

Applicability of Control Materials To Support Gene Promoter Characterization and Expression in Engineered Cells Using Digital PCR

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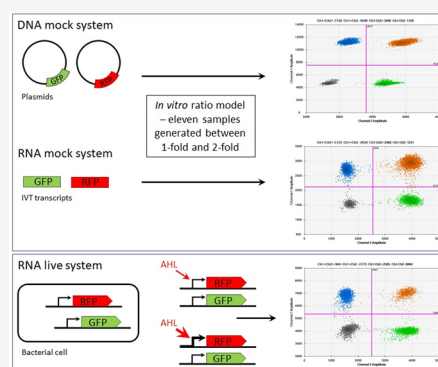


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ABSTRACT: The use of standardized components and processes in engineering underpins the design-build-test model, and the engineering of biological systems is no different. Substantial efforts to standardize both the components and the methods to validate the engineered biological systems is ongoing. This study has developed a panel of control materials encoding the commonly used reporter genes GFP and RFP as DNA or RNA molecules. Each panel contained up to six samples with increasingly small copy number differences between the two reporter genes that ranged from 1- to 2-fold differences. These copy number differences represent the magnitude of changes that may need to be measured to validate an engineered system. Using digital PCR (dPCR), we demonstrated that it is possible to quantify changes in both gene and gene transcript numbers both within and between samples down to 1.05-fold. We corroborated these findings using a simple gene circuit within a bacterial model to demonstrate that dPCR was able to precisely identify small changes in gene expression of two transcripts in response to promoter stimulation. Finally, we used our findings to highlight sources of error that can contribute to the measurement uncertainty in the measurement of small ratios in biological systems. Together, the development of a panel of control materials and validation of a high accuracy method for the measurement of small changes in gene expression, this study can contribute to the engineering biology “toolkit” of methods and materials to support the current standardization efforts.



Advances in engineering biology (also referred to as synthetic biology) are currently transforming our ability to produce new chemicals, energy, food, and medicines.^{1,2} The field covers all aspects of intended manipulation and modification of living organisms. Standardization of the components and processes used is central to enhancing productivity and predictability, and to generate sustainable bioengineering of organisms that will ensure the development of repeatable high-quality products.^{3,4}

Broadly, biological systems are engineered within host cells that have had their existing genetic material modified using editing methods such as CRISPR-Cas9, or by the addition of new genetic material in the form of expression vectors. Similar to that of natural cellular networks, the modified genetic region(s) encodes RNA that encode protein molecules that respond to environmental stimuli or control other genetic regions with positive and negative feedback loops, comparable to the logic gates in an electronic circuit, that consequently produce biochemicals or biomaterials.⁵

A crucial principle in all fields of engineering is the use of standardized components and processes; the engineering of biological systems is no different.^{6,7} Standardization in engineering biology is known to be currently insufficient, but

there is a substantial effort to overcome this with initiatives to support implementation of standards and provide recommendations.^{8–11} Repositories containing standardized parts and components, such as highly characterized plasmid vectors, have been established to utilize a “plug and play” model. Examples include the Standard European Vector Architecture (SEVA)¹² and BioBricks.^{13,14}

A second aspect in need of standardization are the methods used to build, validate, or measure the output of the novel biological system. Depending on the system, it may be necessary to determine the gene cargo delivery efficiency, integration efficiency, specificity in terms of the correct modifications being made, and identification of off-target effects that encompass any unintended change to the system. Polymerase chain reaction (PCR) and next-generation

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sequencing (NGS) are commonly used methods as they can detect large changes in nucleic acid copy number (>2-fold) and can confirm the presence/absence of an intended change within a cell, as well as informing on off-target effects.¹⁵

Functional characterization of the biological system is achieved by measurement of the genetic product, most commonly by the detection of fluorescent protein reporters, in response to the stimulation of the gene circuit. While this approach is noninvasive, relatively quick and can be used on living cells in real-time, it gives no mechanistic information about the relationship between the transcription and translation of the gene circuit and its context in the cell. Alternative methods for functional characterization target the transcriptome with RNA sequencing enabling the full transcriptome to be identified.¹⁶ However, it can be challenging and costly to reproduce sequencing-based results across experimental conditions.¹⁵

Reverse-transcription quantitative PCR (RT-qPCR), which directly targets specific RNA molecules based on their sequence, is capable of reproducibly measuring >2-fold changes in transcript levels.¹⁷ However, the sheer number of synthetic gene transcripts within a cell may need a method that can resolve much smaller fold changes. For example, promoter stimulation could result in a change from 90 000 transcripts to 100 000 and so would require a method that can accurately and precisely measure a 1.1-fold change in RNA copy numbers.

Advanced measurement tools that can measure small changes (<1.5-fold) in nucleic acid copy number include digital PCR (dPCR).¹⁸ Quantification is performed by counting amplification events and is not reliant on a calibration curve to convert the method output into copy number concentration.¹⁹ This results in increased accuracy, sensitivity, robustness, and reproducibility in the measurements for both DNA molecules, and RNA molecules when the method is preceded by a reverse-transcription step (RT-dPCR).²⁰ dPCR can be used to directly measure challenging samples, such as those where the requirement is to quantify subtle differences in gene expression,²¹ the precise quantification of the gene edit,²² or quantification of vector insertions.^{23,24}

Furthermore, dPCR is capable of SI traceable measurement of DNA^{25–27} and, therefore, could support existing PCR and NGS methods by using a reference measurement procedure (RMP). A RMP is a procedure that has been accepted as providing true and unbiased measurement with defined measurement uncertainties. It can be used to value assign reference or control materials (not to be confused with standardized parts and components described earlier) that, in turn, can be used to support and improve measurements from existing methods²⁸ such as copy number value assignment of calibrants by dPCR for use in standard curves in quantitative PCR (qPCR).

