Direct elicitation of template concentration from quantification cycle (C_q) distributions in digital PCR

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Supplementary Text

Derivation of model equation

Here we outline the reasoning that leads to the probabilistic model of quantification cycles $\rho_Y(y;\lambda,k_t,\varepsilon)$ in the main text. Starting point is the Poisson distribution $p(k_0;\lambda)$. When uniformly distributing a volume containing n molecules over N reactions, it describes the probability of finding k_0 molecules in any given reaction volume, while $\lambda = n/N$ are expected:

$$p(k_0;\lambda) = \mathrm{e}^{-\lambda}\frac{\lambda^{k_0}}{k_0!}.$$

To afford for a simpler analytical treatment, it is tempting to rewrite the factorial as a Gamma function. Doing this in the Poisson distribution, however, to maintain its norm. A correct analytical extension of the above formula is directly given via its cumulative distribution function (CDF), which can be rewritten as the ratio of the (upper) incomplete and complete Gamma functions:

$$Pr(k_0 \geq x;\lambda) = \frac{\Gamma_\lambda(x)}{\Gamma(x)} =: P_X(x)\,.$$

For integer x, this function recovers the usual Poissonian CDF, as can be verified, and is well defined on the same support of $[0,\infty)$. The derivative of $P(x)$ yields a continuous, interpolating version of the Poisson distribution that retains its norm and thus remains a probability distribution:

$$\begin{aligned}
\rho_X(x) &= \partial_x P_X(x) \\
&= \Gamma^{-2}\cdot(\partial_x\Gamma_\lambda\cdot\Gamma - \Gamma_\lambda\cdot\partial_x\Gamma_\lambda) \\
&= \Gamma^{-2}\cdot\left(\int_\lambda^\infty \log t\,\mathrm{e}^{-t}t^{x-1}\mathrm{d}t\cdot\Gamma - \Gamma_\lambda\cdot\int_0^\infty \log t\,\mathrm{e}^{-t}t^{x-1}\mathrm{d}t\right) \\
&= \Gamma^{-2}\cdot\left(\int_\lambda^\infty \log t\,\mathrm{e}^{-t}t^{x-1}\mathrm{d}t\int_0^\infty \mathrm{e}^{-t}t^{x-1}\mathrm{d}t - \int_\lambda^\infty \mathrm{e}^{-t}t^{x-1}\mathrm{d}t\int_0^\infty \log t\,\mathrm{e}^{-t}t^{x-1}\mathrm{d}t\right) \\
&= \Gamma^{-2}\cdot\left(\iint_{\lambda,0}^{\infty,\infty} \mathrm{e}^{-s}\,s^{x-1}\log t\,\mathrm{e}^{-t}t^{x-1}\mathrm{d}s\mathrm{d}t - \iint_{\lambda,0}^{\infty,\infty} \mathrm{e}^{-t}\,t^{x-1}\log s\,\mathrm{e}^{-s}s^{x-1}\mathrm{d}s\mathrm{d}t\right) \\
&= \Gamma^{-2}\cdot\left(\iint_{\lambda,0}^{\infty,\infty} \mathrm{e}^{-(s+t)}\,(st)^{x-1}\log\frac{t}{s}\mathrm{d}t\mathrm{d}s\right).
\end{aligned}$$

To find the probability distribution of amplification cycles, ρ_X is transformed using the relation imposed by exponential amplification $x(y) = k_0\varepsilon^y$, with the quantification threshold $k_\mathrm{t} = x(C_\mathrm{q})$. In terms of continuous variables the transformation reads:

$$\begin{aligned}\rho_Y(y) &= \rho_X(x(y)) \cdot \left|\frac{\partial x}{\partial y}\right| \\ &= \log\varepsilon \frac{k_\mathrm{t}\varepsilon^{-y}}{\Gamma(k_\mathrm{t}\varepsilon^{-y})^2} \int_\lambda^\infty \mathrm{d}r \int_0^\lambda \mathrm{d}s\, \mathrm{e}^{-(r+s)}(rs)^{k_\mathrm{t}\varepsilon^{-y}-1} \log\frac{r}{s} \\ &=: \rho_Y(y;\lambda,k_\mathrm{t}\varepsilon)\,.\end{aligned}$$

