



# Use of synthesized double-stranded gene fragments as qPCR standards for the quantification of antibiotic resistance genes

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## ABSTRACT

Pollution of various environmental matrices by antibiotic resistance genes (ARGs) has become a growing threat to human health. For the quantitative analysis of the presence of ARGs, there is a need for sensitive and robust qPCR assays which can detect various genes from different types of DNA extracts. Fourteen ARGs were selected as target genes in this study including: *bla<sub>TEM</sub>*, *bla<sub>OXA-1</sub>* and *bla<sub>CTX-M</sub>* coded for resistance to  $\beta$ -lactams; *ermB* for macrolides; *tetA*, *tetG*, *tetM*, *tetQ*, *tetW* and *tetX* for tetracyclines; *sul I* and *sul II* for sulfonamides; *drfA1* and *drfA12* for trimethoprim; and integron gene *intI 1* and *intI 2*. Chemically synthesized double-stranded gene fragments were modified using molecular biology methods and used as real-time PCR standards as well as to establish in-house qPCR assays. The *ermB* gene from a naturally occurring plasmid was used to compare the performance of qPCR assay with the chemically synthesized *ermB*. Additionally, environmental water, soil and faeces samples were used to validate the established qPCR assays. Importantly, the study proves the usefulness of rapidly synthesized oligonucleotides serving as qPCR standards for ARG analysis and provides comparable sensitivity and reliability to a traditional amplicon standard.

## 1. Introduction

Antibiotic resistance genes (ARGs) are recognised as emerging environmental micropollutants (Pruden et al., 2006). These genes are diverse and ubiquitous in natural environments and an increasing number of reports have been published on the prevalence of ARGs in various environmental matrices, including surface water, drinking water, soil, aquaculture and agriculture (Cheng et al., 2016; Fernando et al., 2016; Wang et al., 2014; Xu et al., 2016). In order to address antimicrobial resistance (AMR), including antibiotic resistance, one of the multiple initiatives led by the WHO is to establish the Global Antimicrobial Resistance Surveillance System (GLASS), calling for more international participants and data to fully assess the resistance gene prevalence worldwide, which necessitates a standardised approach to the analysis and sharing of the data related to antibiotic resistance at a global level (World Health Organization, 2018a). It is reported that many of the same microbes affect both animals and humans via the environment they share and 60% of human infectious diseases are spread from animals (World Health Organization, 2018b). Despite the knowledge of environmental influences on AMR, current surveillance systems often neglect environmental sampling (Thakur and Gray, 2019). The role the environment plays as a reservoir of maintaining AMR genes is as equal important as AMR in human and animal populations. Hence, it is necessary to apply

the One Health approach and study environmental reservoirs more closely (Thakur and Gray, 2019; World Health Organization, 2017), linking the health of people to the health of animals and the environment in order to establish effective surveillance systems to combat AMR.

An ARG is a specific gene which, when expressed, renders an otherwise susceptible host bacterium more resistant to a particular antibiotic (Sukumar et al., 2016). In general, classic molecular techniques such as PCR (polymerase chain reaction), are still of great importance for defining the dissemination of known ARGs in environmental samples (Allen, 2014). The absolute quantity of an ARG in a system is usually expressed as gene 'copy number', indicating the number of copies of a gene in the genome, including any mobile genetic elements (MGEs). For both quantitative and qualitative ARG analysis, standard templates are needed to initially set up the assay and then for use as a positive control. Materials that can be used as standards include PCR-amplified target sequences, plasmids containing the target gene sequence, or commercially prepared DNA (Dhanasekaran et al., 2010). A known ARG sequence can be amplified by PCR from genomic or mobile element DNA using gene specific primers and visualised by gel electrophoresis. The amplified gene fragment can be ligated to a known-sized vector and then transformed to competent cells for the reproduction of vector containing target resistance gene. Positive clones carrying target ARG inserts are usually used as standards for absolute