The aim of this study was to develop and demonstrate the utility of improved methods and control materials for traceable and standardized measurements for the characterization of emerging biobased processes. We developed control materials based on a plasmid or in vitro transcription (IVT) of two reporter constructs encoding the green fluorescent protein (GFP) and red fluorescent protein (RFP) reporter genes. These materials were used to determine the minimum change in plasmid or transcript numbers that could be detected with dPCR or RT-dPCR, respectively, both within and between samples.

We then validated the method by measurement of cellular RNA extracts and demonstrated the utility of the developed control materials to support the quantification of the two reporter genes in a relatively simple gene circuit.²⁹ This circuit contained GFP that was expressed under the control of a constitutive promoter and RFP that was expressed under the control of an inducible promoter that responds positively to increasing concentrations of N-Acyl homoserine lactones (AHLs). Using RT-dPCR, we demonstrated the high level of accuracy of this gene analysis approach (with CVs < 10%) for functional characterization of engineered cells.

EXPERIMENTAL SECTION

Preparation of DNA and RNA Control Materials. Two DNA control materials and two RNA control materials were prepared for this study. Full details of the preparation and characterization, including the gravimetric protocol, are provided in the [Supporting Information](#). Briefly, the two DNA control materials were linearized pET28a plasmids that contained either the RFP or GFP coding sequences under the control of the T7 promoter ([Figure S1](#) in the Supporting Information). Twenty “1E5” units of each, containing $\sim 1 \times 10^5$ copies/ μL of linearized pET28a-GFP or pET28a-RFP in carrier (25 ng/ μL yeast tRNA in Tris-EDTA, pH 8.0; Thermo Fisher Scientific) in a final volume of 50 μL , were prepared by gravimetric dilution and stored at -80°C . The two RNA control materials were generated by in vitro transcription (IVT) of the linearized pET28a-RFP and pET28a-GFP using the MEGAscript T7 kit (Ambion Life Technologies) ([Figure S2](#) in the Supporting Information). Twenty-eight (28) 1E5 units were prepared three times from the stocks of $\sim 1 \times 10^7$ copies/ μL (“1E7 stocks”) at three time points: the initial time (T₀), a month later (T₁), and an additional 11 months later (T₁₂). Each time the units were prepared by gravimetric dilution from the 1E7 stocks and diluted to $\sim 1 \times 10^5$ copies/ μL of GFP or RFP transcripts in carrier (25 ng/ μL yeast tRNA in RNA storage solution; Thermo Fisher Scientific) in a final volume of 50 μL and stored at -80°C .

Digital PCR (dPCR). All dPCR experiments were performed with the QX200 Droplet Digital PCR System (Bio-Rad) and were followed the guidelines of the updated minimum information for publication of digital quantitative PCR experiments (dMIQE2020).²⁰ Full details of the assay optimization and dPCR procedure for quantification of both DNA and RNA templates are provided in the [Supporting Information](#) ([Table S1](#), [Figures S3](#) and [S4](#)). Specific details for the reactions are given in the relevant sections below.

Value Assignment of the 1E5 Units. Copy number value assignment of the DNA 1E5 units was performed using dPCR with the matched RFP or GFP assay in uniplex using a gravimetric 1:10 dilution in carrier of three 1E5 units with six replicate measurements in a single plate to obtain a λ value between 0.18 and 4.7, where the uncertainty based on the Poisson distribution alone is <2%.¹⁷ This was repeated on three separate days over the course of a week (nine units in total measured for each material). For the RNA 1E5 units, the value assignment was performed on three units with three replicate measurements from the three time points (0, 1, and 6 months). The value assignment of each material and calculation of the uncertainty was based on the average of the calculated copy number concentration of replicate reactions and the variance as determined using the one-way ANOVA results and uncertainty component from the