Supplementary discussion: circular vs linearized plasmids in PCR

There is a discrepancy in recommendations regarding the use of linearized or circular plasmids in quantitative PCR. Circular plasmids have been reported to have negative effect on PCR efficiency relative to linearized plasmids (Lin et al., 2011). Amplification dropout has been observed with non-linearized plasmid molecules, which could be due to delayed onset of amplification at early cycles or reduced amplification efficiency (Bhat, Herrmann, Armishaw, Corbisier, & Emslie, 2009). On the other hand, it has been reported that linearized plasmids can result in overestimating target copy numbers in dPCR: linearized plasmid template is potentially present in both double stranded (ds) or denatured single stranded (ss) forms; this gives rise to differences in quantification as high as 2-fold, depending on the denaturation state (Sanders et al., 2011). Moreover, it has been reported that circular plasmid can survive repeated freeze and thaw and handling of serial dilution in compare with linearized plasmids (Dhanasekaran et al., 2010). This may introduce unaccounted variability in copy number of templates at different dilution series prepared as replicates.

As precision in quantification is important to us, we decided to use circular plasmid for this study. We tried to reduce the possible effects of circular plasmid template by measuring the amplification efficiency of circular plasmid as well as taking into account the late reported quantification cycle (C_q) in our calculation to compensate for possible delay in amplification initiation using circular plasmid. Negative template control allows us to discard possible non-specific amplification reaction.

References cited here

Bhat, S., Herrmann, J., Armishaw, P., Corbisier, P., & Emslie, K. R. (2009). Single molecule detection in nanofluidic digital array enables accurate measurement of DNA copy num-

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Dhanasekaran, S., Doherty, T. M., Kenneth, J., & Group, T. T. S. (2010). Comparison of different standards for real-time PCR-based absolute quantification. *Journal of immunological methods*, *354*(1-2), 34–39.
Lin, C.-H., Chen, Y.-C., & Pan, T.-M. (2011). Quantification bias caused by plasmid DNA conformation in quantitative real-time PCR assay. *PLoS ONE*, *6*(12), e29101.
Sanders, R., Huggett, J. F., Bushell, C. A., Cowen, S., Scott, D. J., & Foy, C. A. (2011). Evaluation of digital PCR for absolute DNA quantification. *Analytical chemistry*, *83*(17), 6474–6484.

Table S1: Standard calculation for dPCR analysis

		Unit	Note
Each dPCR array			
Number of subarrays	48	----	
Number of reactions (through-holes / subarray)	64	----	Total 3072 (48x64) reactions / array
Per inlet (experimental design)			
Total volume	5	µl	
Master mix volume	3	µl	
Target volume	2	µl	
Per subarray			
Number of reactions (through-holes / subarray)	64	----	
Total volume	2.1*	µl	64x33nl reaction volume / subarray**
Master mix volume	1.26	µl	
Target volume	0.84	µl	
Per through-hole (chamber)			
Total volume	33	nl	As quoted by manufacturer
Master mix volume	20	nl	
Target volume	13	nl	Target volume per subarray (64 chambers)

Nomenclature:

dPCR array: A 48-subarray dPCR array, where each subarray is partitioned into 64 reaction through-holes

Inlet: Individual well of 384-well plate for sample loading (distinct for each subarray)

* In our experimental design, the reaction volume per subarray is 42% of the reaction volume prepared per inlet (total volume per subarray/total volume per inlet).

**The total volume per subarray is calculated as 2.1µl based on the manufacturer quoted values of: 33nl reaction chambers and 64 individual reaction chambers (through-holes) per subarray.

Table S2: GATA1 (**a**) and PU1 (**b**) target copy number calculation

(**a**)

A	B	C	D
Estimated GATA1 target copies/µl @ different dilutions	Estimated GATA1 target copies/inlet [2µl x A]	Estimated GATA1 target copies/subarray [0.42 x B]	Estimated GATA1 target copies/chamber [C/64]
950	1900	798	12.46
475	950	399	6.23
237.5	475	199.5	3.11
118.75	237.5	99.75	1.55
59.37	118.75	49.87	0.77
29.68	59.37	24.93	0.38
14.84	29.68	12.46	0.19

(**b**)

