

RESEARCH ARTICLE

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Quantification of plasmid DNA reference materials for Shiga toxin-producing *Escherichia coli* based on UV, HR-ICP-MS and digital PCR

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Abstract

Background: The accuracy and metrology traceability of DNA quantification is becoming a critical theme in many fields, including diagnosis, forensic analysis, microorganism detection etc. Thus the research of DNA reference materials (RMs) and consistency of DNA quantification methods has attracted considerable research interest.

Results: In this work, we developed 3 plasmid candidate RMs, containing 3 target genes of *Escherichia coli* O157:H7 (*E. coli* O157:H7) and other Shiga toxin-producing *Escherichia coli* (STEC): *stx1*, *stx2*, and *fliC* (*h7*) respectively. Comprehensive investigation of the plasmid RMs was performed for their sequence, purity, homogeneity and stability, and then the concentration was quantified by three different methods: ultraviolet spectrophotometer (UV), high resolution inductively coupled plasma mass spectrometry (HR-ICP-MS) and digital PCR. As a routinely applied method for DNA analysis, UV was utilized for the quantification (OD₂₆₀) and purity analysis for the plasmids. HR-ICP-MS quantified the plasmid DNA through analysing the phosphorus in DNA molecules. Digital PCR distributed the DNA samples onto a microarray chip containing thousands of reaction chambers, and quantified the DNA copy numbers by analysing the number of positive signals without any calibration curves needed.

Conclusions: Based on the high purification of the DNA reference materials and the optimization of dPCR analysis, we successfully achieved good consistency between UV, HR-ICP-MS and dPCR, with relative deviations lower than 10%. We then performed the co-quantification of 3 DNA RMs with three different methods together, and the uncertainties of their concentration were evaluated. Finally, the certified values and expanded uncertainties for 3 DNA RMs (pFliC, pStx1 and pStx2) were $(1.60 \pm 0.10) \times 10^{10}$ copies/ μ L, $(1.53 \pm 0.10) \times 10^{10}$ copies/ μ L and $(1.70 \pm 0.11) \times 10^{10}$ copies/ μ L respectively.

Keywords: Plasmid DNA, Reference material, Digital PCR, *Escherichia coli* O157:H7

Background

Shiga-toxin-producing *Escherichia coli* (STEC) [1–3] is widely implicated to sporadic cases and serious outbreaks all over the world. Enterohemorrhagic *E. coli* O157:H7 (*E. coli* O157:H7) is one of its most threatening serotypes, which has been reported in over 30 countries causing severe infections [4]. Detection of *E. coli* O157:H7 [5] and

other non O157 STEC [6] is playing a key role in diagnostics, environmental protection and food safety.

DNA analysis is taking a more and more important position in pathogenic microorganism detection, for their remarkable advantages like analysis speed, specificity, sensitivity and high-throughput. Currently, most of the DNA analysis methods are semi-quantitative, including polymerase chain reaction (PCR), sequencing, DNA chip and biosensors, most of which rely on the calibration curves or the comparing threshold value comparison. In this situation, DNA reference materials (RMs) are urgently needed, to guarantee the reliability and traceability of the quantification results. The importance

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of DNA RMs has been more and more highlighted for method calibration and proficiency testing. Scientists in U.S. National Institute of Standards and Technology (NIST) reported that, stable DNA quantitation RMs could obviously help to reduce the within- and among-laboratory quantitation variability [7]. However, for DNA RM [8] development, basic research of quantification methods was needed, in order to study the consistency of these methods and analyse the uncertainty sources [9].

UV spectrophotometry (UV) is commonly used for convenient DNA routine quantification by measuring the absorbance at 260 nm (OD260) [10] based on Beer–Lambert's law for high concentration and pure DNA samples. Some other physicochemical methods have high sensitivity, accuracy and clear metrology traceability [11], however they are still often hampered by the efficiency of the phosphodiesterase enzyme digestion. HR-ICP-MS has higher sensitivity and specificity, which can accurately analyse the mass fraction of phosphorus, that stoichiometrically presents in DNA molecule [12], and consequently achieve the DNA concentration with high precision and clear traceability to the International system of units (SI) [12–15]. However, a disadvantage of UV and HR-ICP-MS is their incapability of specifically distinguishing different DNA sequences.

Real-time quantitative PCR (qPCR) is capable of sensitive and specific nucleotide acid analysis even under very low concentration. However, as a relative method, the quantification results is always traced back to UV [16], when always using a working curve. In contrast, digital PCR (dPCR) [17] as a novel promising DNA absolute quantification method, is more accurate and precise than qPCR, and most importantly, it can independently quantify DNA without calibration or internal control [18]. The sample is partitioned onto a microarray chip with thousands of separate reaction chambers, so that each chamber contains 1 or 0 target molecule. After a PCR amplification step, we can accurately determine the copy number of the original DNA samples [18, 19], by analysing the number of positive partitions (where an amplified signal is found).

When using different methods together for DNA quantification, the consistency is always a critical challenge, which seriously impedes the reliability of the results [20, 21]. Thus, it is becoming more and more important to establish method standard and DNA reference materials. In this work, we developed 3 plasmid candidate RMs for STEC analysis, containing 3 main target genes respectively: Shiga toxin 1 (stx1), Shiga toxin 2 (stx2) and fliC (h7) [22]. In this work, we investigated three methods for DNA quantification: UV, HR-ICP-MS and dPCR, each of which represents a distinctive analysis principle: spectrophotometry, element assay and DNA amplification

respectively. Key conditions were optimized to finally achieve improved consistency and reliable RM quantification results.