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quantification of ARG from various sample types (Calero-Caceres et al., 2014; Chen and Zhang, 2013a). This traditional method, however, is limited to the availability of positive isolates, especially for those very rare or newly-discovered ARGs. Additionally, when a research project has many ARGs of interest, it is costly to purchase the commercially available strains or plasmids harbouring target ARGs. Synthetic oligonucleotides, on the other hand, can be a useful alternative to obtain specific gene fragments. Relying on well-developed synthesis platform, double-stranded DNA fragments with various lengths can be easily purchased from biotechnology companies at an affordable price and used for a wide range of applications, such as antibody research (Dickinson et al., 2013), genome engineering (Cobb et al., 2015; Ghorbal et al., 2014), and qPCR standards (Greiman and Tkach, 2016; Gunawardana et al., 2014). For instance, Greiman and Tkach utilised a 224-bp laboratory synthesized fragment of the *Neorickettsia risticii* GroEL gene to generate a standard curve for the determination of the prevalence of *Neorickettsia* infection within multiple stages of the digenae life cycle (Greiman and Tkach, 2016); According to Krüttgen et al., a chemically synthesized *bla*<sub>NDM-1</sub> gene was introduced as a convenient positive control for the setup of in-house assays for *bla*<sub>NDM-1</sub> detection (Krüttgen et al., 2011).

In this study, we aim to establish a convenient and cost-effective method for those laboratories wishing to setup in-house assays for the quantitative analysis of ARGs in different environmental matrices. The selection of the target ARGs was based on: 1) the antibiotic to which they confer resistance; 2) the mechanism of resistance and, 3) the presence in different environmental matrices. Fourteen ARGs, including *bla*<sub>TEM</sub>, *bla*<sub>OXA-1</sub> and *bla*<sub>CTX-M</sub> coding for resistance to  $\beta$ -lactams; *ermB* for macrolides; *tetA*, *tetG*, *tetM*, *tetQ*, *tetW* and *tetX* for tetracyclines; *sul I* and *sul II* for sulfonamides; *drfA1* and *drfA12* for trimethoprim; and the integron genes *intI 1* and *intI 2* were selected as target genes in this study. A traditional qPCR assay for *ermB* was used for comparison using a naturally occurring plasmid encoding *emrB*. Importantly, this study proves the usefulness of rapidly synthesized gene fragments serving as qPCR standards for ARGs when biological isolates are not commonly available. To the best of our knowledge, this is the first research using synthetic gene fragments as qPCR standards for the quantitative analysis of multiple ARGs in various environmental samples.

## 2. Materials and methods

### 2.1. ARG fragment design and cloning

Nucleic acid sequence for individual ARG were downloaded from the NCBI website (<https://www.ncbi.nlm.nih.gov/nucleotide>). Specific pair of primers for each ARG (Table 1) was used to trim both sides of the sequence that obtained from NCBI website. ARG sequences selected in this study were supplied in Table S1. Chemically synthesized double-stranded ARGs (refer to hereafter as 'gBlocks' gene fragments) were obtained from Integrated DNA Technology (UK) in dry form, ranging from 103 to 516 bp in length. Once received, gBlocks gene products were re-suspended in Tris-EDTA buffer (10 mM Tris-HCl, 1 mM disodium EDTA, pH 8.0, Sigma-Aldrich, UK) according to the manufacturers' instructions to reach a final concentration of 10 or 20 ng/ $\mu$ L based on the length of gene fragment and stored at  $-20^{\circ}\text{C}$  for further process.

### 2.2. gBlocks cloning

As gBlocks are blunt-end DNA fragments, it is necessary to add adenosine (A) overhangs to gBlocks for the compatibility with T/A cloning vectors. The gBlocks DNA suspensions were incubated at  $50^{\circ}\text{C}$  for 20 min prior to use. The A-tailing experiment was conducted at room temperature. 0.6  $\mu$ L Taq DNA polymerase (5 units/ $\mu$ L), 1.5  $\mu$ L  $10\times$  PCR buffer (Taq PCR Core Kit, QIAGEN, UK), 0.05 mM dATP (BIOLINE, UK), 50 ng gBlocks DNA fragments, and PCR grade water were combined to a final volume of 15  $\mu$ L. A reaction tube adding PCR grade water instead of

gBlocks was used as a negative control. After 30 minutes' incubation at  $70^{\circ}\text{C}$ , the A-tailing products were ready for T/A cloning.

In order to compare the performance of gene fragment originated from both chemically synthesis and resistance plasmid, a plasmid pMTL9301 DNA carrying *ermB* was also used for gene cloning. Fresh PCR product with the confirmed presence of *ermB* gene was excised and purified using QIAquick Gel Extraction Kit (QIAGEN, UK).