A	B	C	D
Estimated PU1 target copies/µl @ different dilutions	Estimated PU1 target copies/inlet [2µl x A]	Estimated PU1 target copies/subarray [0.42 x B]	Estimated PU1 target copies/chamber [C/64]
900	1800	756	11.81
450	900	378	5.90
225	450	189	2.95
112.5	225	94.5	1.47
56.25	112.5	47.25	0.73
28.12	56.25	23.62	0.36
14.06	28.12	11.81	0.18

Table S3: Analyzed sample dilutions measured by UV spectrophotometry

Experiment	Assay	DNA target copies/reaction	Cell number serial dilution
Plasmid standard curve	GATA1	$1.9 \cdot 10^7$ $1.9 \cdot 10^6$ $1.9 \cdot 10^5$ $1.9 \cdot 10^4$ $1.9 \cdot 10^3$ $1.9 \cdot 10^2$	
Plasmid standard curve	PU1	$1.8 \cdot 10^7$ $1.8 \cdot 10^6$ $1.8 \cdot 10^5$ $1.8 \cdot 10^4$ $1.8 \cdot 10^3$ $1.8 \cdot 10^2$	
EML sample	GATA1 & PU1		2000 ~1000 ~250 ~62 ~15 ~4 ~1
ERY & MYL samples	GATA1 & PU1		2000 ~250 ~62 ~15 ~4 ~1

Table S4: Summary of plasmid dilution experiments

Sample	n / 64 rx	Subarrays	Expected	Poisson	Retroflex	E/P	E/R
Water	0	23	0.00	7.10	11.89	0.00	0.00
NTC	0	23	0.00	4.02	30.40	0.00	0.00
GATA1	7	8	23.52	7.05	7.70	3.34	3.06
GATA1	14	8	47.04	13.17	17.41	3.57	2.70
GATA1	29	52	633.36	451.16	505.47	1.40	1.25
GATA1	59	52	1288.56	732.81	818.87	1.76	1.57
GATA1	118	52	2577.12	1456.54	1651.51	1.77	1.56
GATA1	237	63	6271.02	3418.48	3185.08	1.83	1.97
GATA1	475	22	4389.00	1746.44	1824.13	2.51	2.41
GATA1	950	22	8778.00	3988.73	4827.47	2.20	1.82
GATA1	1900	22	17556.00	7482.57	10584.15	2.35	1.66
PU1	7	24	70.56	10.04	25.38	7.03	2.78
PU1	14	24	141.12	36.43	71.17	3.87	1.98
PU1	28	57	670.32	385.78	327.69	1.74	2.05
PU1	45	22	415.80	128.71	176.29	3.23	2.36
PU1	56	57	1340.64	670.58	725.97	2.00	1.85
PU1	90	22	831.60	299.76	403.92	2.77	2.06
PU1	112	57	2681.28	1161.70	1384.03	2.31	1.94
PU1	180	22	1663.20	702.34	978.91	2.37	1.70
PU1	225	57	5386.50	2752.37	3046.68	1.96	1.77
PU1	360	22	3326.40	1275.71	1666.30	2.61	2.00
PU1	450	22	4158.00	1521.54	2656.76	2.73	1.57
PU1	900	22	8316.00	3842.55	6129.92	2.16	1.36
PU1	1800	22	16632.00	6770.40	13603.78	2.46	1.22

Table S5: MIQE checklist

ITEM TO CHECK	IMPORTANCE	CHECKLIST
EXPERIMENTAL DESIGN		
Definition of experimental and control groups	E	✓
Number within each group	E	✓
Assay carried out by core lab or investigator's lab?	D	N/A
Acknowledgement of authors' contributions	D	✓
SAMPLE		
Description	E	✓
Volume/mass of sample processed	D	✓
Microdissection or macrodissection	E	N/A
Processing procedure	E	✓
If frozen - how and how quickly?	E	N/A
If fixed - with what, how quickly?	E	N/A
Sample storage conditions and duration (especially for FFPE samples)	E	✓
NUCLEIC ACID EXTRACTION		
Procedure and/or instrumentation	E	✓
Name of kit and details of any modifications	E	✓
Source of additional reagents used	D	✓
Details of DNase or RNAse treatment	E	✓
Contamination assessment (DNA or RNA)	E	✓
Nucleic acid quantification	E	✓
Instrument and method	E	✓
Purity (A260/A280)	D	✓
Yield	D	✓
RNA integrity method/instrument	E	✓
RIN/RQI or Cq of 3' and 5' transcripts	E	N/A
Electrophoresis traces	D	✓
Inhibition testing (Cq dilutions, spike or other)	E	✓
REVERSE TRANSCRIPTION		
Complete reaction conditions	E	✓
Amount of RNA and reaction volume	E	✓
Priming oligonucleotide (if using GSP) and concentration	E	✓
Reverse transcriptase and concentration	E	✓
Temperature and time	E	✓
Manufacturer of reagents and catalogue numbers	D	✓