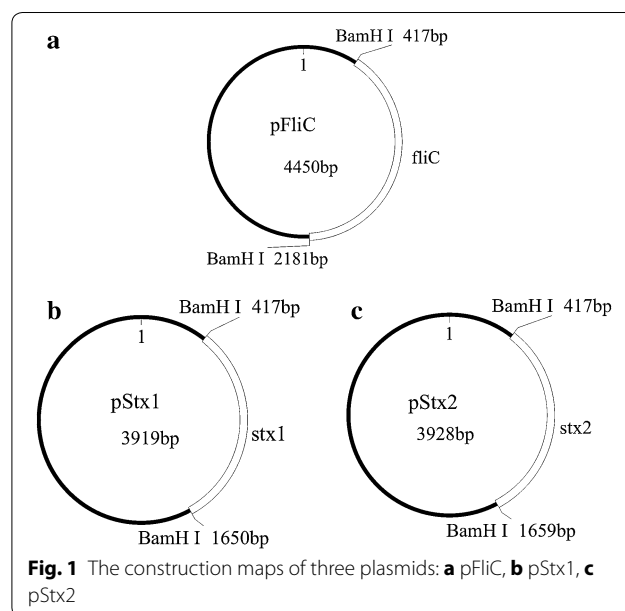
Results

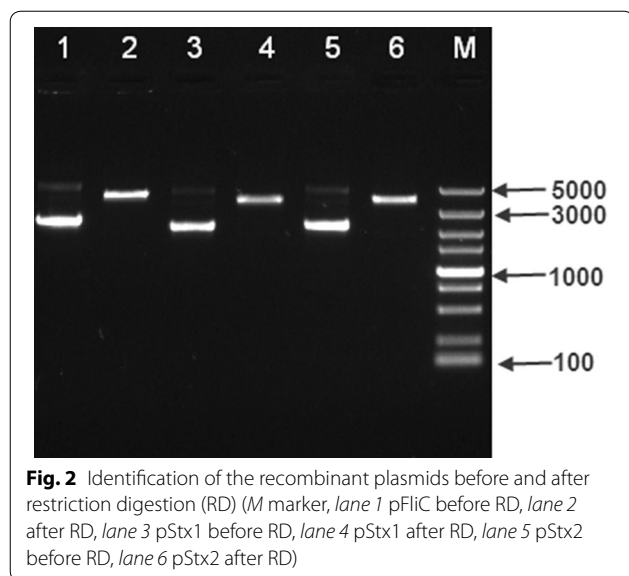
Plasmid DNA construction

The target fragments stx1 (1227 bp), stx2 (1236 bp) and fliC (1758 bp) were ligated into pUC19 vector, and then transformed into *E. coli* JM109. The size of these recombinant plasmids were 4450 bp (pFliC), 3919 bp (pStx1), and 3928 bp (pStx2) respectively. The construction maps (Fig. 1) showed the structures of the recombinant plasmids, each of which carried a single copy of one target gene. The sequencing results confirmed that they were 100 % consistent with the initial design (data not shown).

Then the produced plasmids were investigated by 1 % agarose gel electrophoresis. In order to eliminate the effect of the molecular secondary structures in electrophoresis, our circular plasmids were cut to linear using an enzyme digestion step. As shown in Fig. 2, the size of the digested linear plasmids was demonstrated to be the same as our design (between 3000 and 5000 bp), and no RNA and low-mass fragments were found.

For well purified double-stranded DNA (dsDNA) solution, A260/A280 should be between 1.8 and 2.0 and A260/A230 should be higher than 2.0 [23]. As our UV results showed, ratios of A260/A280 for pFliC, pStx1 and pStx2 were 1.89, 1.88 and 1.83, and ratios of A260/A230 were 2.06, 2.08 and 2.01 respectively, indicating good purity of the DNA samples (Additional file 1: Figure S1). 3 plasmids were then quantified by UV (OD260).





Homogeneity and stability study

For each plasmid candidate RM, 300 bottles of replicates were produced, and the homogeneity of the sub-packed candidate RMs was investigated by UV. For the between-bottle homogeneity, 15 bottles of one plasmid were randomly selected from the whole batch, and 3 test portions (1 μ L) from each bottle were analysed. For the within-bottle homogeneity study, 16 test portions (1 μ L) from one same bottle were analysed. The sample homogeneity was assessed by a one-way analysis of variance (ANOVA) [29], and finally the F test values (F_{cal}) were calculated to be lower than the critical values at 95 % confidence for all 3 candidate plasmid between- and within-bottle (Additional file 1: Table S1), which clearly demonstrated their good homogeneity with the minimum sampling volume as low as 1 μ L. For short-term stability study, 3 bottles of each plasmid were placed at 4 $^{\circ}$ C, for different storage time (0, 1, 3, 7 and 15 days) and quantified by UV, then the analysis data was analysed by the classic linear model and t test. The result showed that all of the three candidate RMs were stable for 15 days under 4 $^{\circ}$ C, which is adequate for the sample delivery (Additional file 1: Table S2).

The long-term stability of the candidate RMs was then continuously investigated for 12 months under -20° C storage. One bottle of each plasmid was randomly taken out from the batch and analysed for 3 times ($n = 3$) by UV at 0.5, 1, 2, 4, 6, 9 and 12 months. The classic linear model and t test was used for the stability analysis. The significance factors (t) of the slopes (β_1) were calculated by equation $t = |\beta_1|/s(\beta_1)$, where $s(\beta_1)$ is the standard deviation (SD) of the slope, t represents the change of the sample concentration. Our results demonstrated 3

candidate RMs were stable in 12 months, because t was lower than $t_{0.95, n-2}$ (under 95 % confidence level, $n - 2$ is the degree of freedom of data analysis (Additional file 1: Table S3).

In summary, we produced 3 plasmid candidate RMs, each of which had 300 bottles of duplicates with well demonstrated purity, homogeneity and stability.

HR-ICP-MS quantification

HR-ICP-MS was applied to quantify the purified plasmids by analyzing the phosphorus in DNA molecules. ELEMENT2 is a double focusing magnetic sector field HR-ICP-MS, which is capable of separate possible polyatomic interferences even with a very small mass difference like ^{31}P (30.97376) and $^{15}\text{N}^{16}\text{O}$ (30.99502).

Based on the excellent resolution of HR-ICP-MS, phosphorus in DNA molecule was accurately quantified. We statistically compared the analysis results of the DNA samples before and after digestion, and the difference was demonstrated to be insignificant (Additional file 1: Figure S4) due to t-test. Thus in our work, all the HR-ICP-MS analysis was performed without any digestion treatment (Fig. 3a).

The working curve with good linear correlation was established by using a phosphorus solution certified RM (SRM3139a) from NIST (Fig. 3b). Then eight aliquots of each plasmid solution were analysed to achieve an average phosphorus concentration value. Based on P % in DNA molecules, the copy number concentrations of three plasmids were calculated.

Digital PCR quantification

Before dPCR was performed, the primer design and sample pretreatment was investigated by qPCR. The candidate RMs were treated by restriction enzyme, and the samples before (circular DNA molecules) and after (linear DNA molecules) enzyme treatment were analysed by qPCR. The result indicated that C_t values of all the samples were delayed by 1.0–2.4 cycles without enzyme digestion, due to the inhibiting effect of the plasmids' circular conformation [24]. Additional file 1: Figure S2 showed the qPCR result of pFliC as an example.