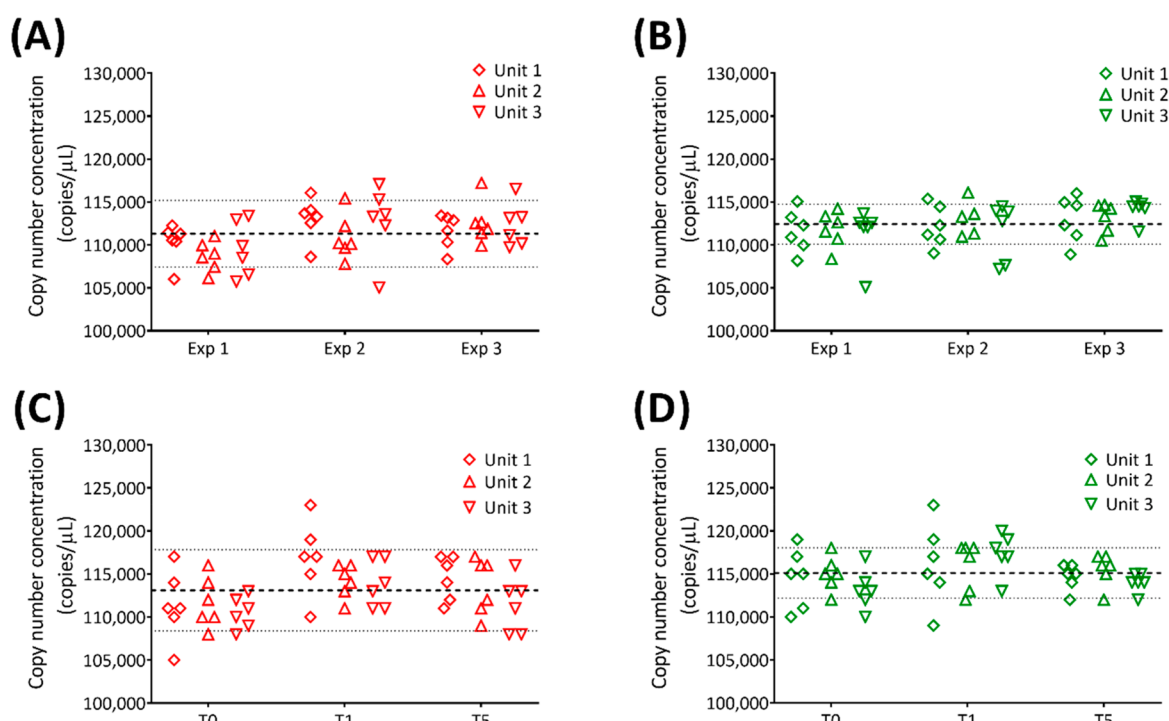


Figure 1. Value assignment of the precursor control materials. Four materials were prepared containing (A) RFP linearized plasmid molecules, (B) GFP linearized plasmid molecules, (C) RFP in vitro transcribed molecules and (D) GFP in vitro transcribed molecules. In all experiments, three units were measured (denoted by the diamond (\diamond), triangle (Δ), and upside-down triangle (∇) symbols) with six replicate dPCR. The horizontal dashed line is the copy number value of each material. The two dotted lines represent the expanded uncertainty limits.

gravimetry. Full details are provided in the [Supporting Information](#).

Generation of the In Vitro Ratio Models. Keeping the copy number concentration of the GFP plasmid or transcript constant at ~ 6200 copies/ μL , 11 different RFP:GFP ratios were generated between 1:1 and 2:1 in two panels (DNA panel I and RNA panel I ratios: 1, 1.2, 1.4, 1.6, 1.8, and 2, DNA panel II and RNA panel II: 1, 1.05, 1.1, 1.15 and 1.2). Each panel contained two controls containing either RFP or GFP plasmid or transcripts only. For the RNA materials, the final batch of 1E5 units was used to prepare the ratios in both phases. Using the value assigned RFP and GFP 1E5 units, each ratio was prepared by gravimetric dilution into diluent, following the developed gravimetric protocol for either DNA or RNA materials (see [Figure S5](#) in the Supporting Information), containing the appropriate carrier, within a total volume of 700 μL . For all ratios, 16 40- μL units were prepared and stored at -80 $^{\circ}\text{C}$.

Copy Number Analysis of the Ratio Models. Each of the four panels of ratios were measured by dPCR independently of each other. Each panel was analyzed in three replicate experiments; each experiment contained three units of each ratio that were measured with triplicate dPCR using duplex assays for GFP and RFP. For all samples and targets, the expected λ was between 1.2 and 2.4, to minimize the uncertainty contribution from the Poisson distribution. For the RFP:GFP ratios, the ratio was calculated using the naturally paired copy numbers of the RFP and GFP molecules in each duplex dPCR. Since copy number ratios follow a log-normal distribution, the data were log-transformed to produce a normal distribution. Two-way ANOVA was then performed to identify the sources of variation. No interaction term was included, since no interaction effect was observed between the

two factors (sample and unit), so a two-way ANOVA containing only main effects was used. For each panel, the difference between each sample containing different numbers of RFP and GFP molecules was compared to the sample that had a ratio of 1 between the RFP and GFP molecules (DNA panel I: sample DNA_6, DNA panel II: sample DNA_11, RNA panel I: sample RNA_6 and RNA panel II: sample RNA_11) (see [Table 2](#), presented later in this paper).

For the RFP:RFP ratio, the absence of natural pairing between the replicates meant that the ratios and comparison were based on the average copy number concentrations for each sample with the variation calculated by log transformation of the ratio values, as described previously.¹⁸ The calculated ratios were then compared as described for the RFP:GFP ratios but using the RFP only sample as the comparator (DNA panel I: DNA_RFP_I, DNA panel II: DNA_RFP_II, RNA panel I: RNA_RFP_I and RNA panel II: RNA_RFP_II) (see [Table 2](#), presented later in this work). To identify the smallest copy number ratio that could be detected between samples, the ratio between each sample was calculated and an ordinary one-way ANOVA was performed. *P*-values were adjusted for multiple comparisons using the Tukey method.