Cqs with and without RT	D*	✓
Storage conditions of cDNA	D	✓
qPCR TARGET INFORMATION		
If multiplex, efficiency and LOD of each assay.	E	N/A
Sequence accession number	E	N/A
Location of amplicon	D	✓
Amplicon length	E	✓
In silico specificity screen (BLAST, etc)	E	✓
Pseudogenes, retropseudogenes or other homologs?	D	N/A
Sequence alignment	D	N/A
Secondary structure analysis of amplicon	D	N/A
Location of each primer by exon or intron (if applicable)	E	✓
What splice variants are targeted?	E	N/A
qPCR OLIGONUCLEOTIDES		
Primer sequences	E	N/A
RTPrimerDB Identification Number	D	N/A
Probe sequences	D**	N/A
Location and identity of any modifications	E	N/A
Manufacturer of oligonucleotides	D	✓
Purification method	D	N/A
qPCR PROTOCOL		
Complete reaction conditions	E	✓
Reaction volume and amount of cDNA/DNA	E	✓
Primer, (probe), Mg++ and dNTP concentrations	E	✓
Polymerase identity and concentration	E	✓
Buffer/kit identity and manufacturer	E	✓
Exact chemical constitution of the buffer	D	✓
Additives (SYBR Green I, DMSO, etc.)	E	✓
Manufacturer of plates/tubes and catalog number	D	✓
Complete thermocycling parameters	E	✓
Reaction setup (manual/robotic)	D	✓
Manufacturer of qPCR instrument	E	✓
qPCR VALIDATION		
Evidence of optimization (from gradients)	D	✓
Specificity (gel, sequence, melt, or digest)	E	✓
For SYBR Green I, Cq of the NTC	E	N/A
Standard curves with slope and y-intercept	E	✓

PCR efficiency calculated from slope	E	✓
Confidence interval for PCR efficiency or standard error	D	✓
r2 of standard curve	E	✓
Linear dynamic range	E	✓
Cq variation at lower limit	E	✓
Confidence intervals throughout range	D	✓
Evidence for limit of detection	E	✓
If multiplex, efficiency and LOD of each assay.	E	N/A
DATA ANALYSIS		
qPCR analysis program (source, version)	E	✓
Cq method determination	E	✓
Outlier identification and disposition	E	✓
Results of NTCs	E	✓
Justification of number and choice of reference genes	E	N/A
Description of normalization method	E	N/A
Number and concordance of biological replicates	D	N/A
Number and stage (RT or qPCR) of technical replicates	E	✓
Repeatability (intra-assay variation)	E	✓
Reproducibility (inter-assay variation, %CV)	D	✓
Power analysis	D	N/A
Statistical methods for result significance	E	✓
Software (source, version)	E	✓
Cq or raw data submission using RDML	D	N/A

All essential information (E) must be submitted with the manuscript. Desirable information (D) should be submitted if available. If using primers obtained from RTPrimerDB, information on qPCR target, oligonucleotides, protocols and validation is available from that source.

*: Assessing the absence of DNA using a no RT assay is essential when first extracting RNA. Once the sample has been validated as RDNA-free, inclusion of a no-RT control is desirable, but no longer essential.

**: Disclosure of the probe sequence is highly desirable and strongly encouraged. However, since not all commercial pre-designed assay vendors provide this information, it cannot be an essential requirement. Use of such assays is advised against.