We established the qPCR calibration curves for 3 genes (Fig. 4). The PCR efficiencies (E) of PCR were calculated to be between 95 and 110 % using the equation $E = 10^{(-1/k)} - 1$, where k is the slope of the calibration curves [25], and the R^2 was larger than 0.99. The limit of detection (LOD) was calculated to be lower than 30 copies ($3 \times \text{SD}$, SD was the standard deviation of the lowest C_t detected by qPCR), and no unspecific amplification was found in negative controls. These results demonstrated the validity of the plasmid production and primer

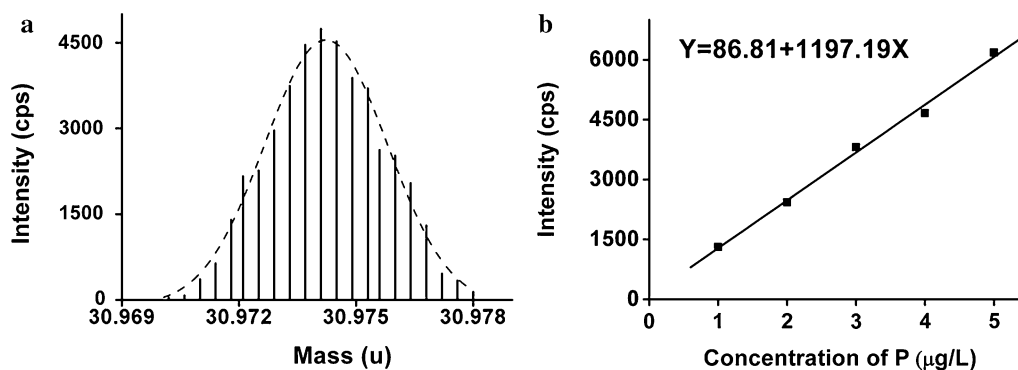


Fig. 3 HR-ICP-MS analysis results: **a** HR-ICP-MS spectrum of phosphorus in DNA (pStx2), **b** the standard curve for phosphorus analysis using phosphorus solution certified RM (SRM3139a)

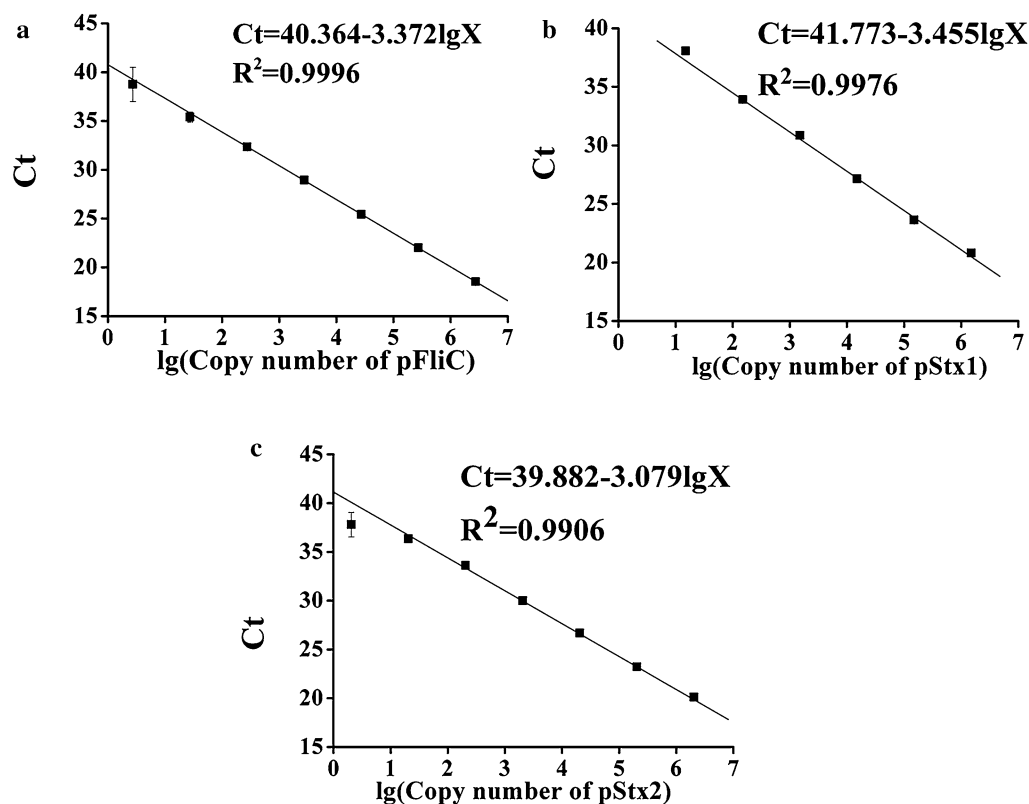


Fig. 4 Real-time PCR calibration curves for 3 plasmid RMs: **a** pFliC, **b** pStx1 and **c** pStx2. The X was the concentration of the plasmids

designing, which was important for the following dPCR analysis.

Then, dPCR was performed based on the optimized conditions. Compared to the droplet digital PCR (ddPCR) which only detects the PCR end-point fluorescence signal, the chip digital PCR in our work recorded the whole PCR amplification curves (Fig. 5)

with important information of the PCR amplification, and statistically recognized positive signals based on a threshold C_{rt} value. The limit of C_{rt} values (green vertical bars in Fig. 5) were set based on 95 % confidence interval of the numbers of positive signals. The amplification curves with Ct values between the green vertical bars were taken as positive amplifications, while those beyond