1  $\mu$ L purified PCR product or A-tailed gBlocks was ligated into pGEM Easy Vector and then transformed into *Escherichia coli* JM109 competent cells using pGEM Easy Vector Systems (Promega, UK) according to the manufacturers' instructions. Successful recombinant cells (blue colonies) were picked from LB agar plate containing 100 mg/L Ampicillin (Sigma-Aldrich, UK) and Blue/White Select Screening reagent (Sigma-Aldrich, UK) and screened by PCR (TECHNE, UK) using the primers listed in Table 1 to evaluate cloning of the target genes. Details about PCR conditions can be found in SI. 6  $\mu$ L of each PCR product were verified by 1.5% agarose gel electrophoresis. All PCR products were sequenced for the verification of the presence of ARGs. The sequence results were compared with existing sequences using BLAST alignment tool (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Plasmid DNA were extracted from vector containing the insert using the QIAprep Spin Miniprep Kit (QIAGEN, UK) and the concentration of the vector was measured by Qubit 3.0 Fluorometer (Invitrogen, UK) using dsDNA Broad Range Assay Kit (Invitrogen, UK).

### 2.3. qPCR procedures

Plasmid DNA containing target genes were used to generate standard curves. The numbers of copies of plasmid DNA per microliter were calculated using the following formula (Zhang et al., 2009).

$$\frac{\text{Copies}}{\mu\text{L}} = \frac{\text{DNA mass concentration (ng}/\mu\text{L}) \times 10^{-9} \times 6.022 \times 10^{23}}{(3015 \text{ bp}^* + \text{amplicon size bp}) \times 660}$$

\* The length of the pGEM Easy vector is 3015 bp.

Primer sequences and amplicon size of individual ARGs are described in Table 1. Seven-point standard curves with copy numbers ranging from  $10^2$  to  $10^8$  for qPCR were generated using 10-fold serial dilutions of the plasmid DNA carrying target ARGs. A final volume of 20  $\mu$ L reaction mixture was used, consisting of 10  $\mu$ L of Luna Universal qPCR Master Mix (New England Biolabs, UK), 0.5  $\mu$ L of each primer (10  $\mu$ M), 1  $\mu$ L DNA template, and 8  $\mu$ L of PCR grade water. The PCR protocol was as follows: 1 min at  $95^{\circ}\text{C}$ , followed by 40 cycles of 15 s at  $95^{\circ}\text{C}$ , 30 s at  $60^{\circ}\text{C}$ , and then a final melt curve stage with temperature ramping from 60 to  $95^{\circ}\text{C}$ . Each reaction was run in triplicate and a non-template control was included. All the qPCR assays were performed in 96-well plates under standard conditions, as per the instructions of the manufacturer, in a 7500 Real-Time PCR system (Applied Biosystems). The qPCR efficiencies were generated by the software (Applied Biosystems 7500 v2.3) on the basis of the standard curves.

### 2.4. Method validation

Different types of environmental samples were used to validate the qPCR assays. Water samples were collected from the River Thames, ponds in Regent's Park and Hyde Park in London; soil and duck faeces samples were collected from Regent's Park. The geographical location of sampling sites is provided in Fig. S1. All samples were collected in triplicate in pre-autoclaved amber glass bottles or sterile tubes and kept refrigerated at  $4^{\circ}\text{C}$  without preservatives until they were processed within 24 h of sampling. DNA was extracted in triplicate using the FastDNA SPIN Kit for Soil (MP Bio, UK) according to the manufacturers' instructions. Water samples (500 mL) were filtered using a vacuum filtration apparatus through a 0.22  $\mu$ m mixed cellulose esters membrane filters (Millipore, UK) and DNA was extracted from the membrane. For soil and faeces samples, DNA was extracted directly from 0.5 g (wet) of