Table S6: Digital MIQE checklist

ITEM TO CHECK	IMPORTANCE	CHECKLIST
EXPERIMENTAL DESIGN		
Definition of experimental and control groups	E	✓
Number within each group	E	✓
Assay carried out by core lab or investigator's lab?	D	N/A
Power analysis	D	N/A
SAMPLE		
Description	E	✓
Volume/mass of sample processed	D	✓
Microdissection or macrodissection	E	N/A
Processing procedure	E	✓
If frozen - how and how quickly?	E	N/A
If fixed - with what, how quickly?	E	N/A
Sample storage conditions and duration (especially for FFPE samples)	E	✓
NUCLEIC ACID EXTRACTION		
Quantification-instrument/method	E	✓
Storage conditions of cDNA: temperature, concentration, duration, buffer	E	✓
DNA or RNA quantification	E	✓
Quality/integrity, instrument/method, e.g. RNA integrity/R quality index and trace or 3':5'	E	✓
Template structural information	E	✓
Template modification (digestion, sonification, preamplification, etc.)	E	✓
Template treatment (initial heating or chemical denaturation)	E	✓
Inhibition dilution or spike	E	✓
DNA contamination assessment of RNA sample	E	✓
Details of DNase treatment where performed	E	✓
Manufacturer of reagents used and catalogue number	D	✓
Storage nucleic acids: temperature, concentration, duration, buffer	E	✓
REVERSE TRANSCRIPTION (if necessary)		
cDNA priming method + concentration	E	✓
One or 2-step protocol	E	✓
Amount of RNA used per reaction	E	✓

Detailed reaction components and conditions	E	✓
RT efficiency	D	N/A
Estimated copies measured with and without addition of RTb	D	N/A
Manufacturer of reagents and catalogue numbers	D	✓
Reaction volume (for 2-step RT reaction)	D*	✓
Storage conditions of cDNA: temperature, concentration, duration, buffer	D	✓
dPCR TARGET INFORMATION		
Sequence accession number	E	N/A
Amplicon location	D	✓
Amplicon length	E	✓
In silico specificity screen (BLAST, etc)	E	✓
Pseudogenes, retropseudogenes or other homologs?	D	N/A
Sequence alignment	D	N/A
Secondary structure analysis of amplicon	D	N/A
Location of each primer by exon or intron (if applicable)	E	✓
Where appropriate, which splice variants are targeted?	E	N/A
dPCR OLIGONUCLEOTIDES		
Primer sequences and/or amplicon context sequence[b]	E	N/A
RTPrimerDB Identification Number[b]	D	N/A
Probe sequences	D**	N/A
Location and identity of any modifications	E	N/A
Manufacturer of oligonucleotides	D	✓
Purification method	D	N/A
dPCR PROTOCOL		
Complete reaction conditions	E	✓
Reaction volume and amount of RNA/cDNA/DNA	E	✓
Primer, (probe), Mg++ and dNTP concentrations	E	✓
Polymerase identity and concentration	E	✓
Buffer/kit identity and manufacturer	E	✓
Exact chemical constitution of the buffer	D	✓
Additives (SYBR Green I, DMSO, etc.)	E	✓
Plates/tubes and catalog number and manufacturer	D	✓

Complete thermocycling parameters	E	✓
Reaction setup (manual/robotic)	D	✓
Gravimetric or volumetric dilutions (manual/robotic)	D	✓
Total PCR reaction volume prepared	D	✓
Partition number	E	✓
Individual partition volume	E	✓
Total volume of the partitions measured (effective reaction size)	E	✓
Partition volume variance/SD	D	N/A
Comprehensive details and appropriate use of controls	E	✓
Manufacturer of dPCR instrument	E	✓
dPCR VALIDATION		
Optimization data for the assay	D	✓
Specificity (when measuring rare mutations, pathogen sequence, etc.)	E	N/A
Limit of detection of calibration control	D	✓
If multiplexing, comparison with singleplex assays	D	N/A
DATA ANALYSIS		
Mean copies per partition (λ or equivalent)	E	✓
dPCR analysis program (source, version)	E	✓
Outlier identification and disposition	E	✓
Results of no-template controls	E	✓
Examples of positives and negative experimental results as supplemental data	E	✓
Where appropriate, justification of number and choice of reference genes	E	N/A
Where appropriate, description of normalization method	E	N/A
Number and concordance of biological replicates	D	N/A
Number and stage (RT or qPCR) of technical replicates	E	✓
Repeatability (intra-assay variation)	E	✓
Reproducibility (inter-assay variation, %CV)	D	✓
Experimental variance or CI[d]	E	✓
Statistical methods used for analysis	E	✓
Data submission using RDML	D	N/A

a All essential information (E) must be submitted with the manuscript. Desirable information (D) should be submitted if available.