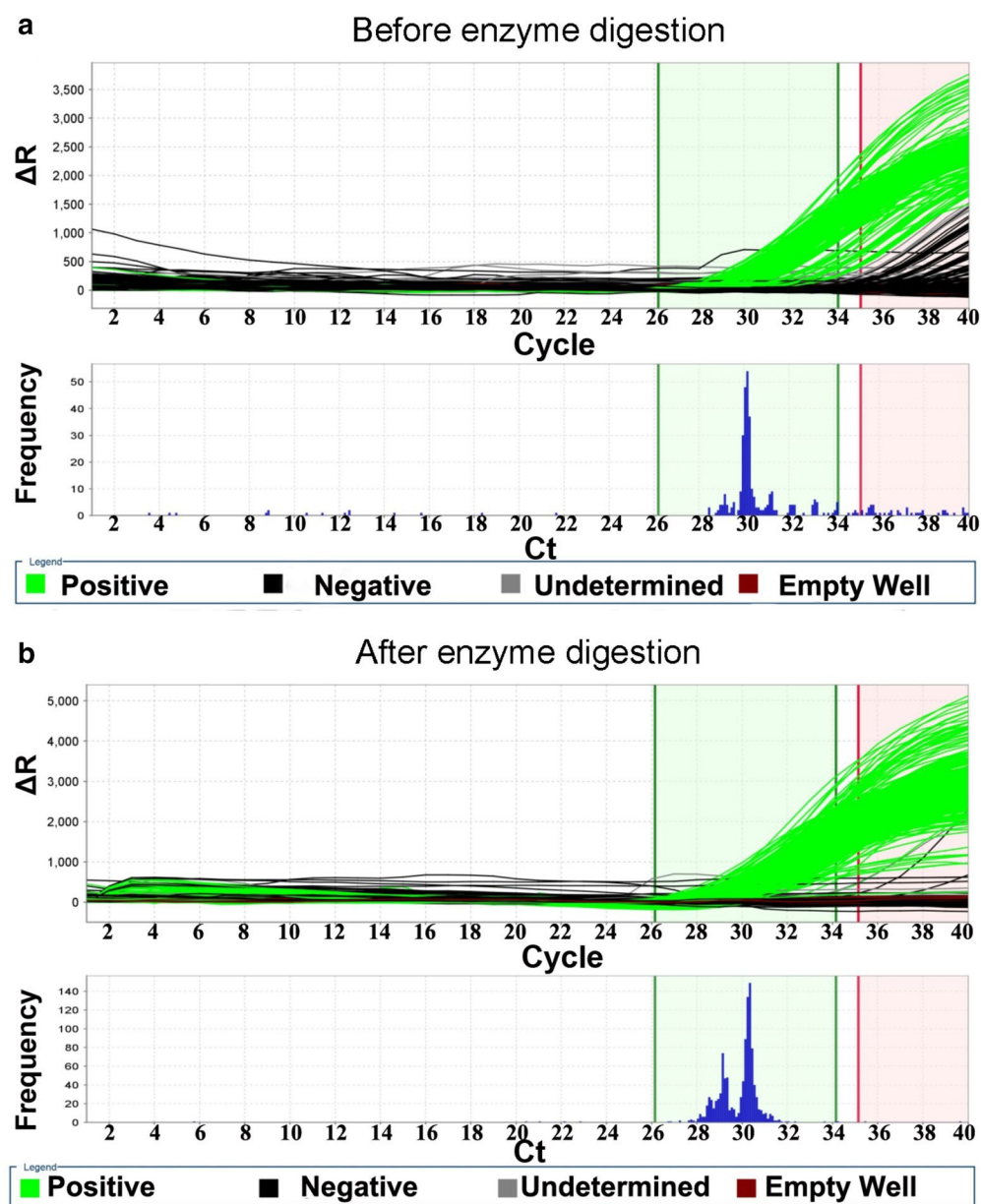


Fig. 5 The dPCR amplification curves of pStx2 before (a) and after (b) enzyme digestion. The relative fluorescence signal (ΔR) was automatically normalized by deducting the background of each chamber. The frequency represented the number of the PCR curves with a certain Ct value

the limits (the red vertical bar in Fig. 5) were regarded as negative signals. Finally, thousands of adopted signals including positive and negative were analysed based on Poisson's distribution, and reliable quantification results were achieved. When we compared the dPCR amplification curves before and after enzyme digestion (Fig. 5 was the results for pStx2), many delayed Ct values were found without enzyme digestion (black curves in Fig. 5a),

causing a 53–66 % decrease of the dPCR quantification results (Additional file 1: Figure S3).

In dPCR quantification, totally 3072 chambers of one microarray chip were divided into 4 groups (see red dashed lines in Fig. 6), and each of the group was uploaded with a diluted concentration of the plasmid separately (from S1 to S4 in Fig. 6). Our dPCR quantification results of three plasmids were shown in Fig. 6.