**Table 1**  
Primers and amplicon size of target ARGs in this study.

Target gene	Primer	Sequence	Amplicon Size (bp)	Reference
<i>bla<sub>CTX-M</sub></i>	<i>bla<sub>CTX-M</sub></i> -F	CTATGGCACCACCAACGATA	103	(Marti et al., 2013)
	<i>bla<sub>CTX-M</sub></i> -R	ACGGCTTCTGCCTTAGGTT		
<i>bla<sub>OXA-1</sub></i>	<i>bla<sub>OXA-1</sub></i> -F	ACCAAAGACGTGGATGCAAT	325	(Tennstedt et al., 2005)
	<i>bla<sub>OXA-1</sub></i> -R	TGCACCAGTTTTCCCATACA		
<i>bla<sub>TEM</sub></i>	<i>bla<sub>TEM</sub></i> -F	CCCCGAAGAAGCTTTTC	516	(Mabilat and Courvalin, 1990)
	<i>bla<sub>TEM</sub></i> -R	ATCAGCAATAAACCCAGC		
<i>ermB</i>	<i>ermB</i> -F	ACGACGAAACTGGCTAAAATAAGT	412	This study
	<i>ermB</i> -R	CTGTGGTATGGCGGGTAAGT		
<i>tetA</i>	<i>tetA</i> -F	GCTACATCCTGCTTGCCTTC	210	(Ng et al., 2001)
	<i>tetA</i> -R	CATAGATCGCCGTGAAGAGG		
<i>tetG</i>	<i>tetG</i> -F	GCTCGGTGGTATCTCTGCTC	468	(Ng et al., 2001)
	<i>tetG</i> -R	AGCAACAGAATCGGGAACAC		
<i>tetM</i>	<i>tetM</i> -F	ACAGAAAGCTTATTATATAAC	171	(Aminov et al., 2001)
	<i>tetM</i> -R	TGGCGTGTCTATGATGTTAC		
<i>tetQ</i>	<i>tetQ</i> -F	AGAATCTGCTGTTTGCCAGTG	169	(Aminov et al., 2001)
	<i>tetQ</i> -R	CGGAGTGCAATGATATTGCA		
<i>tetW</i>	<i>tetW</i> -F	GAGAGCCTGTATATGCCAGC	168	(Aminov et al., 2001)
	<i>tetW</i> -R	GGGCGTATCCACAATGTTAAC		
<i>tetX</i>	<i>tetX</i> -F	CAATAATGGTGGTGGACCC	468	(Ng et al., 2001)
	<i>tetX</i> -R	TTCTTACCTTGGACATCCCG		
<i>sul I</i>	<i>sul I</i> -F	CACCGAAACATCGCTGCA	158	(Luo et al., 2010)
	<i>sul I</i> -R	AAGTTCGCGCGCAAGGCT		
<i>sul II</i>	<i>sul II</i> -F	CTCCGATGGAGCCCGTAT	190	(Luo et al., 2010)
	<i>sul II</i> -R	GGGAATGCCATCTGCCTTGA		
<i>dfrA1</i>	<i>dfrA1</i> -F	TGGTAGCTATATCGAAGAATGGAGT	425	(Grape et al., 2007)
	<i>dfrA1</i> -R	TATGTTAGAGGCGAAGTCTGGGTA		
<i>dfrA12</i>	<i>dfrA12</i> -F	GAGCTGAGATATACACTCTGGCACT	155	(Grape et al., 2007)
	<i>dfrA12</i> -R	GTACGGAATTACAGCTTGAATGGT		
<i>intI 1</i>	<i>intI 1</i> -F	CCTCCGCACGATGATC	280	(Goldstein et al., 2001)
	<i>intI 1</i> -R	TCCACGCATCGTCAGGC		
<i>intI 2</i>	<i>intI 2</i> -F	TTATTGCTGGGATTAGGC	233	(Goldstein et al., 2001)
	<i>intI 2</i> -R	ACGGCTACCCTCTGTTATC		
16S	1369F	CGGTGAATACGTTTCYCGG	143	(Gaze et al., 2011)
	1492R	GGWTACCTTGTACGACTT		

the raw samples. The quality and concentration of the extracted DNA were determined by NanoDrop and 1.5% agarose gel electrophoresis and stored at  $-20^{\circ}\text{C}$  until further analysis. qPCR settings for environmental DNA samples were the same as for qPCR standards as described above.

To assess qPCR inhibitions, dilutions of the standards were spiked with environmental DNA and the threshold cycle (Ct) and copy numbers were compared to the known copy numbers of the target genes in the standards (Colomer-Lluch et al., 2011). No inhibition of qPCR by environmental DNA was detected.

### 2.5. Statistical analysis

In this study, the absolute abundance of ARG was defined as the ARG copies per litre aqueous samples (copies/L) or per gram soil/faeces samples (copies/g). The relative abundance of ARG was defined as the normalised ARG copies to the 16S rRNA copies. Average and standard deviations calculation of all data were done with Microsoft Excel 2016. The results of qPCR were analysed using 7500 software v2.3 (Applied Biosystems, UK). One-way analysis of variation (ANOVA) test was used to evaluate the differences between ARGs detected in environmental DNA samples with significance level of 5% ( $P < .05$ ). ANOVA and Pearson correlation analysis were carried out using OriginPro 2018. All figures were generated by OriginPro 2018. Only samples with three replicates that had been amplified were regarded as positive.