b Disclosure of the probe sequence is highly desirable and strongly encouraged. However, since not all commercial pre-designed assay vendors provide this information, when it's not available assay context sequences must be submitted
c Assessing the absence of DNA using a no RT assay is essential when first extracting RNA. Once the sample has been validated as RDNA-free, inclusion of a no-RT control is desirable, but no longer essential.
d When single dPCR experiments are performed, the variation due to counting error alone should be calculated from the binomial (or suitable equivalent) distribution

Figure S1: Control of plasmid linearization on agarose gel. Lane M: 1 kb plus ladder DNA marker. Lane 1: C, circular GATA1-pSPORT1 plasmid sample. Lane 2: L, linear GATA1-pSPORT1 plasmid sample (NotI treated). Lane 3: C, circular Sfpi1-pCMV-pSPORT6 plasmid sample. Lane 4: L, linear Sfpi1-pCMV-pSPORT6 plasmid sample (NotI treated).

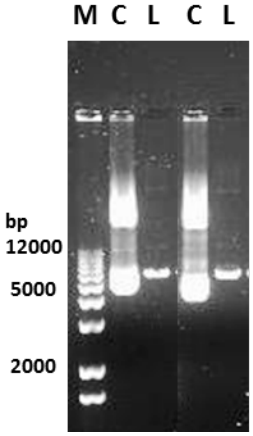
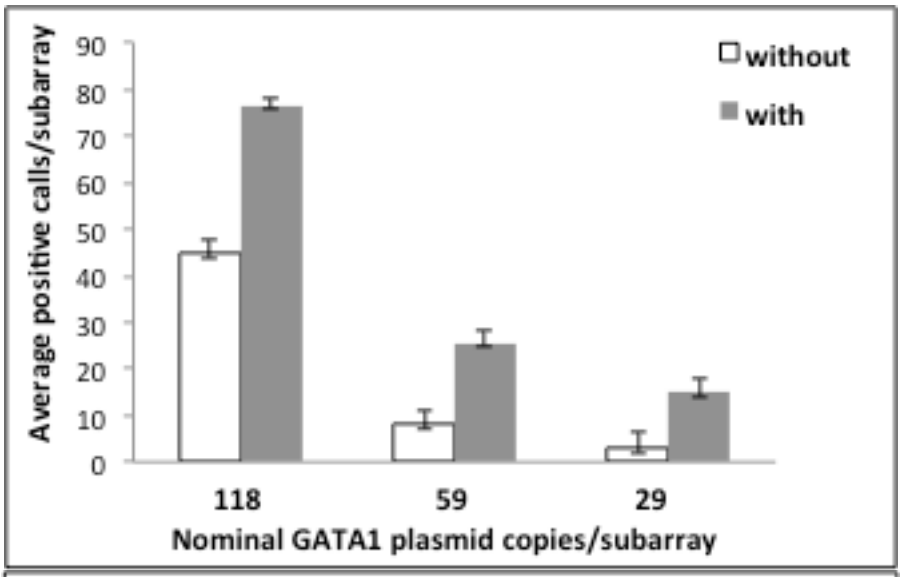


Figure S2: Comparison of using low-binding tips and non-stick tubes vs. regular ones in accurate DNA quantification. GATA1 and PU1 plasmids were diluted to approximately 29/28, 59/56 and 112/118 copies/subarray. Error bars given as standard error, n = 11 subarrays. dPCR was performed on plasmid serial dilution samples prepared using low-binding tips and non-stick tubes (grey bar) or regular tips and tubes (open bar). The average positive calls (reactions)/subarray at different nominal GATA1 plasmid input was plotted at different DNA input for GATA1 plasmid (**a**) and PU1 plasmid (**b**).