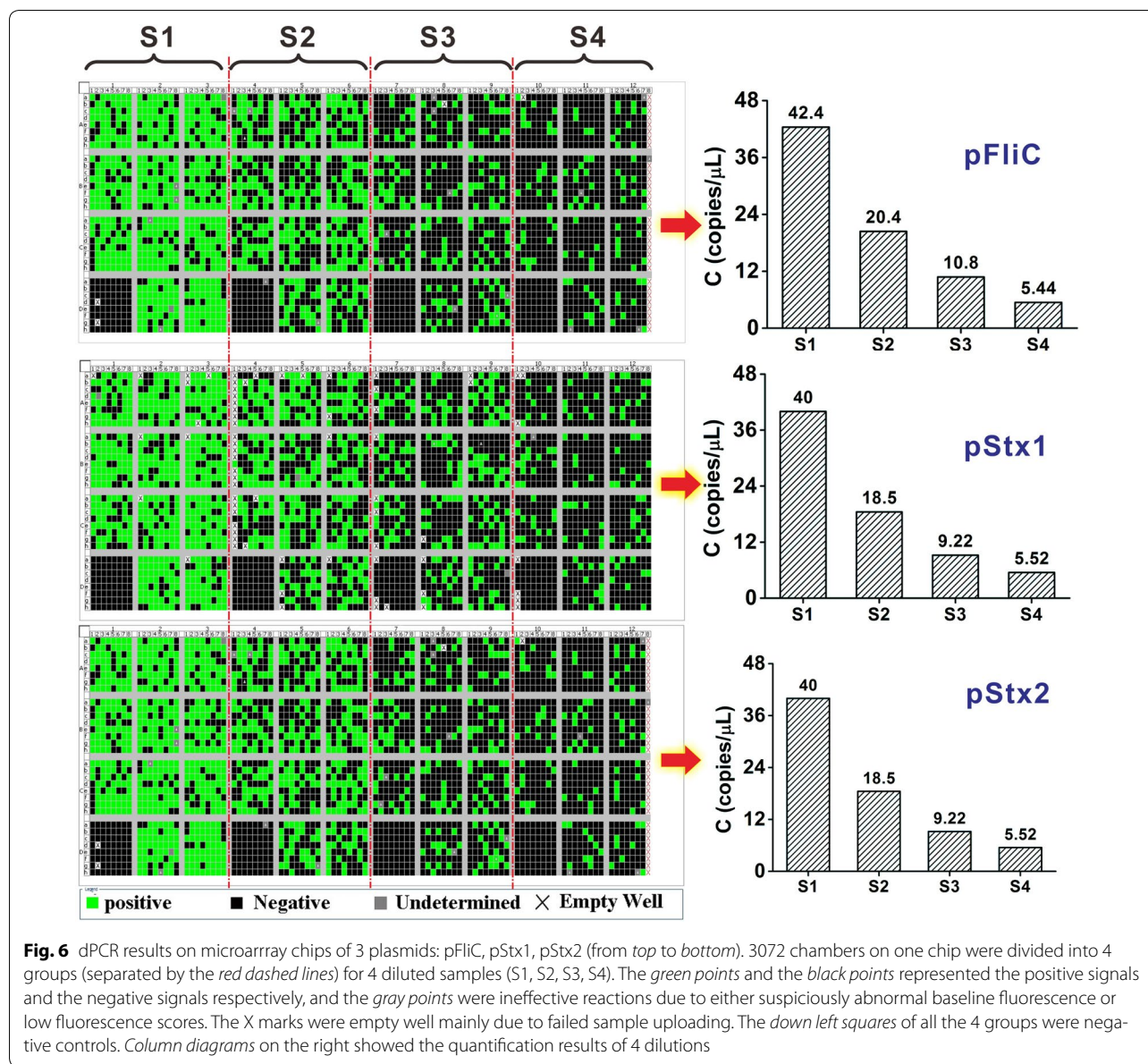


Fig. 6 dPCR results on microarray chips of 3 plasmids: pFliC, pStx1, pStx2 (from top to bottom). 3072 chambers on one chip were divided into 4 groups (separated by the red dashed lines) for 4 diluted samples (S1, S2, S3, S4). The green points and the black points represented the positive signals and the negative signals respectively, and the gray points were ineffective reactions due to either suspiciously abnormal baseline fluorescence or low fluorescence scores. The X marks were empty well mainly due to failed sample uploading. The down left squares of all the 4 groups were negative controls. Column diagrams on the right showed the quantification results of 4 dilutions

From left to right, the number of positive signals (green) decreased obviously with the decrease of the sample concentration. The negative controls (down left of each partition) showed no positive signals, demonstrating the absence of sample pollution or unspecific amplification.

Four quantification results from four partitions (left in Fig. 6) kept good doubling relationship, which strongly demonstrated the reliability of the dPCR quantification. Finally the average of four groups (S1–S4) was taken as the dPCR result for each plasmid.

Method evaluation and RM certification

In order to realize reliable certification of the RM concentration and to evaluate the performance of the

methods, after the quantification of 3 different methods (Additional file 1: Table S5), we assessed the uncertainty of the results, according to the internationally recognised *Guide to the expression of uncertainty in measurement (GUM)* [26], and the uncertainty was derived from two main sources:

(1) Uncertainty of 3 quantification methods:

Firstly, UV analysis was directly performed without sample dilution step, thus the only uncertainty source of UV result was the SD of 8 replicates ($n = 8$), the relative uncertainty of UV analysis was calculated by the equation: $u_{c(UV)} = SD/(\sqrt{n} \cdot \bar{x}_{UV})$, where \bar{x}_{UV} was the average concentration value achieved by UV.

Secondly, RMs were diluted to lower than 10 $\mu\text{g/L}$ (by about 2000 times dilution), then analysed by HR-ICP-MS to quantify P concentration (C_s) in the sample solution, and then DNA concentration was calculated through the $C_{(\text{ICP-MS})} = C_s \cdot V_s / (V \cdot P\%)$, where V_s is the final sample volume, V is the volume of plasmid DNA RMs before dilution, and $P\%$ is the mass fraction of P element in the plasmid DNA molecules. Thus, main uncertainty sources were: first, the uncertainty of C_s including the SD of 8 replicates, the uncertainty of the phosphorus solution RM and the uncertainty of the standard curve fitting; Second, the uncertainty of V_s and V which came from the uncertainty of the pipettors and the volumetric flasks, and the volume change due to the temperature variation.

Thirdly, for dPCR analysis, RMs were diluted to about 18 copies/ μL (by about 10^9 times dilution), then automatically uploaded onto the microarray chips and quantified. The uncertainty sources mainly came from the dilution and the SD of 8 replicates.

Error bars in Fig. 7 represented the uncertainties of 3 methods. The uncertainty of UV was lower than two other methods, because UV was directly performed under high concentration without any dilution. On the contrary, the RMs needed 2000 and 10^9 times dilution before HR-ICP-MS and dPCR quantification respectively. The deep dilution operation and the lower repeatability at low concentration introduced much higher uncertainties to the final results. Owing to the optimization, we achieved very good consistence between different methods with relative deviations lower than 10 %. The black dashed lines in Fig. 7 were the average values of three RMs.

Finally, the quantification uncertainties of 3 plasmid RMs (u_c) were calculated to be 0.052, 0.048 and 0.053

($\times 10^{10}$ copies/ μL) for plasmid pFliC, pStx1 and pStx2, using the following equation:

$$u_c = \bar{X} \cdot \sqrt{\left(\frac{u_c(\text{UV})}{C_{\text{UV}}}\right)^2 + \left(\frac{u_c(\text{HR-ICP-MS})}{C_{(\text{HR-ICP-MS})}}\right)^2 + \left(\frac{u_c(\text{dPCR})}{C_{\text{dPCR}}}\right)^2}$$

(2) Uncertainty of the samples (instability and inhomogeneity):