## 3. Results and discussion

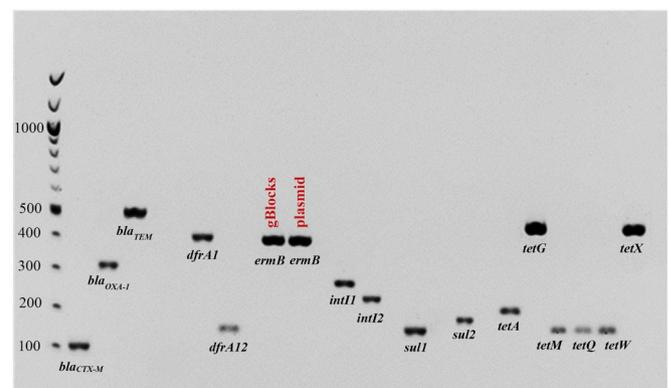
### 3.1. PCR and gel electrophoresis

Recombinant cells were selected and subjected to colony PCR to confirm insertion of the correct DNA fragment. The PCR products were

run on the gel to visualise the specific band for each ARG. As can be seen from Fig. 1, all of the target genes were amplified and formed a single band. The size of each ARG band as it appeared on the gel was in accordance with the amplicon size specified in Table 1, ranging from 103 bp for *bla<sub>CTX-M</sub>* to 516 bp for *bla<sub>TEM</sub>*. Both *ermB* gene bands were the same size and intensity.

### 3.2. Specificity and sensitivity of the qPCR assays

In this study, amplification efficiencies for all target genes ranged from 80.9% to 107.4% with good linearity (Table 2), indicating the reliability of synthetic gene fragments as qPCR standards. Ideally, the qPCR efficiency should be 1.0, however, if consistent, lower efficiency value is also acceptable due to the potential PCR inhibitors in DNA



**Fig. 1.** Electrophoresis bands of target genes. (*ermB* - left: originated from gBlocks; right: originated from plasmid).

**Table 2**  
Standard curves, amplification efficiency, R<sup>2</sup> value of each qPCR array.

Target gene	Standard curve	R <sup>2</sup>	Amplification efficiency	LOD (GC/μL)
<i>bla<sub>CTX-M</sub></i>	Y = -3.44x + 39.19	0.999	95.3%	9.6
<i>bla<sub>OXA-1</sub></i>	Y = -3.53x + 38.05	0.999	92.1%	7.3
<i>bla<sub>TEM</sub></i>	Y = -3.16x + 36.66	0.993	107.4%	6.1
<sup>a</sup> <i>ermB</i> (gBlocks)	Y = -3.30x + 41.55	0.997	100.9%	9.2
<sup>a</sup> <i>ermB</i> (plasmid)	Y = -3.55x + 36.30	0.995	91.3%	4.6
<i>tetA</i>	Y = -3.44x + 36.72	0.999	95.4%	3.2
<i>tetG</i>	Y = -3.84x + 40.25	0.983	82.0%	6.8
<i>tetM</i>	Y = -3.91x + 38.88	0.997	80.9%	11.2
<i>tetQ</i>	Y = -3.26x + 35.25	0.999	102.8%	2.0
<i>tetW</i>	Y = -3.31x + 35.89	0.998	100.3%	7.1
<i>tetX</i>	Y = -3.38x + 40.51	0.997	97.6%	6.3
<i>sul I</i>	Y = -3.43x + 38.11	0.997	95.8%	5.1
<i>sul II</i>	Y = -3.81x + 39.01	0.999	83.0%	7.3
<i>dfrA1</i>	Y = -3.63x + 36.45	0.997	88.5%	4.5
<i>dfrA12</i>	Y = -3.34x + 39.42	0.997	99.4%	6.1
<i>intI 1</i>	Y = -3.51x + 43.04	0.995	92.8%	3.1
<i>intI 2</i>	Y = -3.10x + 39.65	0.998	96.8%	3.8
16S	Y = -3.39x + 36.34	1.000	97.3%	3.9