Bacterial Transformation and RNA Extraction. Full details of the culturing and sampling of the cultures are provided in the [Supporting Information](#). Briefly, the *E. coli* reporter strain MG1655 carrying the pSEVA63-Dual plasmid²⁹ was cultured under different experimental conditions without (referred to as A0) or with treatment of *N*-acyl homoserine lactone (AHL) at two concentrations (A1, 1 nM; A10, 10 nM). A control culture (C) was established with non-transformed cells without the addition of AHL that was grown in parallel to the three transformed experimental cultures. Every hour, each of the four cultures were sampled

Table 1. Sources of Bias and Uncertainty on the Value Assignment of the IES Units^a

sample name	copy number estimate ($\times 10^5$ c/ μ L)	combined expanded uncertainty (%)	Digital PCR		Gravimetry		Bias		overall bias (%)
			copy number estimate	expanded uncertainty (%)	dilution factor	expanded uncertainty (%)	volumetric (%)	UV spectrometry and mass conversion (%)	
RFP_DNA	1.11	3.5	8323	3.5	13.4	0.10	25.2	-52	11
GFP_DNA	1.12	2.1	8296	2.1	13.6	0.10	26.2	-55	12
RFP_RNA	1.13	4.2	11350	4.2	10.0	0.02	-0.1	-12	13
GFP_RNA	1.15	2.5	11561	2.5	10.0	0.02	0.1	-9	15

^aDegrees of freedom = 2; coverage (*k*) factor = 4.303.

Table 2. Design of the In Vitro Ratio Model

phase	sample name	ratio	GFP copies/ μ L	expected GFP λ^a	RFP copies/ μ L	expected RFP λ^a
DNA Panel						
I	DNA_1	2.0	6400	1.2	12800	2.4
	DNA_2	1.8	6400	1.2	11520	2.2
	DNA_3	1.6	6400	1.2	10240	1.9
	DNA_4	1.4	6400	1.2	8960	1.7
	DNA_5	1.2	6400	1.2	7680	1.4
	DNA_6	1.0	6400	1.2	6400	1.2
	DNA_GFP_I	-	6400	1.2	0	0.0
	DNA_RFP_I	-	0	0.0	6400	1.2
II	DNA_7	1.20	6400	1.2	7680	2.2
	DNA_8	1.15	6400	1.2	7360	1.9
	DNA_9	1.10	6400	1.2	7040	1.7
	DNA_10	1.05	6400	1.2	6720	1.4
	DNA_11	1.00	6400	1.2	6400	1.2
	DNA_GFP_II	-	6400	1.2	0	0.0
	DNA_RFP_II	-	0	0.0	6400	1.2
	RNA Panel					
I	RNA_1	2.00	6200	1.2	12400	2.4
	RNA_2	1.80	6200	1.2	11160	2.2
	RNA_3	1.60	6200	1.2	9920	1.9
	RNA_4	1.40	6200	1.2	8680	1.7
	RNA_5	1.20	6200	1.2	7440	1.4
	RNA_6	1.00	6200	1.2	6200	1.2
	RNA_GFP_I	-	6200	1.2	0	0.0
	RNA_RFP_I	-	0	0.0	6200	1.2
II	RNA_7	1.20	6200	1.2	7440	2.2
	RNA_8	1.15	6200	1.2	7130	1.9
	RNA_9	1.10	6200	1.2	6820	1.7
	RNA_10	1.05	6200	1.2	6510	1.4
	RNA_11	1.00	6200	1.2	6200	1.2
	RNA_GFP_II	-	6200	1.2	0	0.0
	RNA_RFP_II	-	0	0.0	6200	1.2

^aAdding 5.5 μ L of template to the 22 μ L prereaction.

for their growth, cell density, and protein expression levels of GFP and RFP (Figure S6 in the Supporting Information). A 1 mL sample of each culture was collected for RNA extraction at four hourly time points once the culture was growing exponentially and the total RNA was extracted using the miRNeasy kit (Qiagen) with the RNA concentration estimated using the BioDrop Duo+ (Biodrop, U.K.) and stored at -80 °C. Each RNA extract was diluted to ~ 5 ng/ μ L in RNA storage solution (ThermoFisher) and stored in aliquots at -80 °C.

Copy Number Quantification of the Bacterial RNA Extracts. Each RNA extract was 5-fold serially diluted in RNA

storage solution and quantified with RT-dPCR. The copy number concentrations and dilution factor were log-transformed (\log_2) and the linear correlation used to estimate the RFP and GFP transcript copy number in the RNA extracts using the method described previously.²⁶ Only concentrations from diluted extracts that returned a λ value between 0.18 and 4.7, that have <2% uncertainty attributed to the molecule partitioning, were included in the linear correlation used in the copy number estimation of the samples. The uncertainty of the linear correlation was used to estimate the uncertainty of the copy number concentrations. Each copy number concentration was transformed by division with the mass concentration of