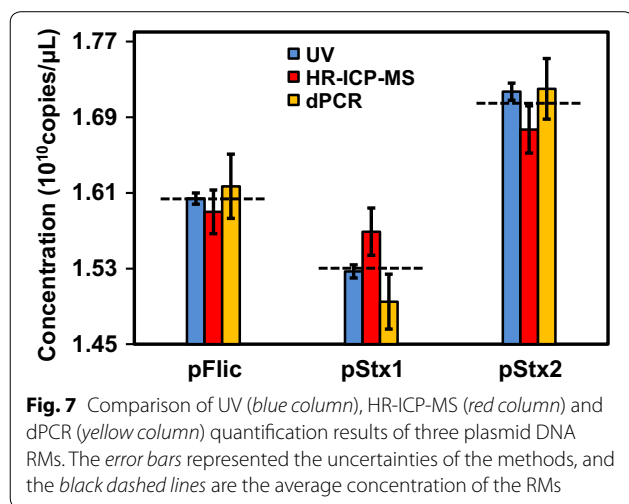
Two other main uncertainty sources of the RM concentration were the uncertainty from instability (u_s) during the storage and the uncertainty from inhomogeneity (u_{bb}) between bottles. The u_s was calculated using the following equation: $u_s = s(\beta_1) \cdot X$, where X is the storage time. The results were summarized in Additional file 1: Table S3. In our work, the mean square of within-bottle analysis data (MS_{within}) was higher than that of between-bottle (MS_{between}) (Additional file 1: Table S1), thus u_{bb} was calculated due to a simplified equation: $u_{bb} = \sqrt{MS_{\text{within}}/n} \times \sqrt{2/V_{\text{within}}}$, where MS_{within} is the mean square of within-bottle analysis data, n is number of analysis replicates for one same bottle, and V_{within} is the degree of freedom of MS_{within}^{25} . All the data is summarized in Additional file 1: Table S1.

The uncertainties of the RM concentrations (u) were then summarized, including u_c , u_{bb} and u_s : $u = \sqrt{u_c^2 + u_{bb}^2 + u_s^2}$. The expanded uncertainty (U) was then calculated by multiplying u by the coverage factor ($k = 2$) ($U = k \times u$). The detailed data of uncertainty evaluation was listed in Additional file 1: Tables S5, S6. Finally, The certified values and expanded uncertainties for plasmid pFliC, pStx1 and pStx2 were $(1.60 \pm 0.10) \times 10^{10}$ copies/ μL , $(1.53 \pm 0.10) \times 10^{10}$ copies/ μL , and $(1.70 \pm 0.11) \times 10^{10}$ copies/ μL respectively.

Conclusions

The lack of DNA RMs and acknowledged DNA quantification standards, hinders the result mutual accreditation and explains the significance of the consistency, uncertainty and traceability of DNA analysis methods. In this work, 3 candidate plasmid RMs for pathogenic *E. coli* detection were prepared with well investigated sequence, homogeneity and stability, and 3 different methods were studied including UV, HR-ICP-MS and dPCR, for the certification of the candidate plasmid RMs.

We investigated the accuracy and the uncertainty of all these methods. UV is simple and stable for purified and high-concentration samples, and the average relative expanded uncertainty of UV for three RMs were only 0.46 %. HR-ICP-MS has clear metrology traceability through phosphorus quantification. The diluted RMs were quantified by HR-ICP-MS, based on the high resolution of phosphorus in DNA molecules. DPCR is capable



of absolute quantification of very low copy numbers of DNA based on the high-throughput microarray chips. For reliable dPCR results, enzyme digestion was researched by qPCR to achieve a distinctively improved PCR performance. However, even with high sensitivity, the complex dilution processes and environmental interferences inevitably enlarged the relative expanded uncertainty of HR-ICP-MS and dPCR to 1.52 and 1.96 % respectively.

Based on detailed optimization of 3 different methods, with very different analysis strategies and detection sensitivity, we finally combined them for the quantification of DNA reference materials. Due to the high resolution of HR-ICP-MS, we eliminated the digestion step to reduce the uncertainty of its results. Although we strongly suggest the importance of sample pretreatment for accurate dPCR to guarantee the amplifying efficiency. Another fact demonstrated by our results is that, the uncertainty from dilution steps is usually a major part, and thus the preparation of standard solutions at low concentrations should be performed very carefully, and the uncertainty should always be counted in. We choose suitable quantification methods and DNA reference materials due to the concentration level, in order to get rid of unnecessary uncertainty sources.

Methods

Reagents and instruments

Primers and DNA sequences were synthesized by Takara (Dalian, China). *Escherichia coli* (*E. coli*) strain JM109 from Takara was used as the host for cloning and plasmid propagation. The plasmid pUC-19 vector, the restriction enzymes *Hind* III and *Sal* I, T4 DNA Polymerase and DL5000 DNA marker used in gel electrophoresis were also purchased from Takara. HR-ICP-MS was performed on the ELEMENT 2 (ThermoFisher Scientific) system. ABI QuantStudio 12K Flex qPCR System (ThermoFisher Scientific) was applied for both qPCR and dPCR (with different analysis modules). All other reagents were at

least analytical grade purity. Ultrapure water (18.2 M Ω) was obtained from a Milli-Q system (Millipore).

All primers and TaqMan probes for PCR analysis were listed in Table 1.

Production of the plasmids

The sequences of three target genes were determined based on the data in GeneBank: The feature gene *fliC* (GenBank: AF228492.1) was a target for the detection of Serotype O157:H7 *E. coli*, while the gene *stx1* (GenBank: EF079675.1) and *stx2* (GenBank: GU126552.1) were specific targets for Shiga-toxin-producing *E. coli*.

We synthesized the sequences containing the target genes, each of which was flanked by two restriction *Bam*H I enzyme sites. Then the synthetic sequences were inserted into pUC19 vectors in a ligation system with a final volume of 20 μ L containing 1 U T4 DNA Polymerase, Tris-acetate 33 mM, CH₃COOK 66 mM, (CH₃COO)₂Mg 10 mM, DTT 0.5 mM, target gene 200 ng and vector DNA 50 ng.

The produced plasmids were then transformed into the host (JM109) mainly following a reported protocol [27], and the positive recombinant bacterial colony was confirmed by PCR reaction and DNA sequencing. Then, the recombinant bacteria was propagated in Luria-Bertani broth [10 g of tryptone, 5 g of yeast extract, and 10 g of sodium chloride in 1 L water (pH 7.4)] for 12–16 h. Bacterial cells were lysed with alkaline-SDS solution, and the produced plasmids were purified with ethanol following a reported protocol [28]. Finally, the plasmids were diluted by TE buffer (10 mM Tris, 0.1 mM EDTA, pH 7.5), and carefully divided into 300 bottles (100 μ L).