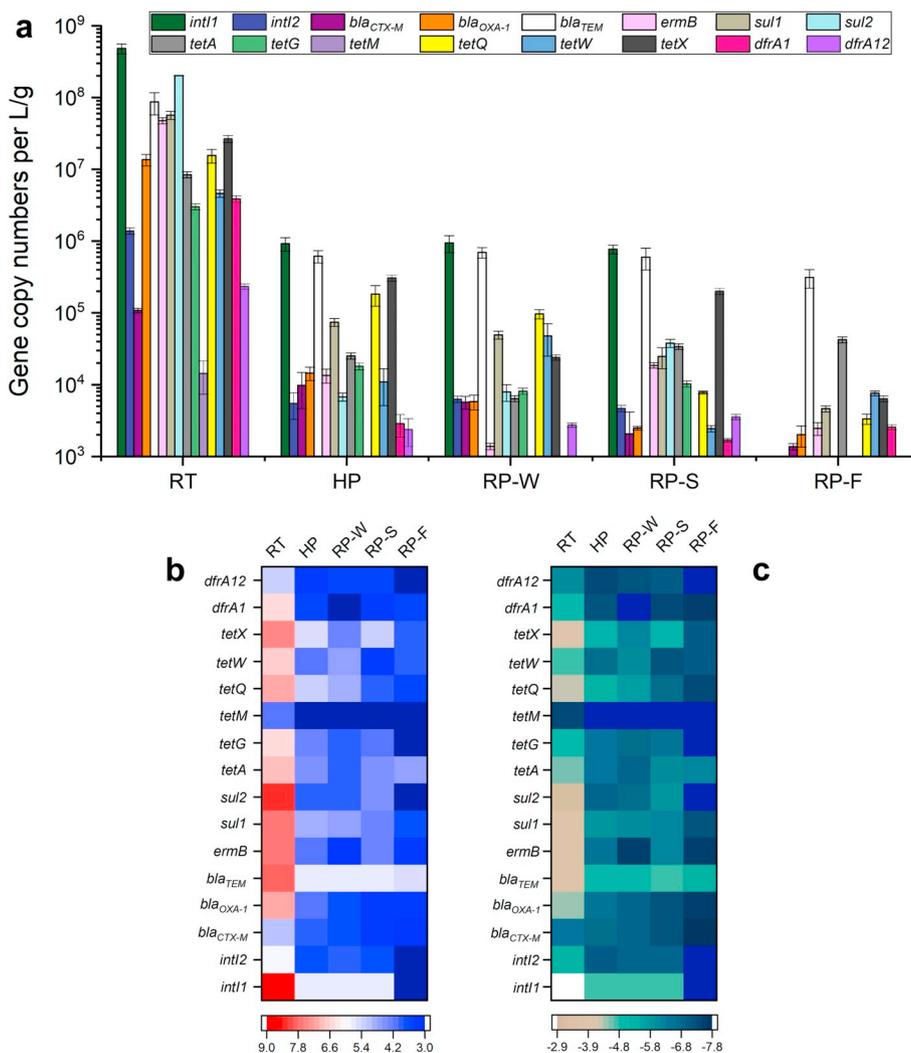
LOD: Limit of Detection; GC/μL: gene copies/μL.

<sup>a</sup> *ermB* (gBlocks): chemically-synthesized *ermB*; *ermB* (plasmid): plasmid-carrying *ermB*.

extracts (Luby et al., 2016). The calculation of limit of detection followed previous research by performing eight replicates of each dilution of the standard for each gene, and the lower gene copies gave results that were accurately reproducible (coefficient of variation < 10%) was considered as limit of detection (Calero-Caceres et al., 2014). Only runs resulting in gene copies higher than the detection limit were applied for the calculation of resistance gene concentrations. The limit of detection of each qPCR assay was shown in Table 2.

The stability of the standards is a critical issue as they are typically used for long-term studies, with different experimental designs where multiple samples need to be compared over time with great accuracy as well as for short term studies (Dhanasekaran et al., 2010). Once extracted, the highly-purified plasmids incorporating gBlocks fragments can be stored in the freezer and used as qPCR standards for extended periods of time. Alternatively, positive clones can also be stored in ampicillin-containing media and plasmid DNA can be extracted sustainably with sufficient quantity and high purity when needed.

In order to compare the performance of synthetic *ermB* (gBlocks) and plasmid-harboured *ermB* serving as qPCR standards, same downstream gene cloning experiments were conducted. Both gBlocks-*ermB* and plasmid-*ermB* achieved good amplification efficiency and linearity (100.9%, 0.997 and 91.3%, 0.995, respectively). Better *ermB* assay efficiency was observed with the gBlocks fragment standard. Sequence alignment results also showed 100% similarity for both *ermB* gene



**Fig. 2.** a: Concentrations of ARGs and integron genes in environmental samples; A heatmap of b: absolute abundance; c: relative abundance (normalised to the corresponding 16S rRNA) of ARGs showing distinct pattern between River Thames (RT), Hyde Park (HP), Regent's Park water (RP-W), soil (RP-S) and faeces (RP-F) sample.

fragments, indicating the performance of gBlocks-*ermB* qPCR assay was comparable with plasmid-harboring *ermB*. In general, gBlocks standards enable more independent qPCR assay development which is not limited to the availability of the positive isolates or plasmids, especially when a research project has several ARGs of interest.