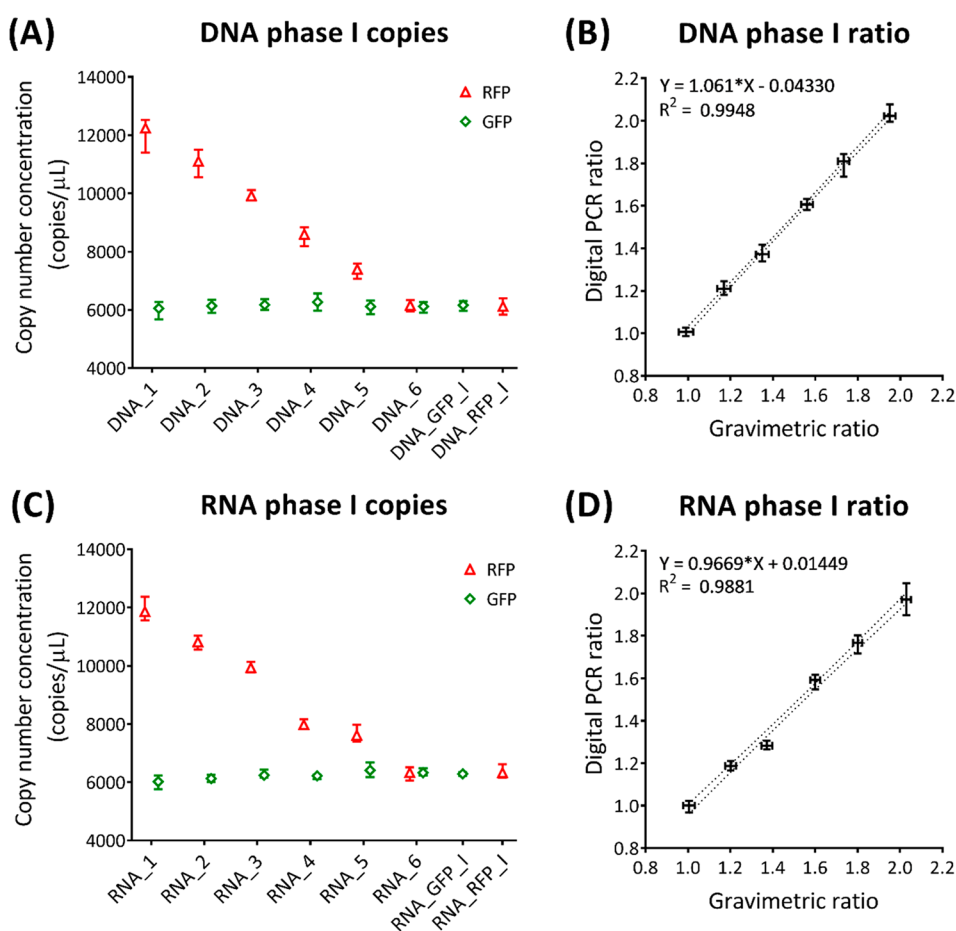


Figure 2. Evaluation of dPCR to measure small changes in ratio of nucleic acid targets. The copy number concentrations for the RFP molecules (red triangles), GFP molecules (green diamonds), and ratio (black symbols) for the eight materials prepared in the phase I panels for (A, B) DNA and (C, D) RNA. The error bars represent the expanded uncertainty of the measurements. The 95% confidence interval of the linear correlation of the measured ratios is shown as two dotted lines for the (B) DNA and (D) RNA phase I panel.

RNA for comparison across the time range and AHL experimental conditions. The RFP:GFP ratios were calculated and analyzed as described for the in vitro ratio model.

RESULTS AND DISCUSSION

Value Assignment of the Precursor Control Materials.

In order to produce control materials that contain small changes in nucleic acid copy numbers, it was necessary to characterize the precursor control materials for their copy number concentration. Frequently, methods such as UV spectrometry, that estimates the ng/ μ L of a target based on absorption at different wavelengths with conversion to copies/ μ L achieved using the molecular weight of the target overestimate the copy number concentration compared with methods such as dPCR that count the molecules directly.²⁶ Using such estimates would result in bias in the true copy numbers of the target molecules in the control material. In this study, copy number estimates were made using gravimetric dilutions and dPCR to minimize the uncertainty for the preanalytical dilution steps and availability of the molecules for amplification based on their sequence rather than by the estimated weight of nucleic acids (Figure 1). Four precursor control materials, referred to as the “1ES units”, were prepared and nine units of each material were used to estimate the copy number concentration for the RFP DNA molecules (Figure 1A), GFP DNA molecule (Figure 1B), RFP transcripts (Figure

1C), or GFP transcripts (Figure 1D). All four of the precursor materials were assigned a copy number concentration that was higher than the nominal concentration by $\sim 15\%$ (between 1.11×10^5 copies/ μ L to 1.15×10^5 copies/ μ L) with a combined expanded uncertainty of $<5\%$ (Table 1).

Although small, the uncertainty calculations identified the experiment or time point as contributing to the main source of the uncertainty, while the repeatability (that includes the unit homogeneity in the replicates) and gravimetry contributed $\leq 4.2\%$ and $\leq 0.1\%$ of the uncertainty, respectively (Table 1). The overall stability of the RNA 1ES units was deemed suitable based on analysis of variance ($p > 0.44$). The contributions of different parameters to the uncertainty observed here are within the reported ranges observed in a previous study, using plasmid control materials.