(**a**)



(**b**)

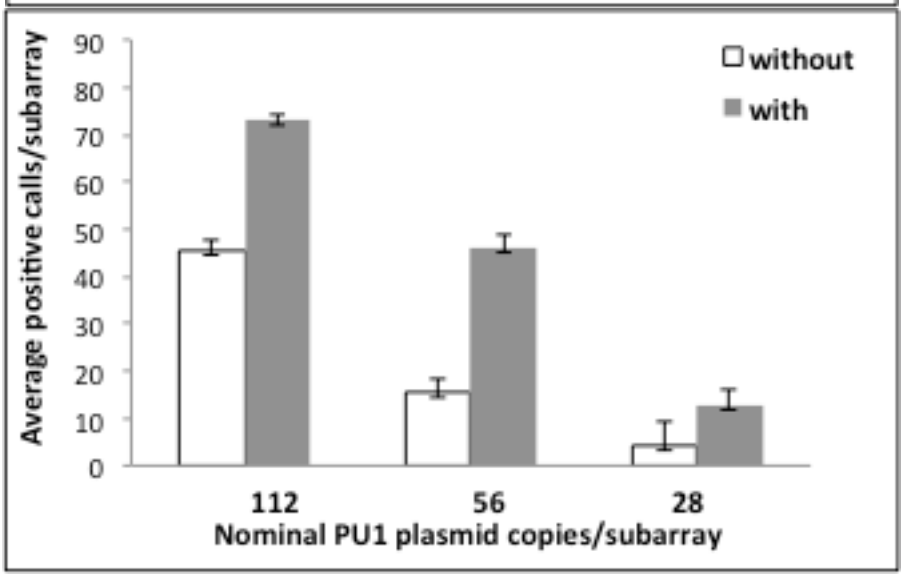


Figure S3: Representative output from chip-based real-time dPCR instrument (Applied Biosystems OpenArray). Real-time amplification plots showing amplification curves for positive and negative partitions: representative amplification plot for positive reactions (**a**), typical amplification plot for negative template control (**b**).

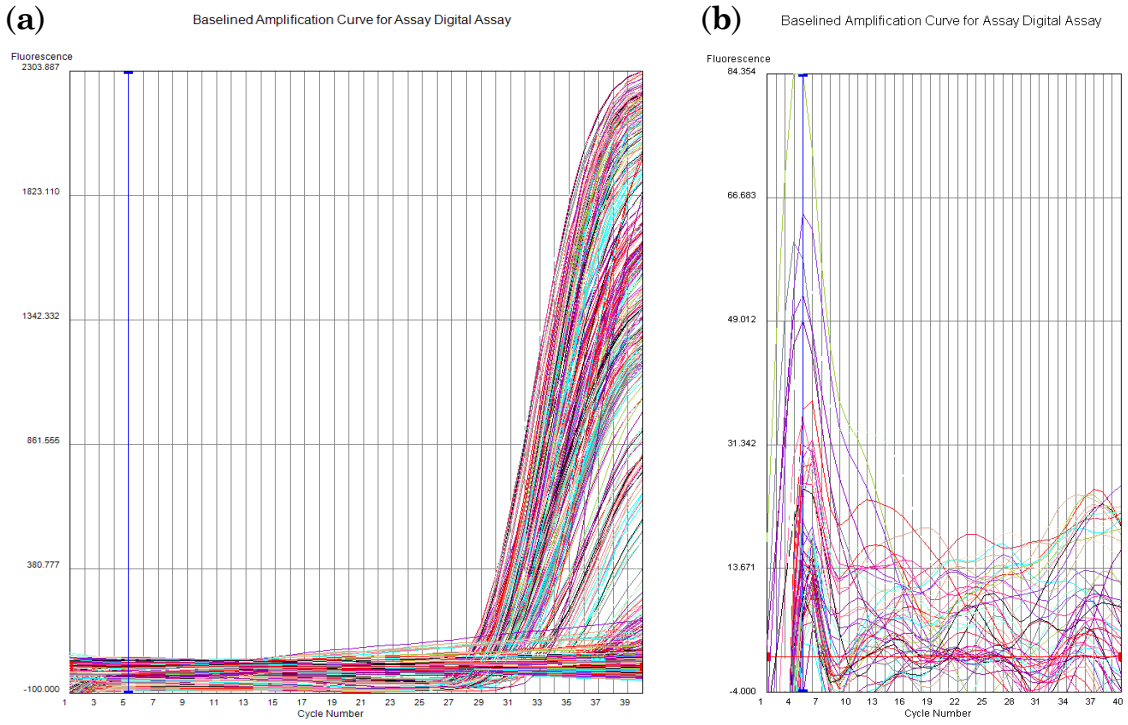
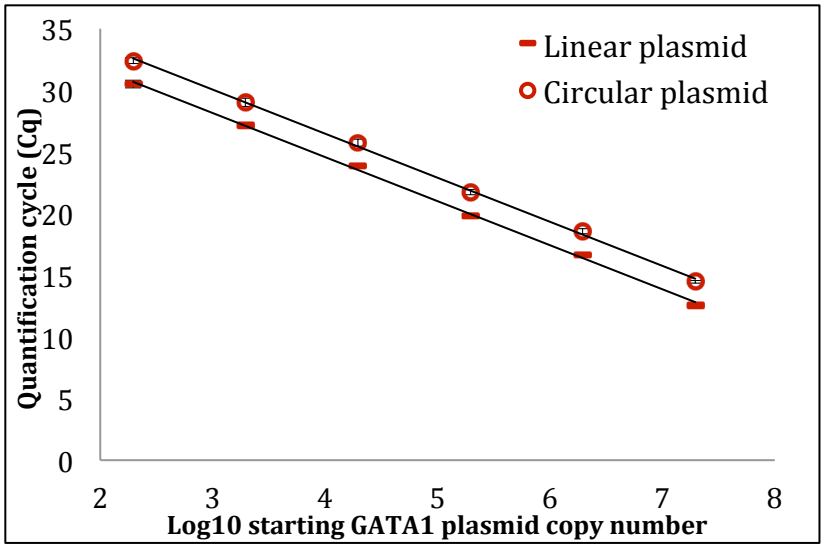