### 3.3. Validation of qPCR assays

Water, soil and duck faeces samples from the natural environment were used to validate the established qPCR assays based on gBlocks modification. For the consistency of data analysis, only the results of *ermB* originated from gBlocks were present in the figures and table in the following context. For comparisons, raw qPCR results of gBlocks-*ermB* and plasmid-*ermB* can be found in Table S2.

#### 3.3.1. ARG abundance

An overview of the absolute abundance of ARGs and integron genes *intI 1* and *intI 2* in different environmental samples is shown in Fig. 2a. The concentration of ARGs among all the samples was between  $10^3$  and  $10^8$  copies/L, with the detection frequencies ranging from 71.42% to 100%.

ARGs are ubiquitous in the environment. All of the fourteen selected ARGs were detected in River Thames water samples. In general, the overall abundance of ARGs in the River Thames was two to three orders of magnitude higher than in the parks' water samples. The order of the average gene copies from low to high was: RP-F ( $3.86 \times 10^4$  copies/g), RP-S ( $7.28 \times 10^4$  copies/g), RP-W ( $7.97 \times 10^4$  copies/L), HP ( $9.91 \times 10^4$  copies/L), and RT ( $3.37 \times 10^7$  copies/L). The result is consistent with the initial hypothesis as the River Thames is much affected by anthropogenic activities. There are many residential and commercial areas along the River Thames, and it is also the receiving water body to the municipal wastewater treatment works. Previous research in Huangpu River has shown that the levels of ARGs in areas with anthropogenic activities was much higher than in areas that were less affected by human activities (Jiang et al., 2013). Considering the River Thames is used as a drinking water source, the high detection frequency and concentration of ARGs in river water may imply a potential health threat to the public.

The parks selected in this study are located in Central London and have been open to public for decades. Not surprisingly, ten out of fourteen ARGs were detected in wild duck faeces samples. It was very common for park visitors to walk dogs along the pond pathways, however, pet animals that live with humans, including cats and dogs, are reservoirs of antibiotic-resistant bacteria due to the antibiotic treatment for diseases and the transfer of resistant bacteria from humans (Guardabassi et al., 2004). Upon release into the soil and pond through surface runoff, those resistant bacteria could have acted as donors of genes encoding antibiotic resistance, or their presence could have been favoured as a result of selection pressure exerted by the presence of antibiotic residues in animal excreta, contributing to the dissemination of ARGs to the wild animals such as ducks and geese (Petersen et al., 2002).

Among all the ARGs targeted in this study, sulfonamide resistance genes, *sul1* and *sul2* had the highest abundance with an average concentration of  $3.11 \times 10^7$  copies  $L^{-1}/g^{-1}$ , followed by *ermB* gene ( $9.58 \times 10^6$  copies  $L^{-1}/g^{-1}$  on average) encoding resistance to macrolides.  $\beta$ -lactams resistance genes were the third most abundant resistance gene family, among which *bla<sub>TEM</sub>* gene had the highest concentration ( $1.79 \times 10^7$  copies  $L^{-1}/g^{-1}$  in average). *tetQ* and *tetX* were the most abundant tetracycline resistance genes, with the average concentration ranging from  $3.18 \times 10^6$  to  $4.57 \times 10^6$  copies  $L^{-1}/g^{-1}$ . Trimethoprim resistance genes, *dfrA1* and *dfrA12*, were the least abundant ( $5.19 \times 10^5$  copies  $L^{-1}/g^{-1}$  in average) among the environmental samples. Heatmaps illustrating distinct patterns of the absolute and relative abundance of ARGs between environmental samples are shown in Fig. 2b & c. The trend for the relative abundance

of ARGs (normalised to the corresponding 16S rRNA gene copy number in the sample) was similar to the absolute gene copies.