Design and Production of the In Vitro Ratio Models.

dPCR is able to measure small fold changes (<1.20) in the number of DNA molecules between samples using 6100 partitions.¹⁸ Using power calculations, dPCR would be able to measure significantly smaller differences if more than 10 000 partitions per reaction were measured. Furthermore, lower concentration samples require more partitions to accurately quantify the same ratio compared to a higher concentration sample.¹⁸ For this study, a dPCR instrument that generated between 10,000 and 20,000 subnanoliter partitions per reaction was used. The aim was to develop a model that

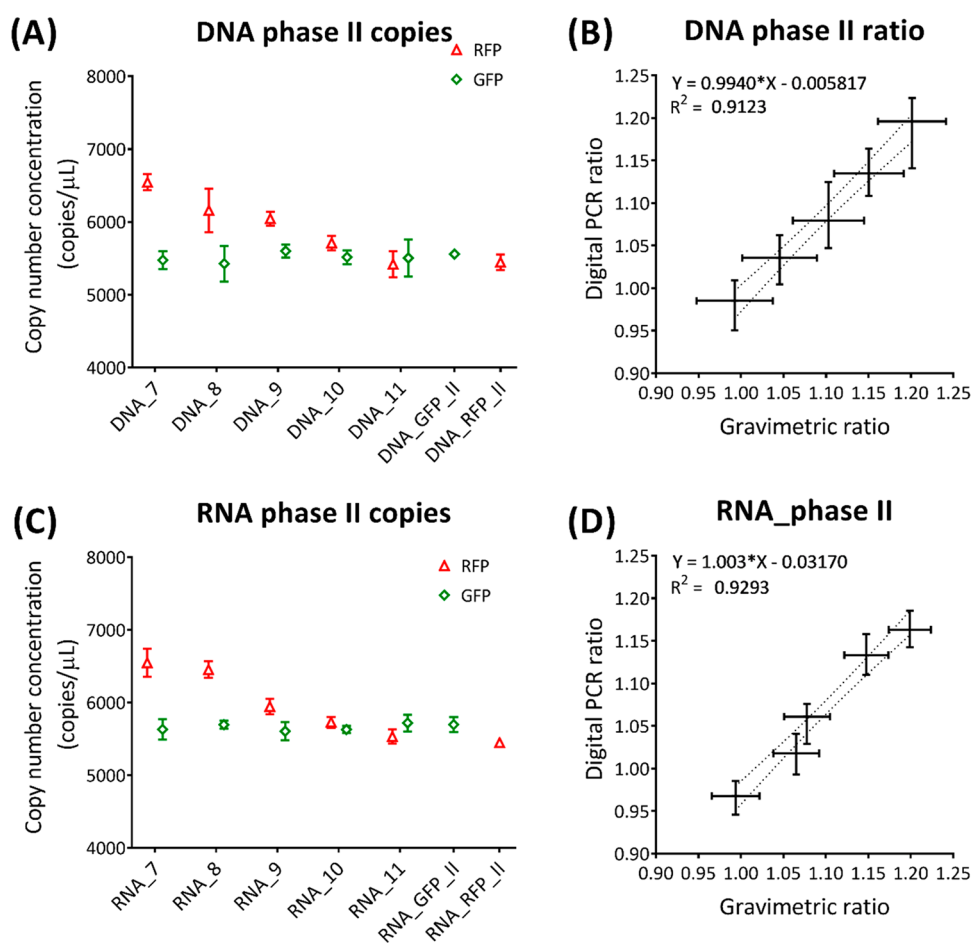


Figure 3. Evaluation of dPCR to measure very small changes in ratio of nucleic acid targets. The copy number concentrations for the RFP molecules (red triangles, Δ), GFP molecules (green diamonds, \diamond), and ratio (black symbols) for the eight materials prepared in the phase II panels for (A, B) DNA and (C, D) RNA. The error bars represent the expanded uncertainty of the measurements. The 95% confidence interval of the linear correlation of the measured ratios is shown as two dotted lines for the (B) DNA and (D) RNA phase II panel.

could determine the smallest ratio that could be quantified by dPCR. As the highest precision is achieved when λ (average number of target molecules per partition in the reaction) is between 1.2 and 2.4, where the precision based on the Poisson distribution alone would be $<1\%$, the two molecules were combined so that their copy numbers were both within this range.

Two panels of ratios were designed, one containing DNA molecules and the other containing RNA molecules (Table 2). Each panel consisted of 11 samples each containing a constant number of GFP molecules ($\lambda \approx 1.2$) and equal or higher numbers of RFP molecules to generate a range of RFP:GFP copy number ratios between 1 and 2 (highest RFP λ of ~ 2.4). Production of the panels was executed in two phases: phase I contained six samples with fold changes between 1 and 2 at 0.2 increments and phase II contained five samples of fold changes between 1 and 1.2 at 0.05 increments. For each panel and phase, control samples were produced containing only GFP molecules in carrier (predicted λ of ~ 1.2), only RFP molecules in carrier (predicted λ of ~ 1.2) and only carrier molecules.

Each of the samples in the *in vitro* ratio model panels were generated by combining different volumes of the GFP and RFP 1E5 units. The use of gravimetry in the production of the *in vitro* ratio models was used to reduce the small, but significant errors that could be introduced by pipet volume transfer (see the Supporting Information (Figure S4)).

Evaluation of dPCR To Measure Small Changes in Ratio of Nucleic Acid Targets. For each sample in the two panels, the copy number concentrations of both the GFP and RFP molecules were measured using triplicate duplex dPCR in three separate experiments ($n = 9$ for each sample). The natural pairing of the two molecules in each sample from the use of duplex reactions enabled the RFP:GFP ratio to be calculated for each reaction. There was no significant difference in the GFP copy number between samples in the same panel (DNA_phase I; $p > 0.1114$, RNA_phase I; $p > 0.0706$). Therefore, the RFP:RFP ratio between samples was calculated by log transforming the ratio before calculating the variation of the ratio values (described in ref 18).