Enzyme digestion

Enzyme digestion solution was as follows: 1 μ L restriction enzyme (15 U/ μ L), 2 μ L digestion buffer, 2 μ L plasmid DNA from previous step (containing about 160 ng plasmid DNA), an 15 μ L H₂O. Purified plasmid pFliC and

Table 1 The sequences of the primers and probes for the qPCR and dPCR analysis of 3 plasmids

primer/probe	Sequence 5'–3'	Amplicon size (bp)
<i>fliC</i> -FP	CCGCGAGCGAAGGTAGTG	75
<i>fliC</i> -RP	CAGGAGTTGCTTTTGCGATAGTATAT	
<i>fliC</i> -probe*	FAM-CGGTGCTTCTCTGACATTCAATGGCA- TAMRA	
<i>stx1</i> -FP	TGCAGATAAATCGCCATTCG	123
<i>stx1</i> -RP	AAGCTTCAGCTGTACAGTAACAAA	
<i>stx1</i> -probe	FAM-ACCTCACTGACGAGTCTGTGGCAAGA- TAMRA	
<i>stx2</i> -FP	CACTGTCTGAAACTGCTCCTGTTT	78
<i>stx2</i> -RP	TGCTGATTCTCCCCAGTTC	
<i>stx2</i> -probe	FAM-CGGTGCTTCTCTGACATTCAATGGCA- TAMRA	

* The Taqman probe was labeled with a FAM fluorophore and a TAMRA quencher

pStx2 were digested with *Hind*III, and pStx1 was digested with *Sal*I. The digestion buffer for pFliC and pStx2 was as following: 100 mM Tris–HCl (pH 7.5), 100 mM MgCl₂, 10 mM Dithiothreitol and 500 mM NaCl. The digestion buffer for plasmids pStx1 was consist of: 500 mM Tris–HCl (pH 7.5), 100 mM MgCl₂, 10 mM Dithiothreitol and 1000 mM NaCl. The digestion was performed under 37 °C for 2 h, then the digestion result was investigated by 1 % agarose gel electrophoresis.

UV spectrophotometry

UV quantification of the plasmids was performed on a UV–VIS spectrophotometer (Cary-100, Varian). The absorbance of the purified plasmid DNA was measured at 230, 260, 280, and 320 nm, and the mass concentration (C_m , ng/μL) of DNA was calculated through the equation: $C_m = (A_{260} - A_{230}) \times 50$, where A_{260} and A_{320} are the absorbance at 260 and 320 nm, and A_{320} was subtracted as background absorption. For the UV quantification of the double-strand DNA (dsDNA), the molar absorption coefficients is 50 ng/μL [29]. The copy number concentration of plasmids (C_c) was calculated using the equation: $C_c = C_m \times N_A / M_{DNA}$, where N_A is the Avogadro constant, and M_{DNA} is the molecular weights of plasmids.

HR-ICP-MS

The key conditions for HR-ICP-MS analysis were as following: the plasma power was 1350 W, the flow rate of cool gas, aux gas and sample gas was 16.86, 0.99 and 1.123 L/min respectively. A phosphorus solution RM from NIST (SRM3139a) was applied as the external standard calibration, so as to guarantee the traceability for the quantification.

The mass fraction of phosphorus (P %) in plasmid DNA was calculated to be 10.22 %, using the equation $P \% = 2 \times \text{bp} \times 30.974 / M_{DNA}$, Where bp is the number of base pairs in plasmid molecules, and M_{DNA} is the molecular weight of the plasmids. Thus, the concentration of DNA (C_m) was achieved from the concentration of phosphorus (P_{mass}) through the equation: $C_m = P_{mass} / P \%$.

qPCR

Seven serially diluted solutions of enzyme digested plasmid DNA (with UV estimated concentration from 10⁶ to 1 copies/μL) were used to establish the PCR calibration curve. The PCR reaction mixture contained: 10 μL 2× Master Mix (TaqMan Universal PCR Master Mix with ROX reference dye pre-mixed, Applied Biosystems), 0.2 μM probe, 0.4 μM forward primer and 0.4 μM reverse primer, 1 μL enzyme digested plasmid DNA, and DNase-free H₂O. The PCR thermal program was as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s; then 60 °C for 60 s; fluorescence was collected at the annealing

and extension step (60 °C) [30]. For each analysis, the same amount of TE buffer was analyzed as the blank.

dPCR

The thermal program, concentration of primers and probes for dPCR assay were all the same with qPCR. The dPCR chip has 3072 reaction chambers with a volume of 33 nL, and for an effective dPCR analysis, each of the chip chamber should contain 0 or 1 copy of target DNA molecule. Thus, In our experiment, four deeply diluted samples were prepared by 1 × 10⁸, 2 × 10⁸, 4 × 10⁸ and 8 × 10⁸ times (S1–S4) dilution of the original RMs. Finally, 1.25 μL of each diluted sample was added to 5 μL PCR reaction mixture and then uploaded onto microarray chips for dPCR analysis. TE buffer was analyzed as blank. After an amplification process, the dPCR curves were statistically analyzed, and the quantification results were achieved without any working standards needed.

Additional file

Additional file 1. Supplementary information.

Authors' contributions

GL and WL conceived and designed the study. LX, ZS, YL, LL, and YW performed the experiments. GL and WL wrote the paper. SR reviewed and edited the manuscript. All authors read and approved the final manuscript.

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Acknowledgements

The authors would like to thank the financial support from the National Natural Science Foundation of China (NSFC Grants 21305091 and 21205079), General Administration of Quality Supervision, Inspection and Quarantine of the People's Republic of China (Grants 201310016 and 2014QK141).

Competing interests

The authors declare that they have no competing interests.

Received: 25 February 2016 Accepted: 1 September 2016

Published online: 12 September 2016

References

1. Paton AW, Paton JC (1998) Detection and characterization of shiga toxigenic *Escherichia coli* by using multiplex PCR assays for stx(1), stx(2), eaeA, enterohemorrhagic *E. coli* hlyA, rfb(O111), and rfb(O157). *J Clin Microbiol* 36:598–602
2. Ateba CN, Mbewe M (2011) Detection of *Escherichia coli* O157:H7 virulence genes in isolates from beef, pork, water, human and animal species in the northwest province, South Africa: public health implications. *Res Microbiol* 162:240–248
3. Zainudin N, Mohd Hairul AR, Yusoff MM, Tan LL, Chong KF (2014) Impedimetric graphene-based biosensor for the detection of *Escherichia coli* DNA. *Anal Methods* 6:7935–7941