#### 3.3.2. Relationship between ARGs and integron genes

Two mobile element genes, class I and class II integrons (*intI 1*, *intI 2*) were targeted in this study. The River Thames water samples had the highest integron gene copy numbers ( $4.83 \times 10^8$  copies/L for *intI 1* and  $1.40 \times 10^6$  copies/L for *intI 2*). As an indicator of horizontal gene transfer (HGT) potential, *intI 1* was reported to integrate and express > 100 types of resistance genes by gene cassettes, most of which were aminoglycoside and trimethoprim resistance genes and  $\beta$ -lactamases (Gillings et al., 2008; Gillings et al., 2015). In this study, *intI 1* showed significant positive correlations with all of the ARGs families ( $p < .05$ ), particularly with sulfonamides ( $R^2 = 0.999$ ,  $p < .001$ ), macrolides ( $R^2 = 0.998$ ,  $p < .001$ ), and trimethoprim ( $R^2 = 0.924$ ,  $p < .001$ ). In contrast with class I integrons, class II integrons were less commonly found in environmental samples. Previous research has shown that the *dfrA* genes encoding resistance to trimethoprim can be found in class II integrons (Antunes et al., 2006). This was consistent with our result as both *dfrA1* and *dfrA12* were found to have significant positive correlations ( $p < .001$ ) with *intI 2*. ARGs that are associated with mobile genetic elements can propagate among species through horizontal gene transfer mechanisms, contributing to the persistence and spread of ARGs in different environmental matrix (Chen and Zhang, 2013b).

#### 3.4. The applicability of gBlocks

In this study, we demonstrate that using gBlocks gene fragments as qPCR standards provides comparable assay performance to a traditional amplicon standard. It allows routine, reliable identification or profiling of ARGs from samples in any research laboratory with access to a real-time PCR instrument. The established in-house qPCR assays for ARGs were applicable to different environmental samples, including surface water, soil and animal faeces. Considering the complexity of DNA extracts from soil and animal faeces, this method can also be applied to a wider range of sample types, for example wastewater, manure, sediment, slurry and sludge. Apart from using as qPCR standards, gBlocks gene fragment can also be used as positive control of conventional PCR due to the unavailability of biological isolates (Krüttgen et al., 2011).

Culture-independent approaches have been successfully developed, among which qPCR, is of great importance to provide an approximation of the dissemination of known ARGs in environmental samples (Berendonk et al., 2015). In accordance with WHO's initiatives for the surveillance of antibiotic resistance, more data across countries are needed to fully assess the prevalence of ARGs worldwide (World Health Organization, 2018a). gBlocks-based qPCR standard method developed in this study provides a potential standardised approach for the comparison of resistance prevalence in different sampling locations to acquire a temporal perspective on resistance dynamics and to assess possible correlations between antibiotic resistance and anthropogenic activities. Furthermore, the simplicity of the gBlocks product format and operating procedure allow easy gene construction or modification, and it saves the cost on commercially available strains/plasmids, or the time needed for lab-exchange strains/plasmids.

There are challenges and limitations to the application of gBlocks-based qPCR standards for environmental ARGs analysis. One drawback is that, similar to conventional qPCR method, the numbers of targeted ARGs in a research project is limited. Theoretically, synthetic ARG fragments can be obtained and constructed as many as needed, but this may come at significant cost and labor. In general, up to 20 ARGs target numbers would be appropriate for a given study. Although the raw ARGs fragments are easily to obtain, the regions selected for individual ARG may vary, and may occasionally not fit the production criteria due to the sequence complexity. The operational procedures of preparing gBlocks standards are straightforward, however, specialised training

will be needed to avoid the potential cross-contamination that might occur during the gene cloning process. Furthermore, qPCR is highly based on the quality and purity of extracted DNA, which will vary in efficiency across environmental matrices and is likely to carry through inhibitors depending on DNA extraction methods that can interfere with qPCR (Luby et al., 2016). Harmonised guidelines regarding the DNA extraction methods; the target resistance genotypes or primer sets will be useful for the direct comparisons between different environmental compartments (Berendonk et al., 2015).

#### 4. Conclusion

In summary, we established in-house qPCR assays using chemically synthesized oligonucleotides (gBlocks) as standards for the quantification of fourteen ARGs and two integron genes. The performance of gBlocks-*ermB* standard was comparable to traditional *ermB* standard from a naturally occurring plasmid with similar sensitivity and amplification efficiency. The qPCR assays have been successfully applied to surface water, soil and animal faeces samples to assess the ARGs prevalence in the environment. Our study provides a routine and reliable method for the identification or profiling of ARGs, especially suitable for a research project that has several ARGs of interest or for those very rare or newly-discovered ARGs.

#### Declaration of Competing Interests

No conflict of interest declared.

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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.2019.105670>.

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