For the phase I panels (measuring ratios between 2 and 1 at 0.2 intervals), dPCR was able to discriminate between the copy number concentrations of the RFP molecules between all the samples (RFP:RFP ratio) within the DNA panel (Figure 2A) and RNA panel (Figure 2C). Evaluation of the RFP:GFP ratios confirmed that dPCR was able to reproducibly measure fold changes of 1.2 within a sample (RFP:GFP ratio) for both the DNA (Figure 2B) and RNA (Figure 2D) molecules. Good linearity was observed between the dPCR and gravimetric ratios over the measurement range. Furthermore, the 95% confidence intervals of both the y - and x -intercepts spanned zero indicating that no significant bias was present.

Table 3. Limit of Detection with Small Ratios between Samples^a

	DNA_8	DNA_9	DNA_10	DNA_11	DNA_RFP_II
DNA_7	1.06	1.08	1.14	1.19	1.15
	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
DNA_8		1.02	1.08	1.14	0.78
		$p = 0.5486$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
DNA_9			1.06	1.12	1.11
			$p = 0.0001$	$p < 0.0001$	$p < 0.0001$
DNA_10				1.05	1.05
				$p = 0.0010$	$p = 0.0034$
DNA_11					1.00
					$p = 0.9985$
	RNA_8	RNA_9	RNA_10	RNA_11	RNA_RFP_II
RNA_7	1.01	1.10	1.14	1.20	1.20
	$p = 0.4724$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
RNA_8		1.09	1.13	1.17	0.78
		$p = 0.5486$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
RNA_9			1.04	1.07	1.09
			$p = 0.0016$	$p < 0.0001$	$p < 0.0001$
RNA_10				1.04	1.05
				$p = 0.0061$	$p < 0.0001$
RNA_11					1.02
					$p = 0.5769$

^aThe observed ratio between the RFP molecules between the two samples is shown to 2 d.p. Using the Tukey adjusted p -values, statistical significance was found when $p < 0.05$ with high significance when $p < 0.01$. Nonsignificance was declared when $p > 0.05$.

Table 4. Sources of Uncertainty in Digital PCR^a

Uncertainty Factor	Description	Approach To Identify Uncertainty	Magnitude in Our Study (%)
Poisson distribution in RT-dPCR	models the random distribution of the molecules into the partitions in a reaction	calculated for each individual reaction based on observed positive and total partition numbers	<2
repeatability of RT-dPCR	pipetting error in reaction preparation, reverse transcription efficiency, PCR efficiency, droplet generation, droplet transfer, droplet reading	triplicate reactions on a single plate; all reactions prepared independently	3–5
intermediate precision of RT-dPCR	replicate experiments, reaction location	replicate plates performed with different randomized plate layouts	<5
preanalytical dilution of RNA extracts	true pipet volume calculated by weighing the tube between all liquid transfer steps	gravimetry used to correct pipetted volumes	<25
biological sampling and processing	1 mL was subsampled that represents 5% of the total culture; total RNA was extracted from each sample.	not evaluated and part of a follow-up study	N/A

^aThe sources of uncertainty in the dPCR method are presented in order of magnitude: low to high.

Identification of the Limit of Quantification of dPCR for Analyzing Small Ratio Differences between Nucleic Acid Targets. dPCR was able to repeatably identify 1.2-fold differences in nucleic acid copy numbers both between and within samples with high precision and accuracy. While this level of precision is more than sufficient for many applications, the phase II panels (measuring ratios between 1.2 and 1.0 at 0.05 intervals) were generated to identify the smallest copy number change that dPCR could measure at this template concentration (Figure 3). As was observed with the phase I panels, there was no significant difference in the GFP copy number between samples in the same panel regardless of molecule type (DNA_phase II; $p > 0.2079$, RNA_phase II; $p > 0.1840$).

The analysis of the between sample RFP:RFP ratios demonstrated that dPCR was able to reproducibly measure all the 1.10-fold RFP:RFP ratios and all but one of the 1.05 ratios (Table 3). Because of the relatively large variation in dPCR measurement of the DNA_8 sample (Figure 3A), there was no significant difference between the measured RFP copy

number of this sample with DNA_9. This was the only instance where dPCR was unable to discriminate between two samples within the panel and amounted to a difference of 1.02-fold (Table 3).

Despite the increasingly small differences between each ratio, dPCR was able to discriminate between fold changes of 1.05 between RFP and GFP within the DNA samples (Figure 3B). As was observed for the phase I panel, good linearity was observed between the dPCR and gravimetric ratios over the measurement range. As anticipated, the natural pairing of the duplex assay within each measurement enabled dPCR to measure the RFP:GFP ratios in all cases (Figure 3B).

