesis for trends in plasmid copy number of our test plasmids. pUC19 had a plasmid copy number of 6.26, pBART had a plasmid copy number of 1.70, and pCAWK had a plasmid copy number of 1.27 (Table 4). pBART was expected to have a plasmid copy number somewhere between that of pUC19 and pBR322 because pBART is a pUC19-derived plasmid with the insertion of the *rop* gene (5). The presence of the *rop* gene in pBART was expected to decrease its plasmid copy number relative to pUC19. The presence of the G to A point mutation in pBART was expected to increase the plasmid copy number of pBART relative to pBR322 (1). Our results demonstrated this trend, as pCAWK had a plasmid copy number between that of pUC19 and pBR322. This data suggests that our assay may be able to measure relative differences in plasmid copy number between plasmids that have different genetic characteristics.

Our experimentally determined plasmid copy number for pCAWK did not follow the expected trend. pCAWK is a pBR322-derived plasmid with insertional

inactivation of the *rop* gene (6). It was therefore expected to have a plasmid copy number below that of pUC19, since it did not have the G to A point mutation. pCAWK is expected to have a plasmid copy number above that of pBR322, since the *rop* gene was inactivated. However, we obtained a plasmid copy number of 1.27 for pCAWK, which is below that of pBR322 (plasmid copy number of pBR322 is 1.60). This unexpected result may be attributed to the fact that we did not test whether or not the *rop* gene was truly inactivated in pCAWK.

We were originally going to use the student-made plasmid pANPT to evaluate the effect of plasmid size of plasmid copy number, by comparing its plasmid copy number to that of pBR322 (4). However, upon confirmation of the size of pANPT, we discovered that it unexpectedly was identical in size to pBR322 (Fig S2) and so we were not able to use pANPT for following experiments.

The novel calibrator plasmid pCHCS contains *bla* and *dxs* and yields repeatable standard curves for the determination of plasmid copy number using a qPCR-based assay. Our qPCR assay to measure plasmid concentration was shown to respond in a dose-dependent manner and may be useful in assessing the effects of genetic elements on plasmid copy number.

FUTURE DIRECTIONS

Future projects should begin by applying a correction factor to our standard curves, as well as widening the range of the curves in order to resolve the discrepancy in calculated plasmid copy number of pCHCS. It is also important to develop a more accurate way to isolate both plasmid and genomic DNA to be used as samples in our qPCR assay. This would allow for more accurately measured plasmid copy numbers that match literature values.

Another project could test the specific modifications to student-made plasmids in order to ensure that these modifications were successful. For example, Rop activity should be tested in both pCAWK and pBART, since its activity is essential to determining the effects that Rop has on plasmid copy number.

Projects could also involve creating a plasmid similar to pANPT in that it is smaller than pBR322, but still contains the *rop* gene and no G to A point mutation in RNA II. This plasmid could be used in our assay, and compared to pBR322 to determine the specific effects of size on plasmid copy number.

A project could be to control for size in our test plasmids, since this could alter the relative effects of other factors in plasmid copy number. For example pBART, when compared with pBR322, can reveal the effects of the G to A point mutation, however the two plasmids differ in size by approximately 600 base pairs. This size difference may alter results, and should be controlled in order to obtain definitive results. Finally, our assay can be applied more broadly to other plasmids, and can be used to test other characteristics/environmental factors on plasmid copy number.

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