- Rangel JM, Sparling PH, Crowe C, Griffin PM, Swerdlow DL (2005) Epidemiology of *Escherichia coli* O157:H7 outbreaks, United States, 1982–2002. *Emerg Infect Dis* 11:603–609
- Barkocy-Gallagher GA, Arthur TM, Rivera-Betancourt M, Nou XW, Shackelford SD, Wheeler TL, Koohmaraie M (2003) Seasonal prevalence of Shiga toxin-producing *Escherichia coli*, including O157:H7 and non-O157 serotypes, and *Salmonella* in commercial beef processing plants. *J Food Prot* 66:1978–1986
- Brooks JT, Sowers EG, Wells JG, Greene KD, Griffin PM, Hoekstra RM (2005) Strockbine NA Non-O157 shiga toxin-producing *Escherichia coli* infections in the United States, 1983–2002. *J Infect Dis* 192:1422–1429
- Kline MC, Duewer DL, Travis JC, Smith MV, Redman JW, Vallone PM, Decker AE, Butler JM (2009) Production and certification of NIST standard reference material 2372 human DNA quantitation standard. *Anal Bioanal Chem* 394:1183–1192
- Haynes RJ, Kline MC, Toman B, Scott C, Wallace P, Butler JM, Holden MJ (2013) Standard reference material 2366 for measurement of human cytomegalovirus DNA. *J Mol Diagn* 15:177–185
- Griffiths KR, Burke DG, Emslie KR (2011) Quantitative polymerase chain reaction: a framework for improving the quality of results and estimating uncertainty of measurement. *Anal Methods* 3:2201–2211
- Bhat S, Curach N, Mostyn T, Bains GS, Griffiths KR, Emslie KR (2010) Comparison of methods for accurate quantification of DNA mass concentration with traceability to the international system of units. *Anal Chem* 82:7185–7192
- O'Connor G, Dawson C, Woolford A, Webb KS, Catterick T (2002) Quantitation of oligonucleotides by phosphodiesterase digestion followed by isotope dilution mass spectrometry: proof of concept. *Anal Chem* 74:3670–3676
- Leclerc O, Fraisse PO, Labarraque G, Oster C, Pichaut JP, Baume M, Jarraud S, Fiscaro P, Vaslin-Reimann S (2013) Method development for genomic *Legionella pneumophila* DNA quantification by inductively coupled plasma mass spectrometry. *Anal Biochem* 435:153–158
- Leclerc O, Fraisse P-O, Labarraque G, Oster C, Pichaut J-P, Baume M, Jarraud S, Fiscaro P, Vaslin-Reimann S (2013) Method development for genomic *Legionella pneumophila* DNA quantification by inductively coupled plasma mass spectrometry. *Anal Biochem* 435:153–158
- Brennan RG, Rabb SA, Holden MJ, Winchester MR, Turk GC (2009) Potential primary measurement tool for the quantification of DNA. *Anal Chem* 81:3414–3420
- Holden MJ, Rabb SA, Tewari YB, Winchester MR (2007) Traceable phosphorus measurements by ICP-OES and HPLC for the quantitation of DNA. *Anal Chem* 79:1536–1541
- Brennan RG, Rabb SA, Holden MJ, Winchester MR, Turk GC (2009) Potential primary measurement tool for the quantification of DNA. *Anal Chem* 81:3414–3420
- Bhat S, McLaughlin JLH, Emslie KR (2011) Effect of sustained elevated temperature prior to amplification on template copy number estimation using digital polymerase chain reaction. *Analyst* 136:724–732
- Burke DG, Dong L, Bhat S, Forbes-Smith M, Fu S, Pinheiro L, Jing W, Emslie KR (2012) Digital polymerase chain reaction measured pUC19 marker as calibrant for HPLC measurement of DNA quantity. *Anal Chem* 85:1657–1664
- Nixon G, Garson JA, Grant P, Nastouli E, Foy CA, Huggett JF (2014) Comparative study of sensitivity, linearity, and resistance to inhibition of digital and nondigital polymerase chain reaction and loop mediated isothermal amplification assays for quantification of human cytomegalovirus. *Anal Chem* 86:4387–4394
- Baume M, Garrelly L, Facon JP, Bouton S, Fraisse PO, Yardin C, Reyrolle M, Jarraud S (2013) The characterization and certification of a quantitative reference material for *Legionella* detection and quantification by qPCR. *J Appl Microbiol* 114:1725–1733
- Caprioara-Buda M, Meyer W, Jeynov B, Corbisier P, Trapmann S, Emons H (2012) Evaluation of plasmid and genomic DNA calibrants used for the quantification of genetically modified organisms. *Anal Bioanal Chem* 404:29–42
- Fratamico P, DebRoy C (2010) Detection of *Escherichia coli* O157:H7 in food using real-time multiplex PCR assays targeting the stx 1, stx 2, wzy O157, and the fliC h7 or eae genes. *Food Anal Methods* 3:330–337
- Kalman L, Tarleton J, Percy A, Aradhya S, Bale S, Bayrak-Toydemir P, Bridges C, Buller-Burckle A, Dag S, Iyer R, Vo T, Zvereff V, Toji L (2013) Development of a genomic DNA reference material panel for rett syndrome genetic testing. *J Mol Diagn* 15:845–846
- Lin CH, Chen YC, Pan TM (2011) Quantification bias caused by plasmid DNA conformation in quantitative real-time PCR assay. *PLoS ONE* 6:e29101
- Brod FCA, Dinon AZ, Kolling DJ, Faria JC, Arisi ACM (2013) Development of plasmid DNA reference material for the quantification of genetically modified common bean embrapa 5.1. *J Agr Food Chem* 61:4921–4926
- International Bureau of Weights and Measures, International Organization for Standardization (1993) Guide to the expression of uncertainty in measurement, 1st edn. International Organization for Standardization, Genève
- Joseph Sambrook DWR (2002) Molecular cloning: a laboratory manual, 3rd ed
- Baoutina A, Bhat S, Emslie KR (2013) Development of synthetic DNA reference material for accurate analysis of gene therapy vectors. *Hum Gene Ther* 24:A63–A64
- ISO21571 (2005) Foodstuffs-methods of analysis for detection of genetically modified organisms and derived products—nucleic acid extraction. Geneva
- Hindson CM, Chevillet JR, Briggs HA, Gallichotte EN, Ruf IK, Hindson BJ, Vessella RL, Tewari M (2013) Absolute quantification by droplet digital PCR versus analog real-time PCR. *Nat Methods* 10:1003–1005

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