A similar pattern was observed with the analysis of the RNA phase II panel (Figure 3C). The analysis of the between sample RFP:RFP ratios demonstrated that dPCR was able to reproducibly measure all the 1.10-fold RFP:RFP ratios but not all of the 1.05 ratios (see Table 3). The relatively larger technical variation in the copy number concentrations in the RNA phase II panel contributed to the increase in variation in the ratio measurements within the samples. dPCR was able to

discriminate between fold changes of 1.05 between RFP and GFP within the samples for four of the five ratio samples and good linearity was observed between the dPCR and gravimetric ratios over the measurement range (Figure 3D). That dPCR was unable to detect all of the 1.05-fold ratios that was attributed to the inability to accurately manufacture such small ratios. For all the samples, the observed precision of the dPCR measurement for each sample was of a similar magnitude to that of the gravimetric dilution, thereby indicating that it was the production of the materials that contributed to the limiting factor as well as the precision of dPCR.

Characterization of the Changes in GFP and RFP in Bacterial Cells in Response to Promoter Stimulation. This study has so far demonstrated that dPCR is capable of accurate and precise copy number characterization of control materials in the absence of a biological matrix. The control materials and dPCR experiments were designed to capture the technical error (Table 4) to enable the identification of true biological changes in response to promoter stimulation. In the case of a gene circuit, the transcripts of interest would be coextracted from the biological sample along with the full cell transcriptome. These background RNA molecules may render the level of precision capably by dPCR out of reach if there are competing or inhibiting molecules present.

To investigate the ability of dPCR to identify small changes in RNA copy number within a biological system, RNA was extracted from bacterial cells that had been transformed to contain a simple gene circuit containing constitutively active GFP and inducible RFP that responds positively to increasing concentrations of AHL.²⁹ The copy number concentration of both the RFP and GFP transcripts in the extracted RNA were measured and normalized to the copies per ng of extracted RNA (Figure 4). Each sample was compared over four time

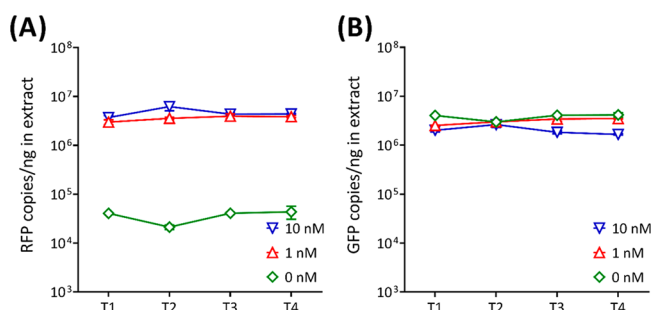


Figure 4. Evaluation of the change in RFP and GFP transcript number in response to addition of AHL. The copy number of (A) RFP and (B) GFP in the bacterial extracts was estimated using RT-dPCR and converted to copies per nanogram in the extracts. The addition of AHL significantly increased the copy number concentration of the RFP transcripts but had a small negligible effect on the GFP copy number.

points while the cells were in the exponential growth phase as identified by OD₆₀₀ measurements (see Figure S6A in the Supporting Information). dPCR was able to identify a significant two-log increase in RFP expression in response to promoter stimulation by AHL compared with the controls (no induction; 0 nM) across all four time points (Figure 4A). Despite a 10-fold difference in AHL added to the culture between the low (1 nM) and the high concentration (10 nM), only a small, but not significant, increase in the number of RFP

transcripts was observed. This trend was observed with the parallel protein expression measurements (Figure S6B in the Supporting Information).

Analysis of the copy numbers of the GFP transcripts demonstrated that the expression remained largely stable over time with no significant difference in copy number between time points ($p = 0.619$). However, an inverse correlation between the level of AHL addition and the number of GFP copies was observed whereby cells exposed to the higher concentration of AHL had a lower GFP transcript number than those with the lower concentration of AHL (Figure 4B). This was corroborated by the protein expression analysis (Figure S6C in the Supporting Information) thereby suggesting that triggering RFP expression could decrease the levels of GFP expression at both the mRNA and protein level and are hypothesized to be due to the competition for expression machinery within the cell.

CONCLUSIONS

This study evaluated the ability of dPCR to detect very small changes in copy number in both DNA and RNA measurements using carefully designed and produced control materials. The model has shown that dPCR is capable of discriminating ratios of two independent molecules with a 1.05-fold difference in their copy number both within a sample and between samples consisting of linearized plasmid or *in vitro* transcripts in a carrier RNA background. The ability to measure such small differences in copy number was initially attributed to relative simplicity of the control materials used to generate the ratios. Quantification of RNA extracted from bacterial cells containing a simple gene circuit have validated the use of dPCR to measure small changes in gene expression within a complex biological matrix. Together these findings have identified and quantified some of the technical sources of uncertainty that can hamper the accurate quantification of small changes in transcript number. By characterizing these sources, the technical noise of the method can either be reduced through experimental design or compensated by background subtraction to increase the ability to identify and confidently quantify true gene transcript changes in a biological system. In this way, this study has expanded the toolbox of methods and materials to support standardization in the field of engineering biology. Furthermore, these control materials could be used to calibrate other molecular methods, such as real-time PCR, where quantification may be needed for high-throughput screening of gene expression targets for the validation of gene circuits.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.analchem.1c05134>.

Additional materials and methods, validation of dPCR method, dMIQE checklist, validation of gravimetric protocol, validation of bacterial model system; Figures S1–S7 and Tables S1–S2 (PDF)

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Notes

The authors declare no competing financial interest.

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