Figure S4: Standard curves between circular and linear (a) GATA1-pSPORT1 (b) Sfpi1-pCMV-pSPORT6 plasmids. Standard curves are linear regression lines between C_q and log10 starting plasmid copy number. All ΔC_q were calculated as the average of C_q difference across serial dilutions and y-intercepts were not significantly different for GATA1-pSPORT1 plasmid (p=0.63) and Sfpi1-pCMV-pSPORT6 (p=0.69). Note that similar slopes of the standard curves indicate similar amplification efficiencies for circular and linear plasmids. The error bars denote the standard deviations of C_q values among n=4 replicates.

(a)



(b)

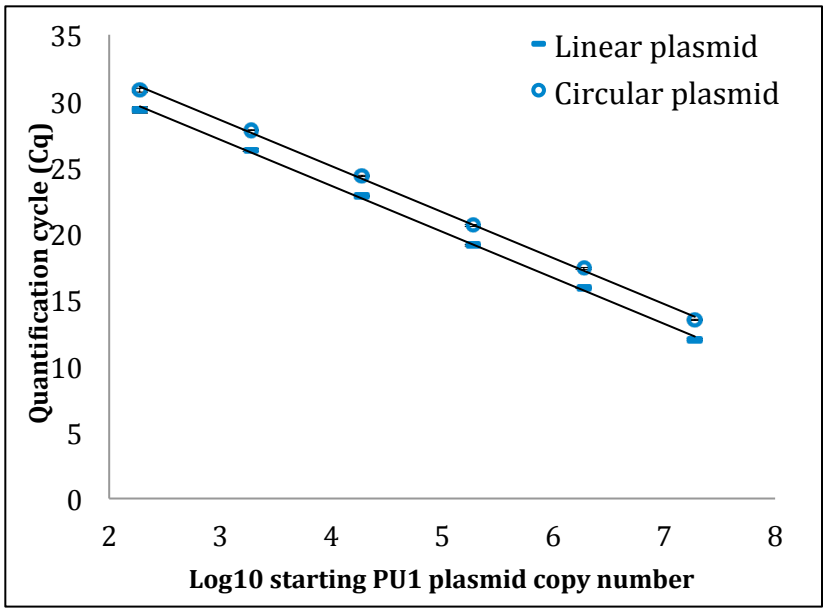


Figure S5: qPCR primer efficiency plots. Mean quantification cycle (C_q) values of each set of 10-fold serial dilution plotted against the logarithm of cDNA template dilution. Two biological replicates of isolated RNA was used to prepare serial dilution. Three qPCR technical replicates were measured at each dilution. The amplification efficiency is given by $\varepsilon = 10^{-1/S}$, where S is the slope of the linear regression line.

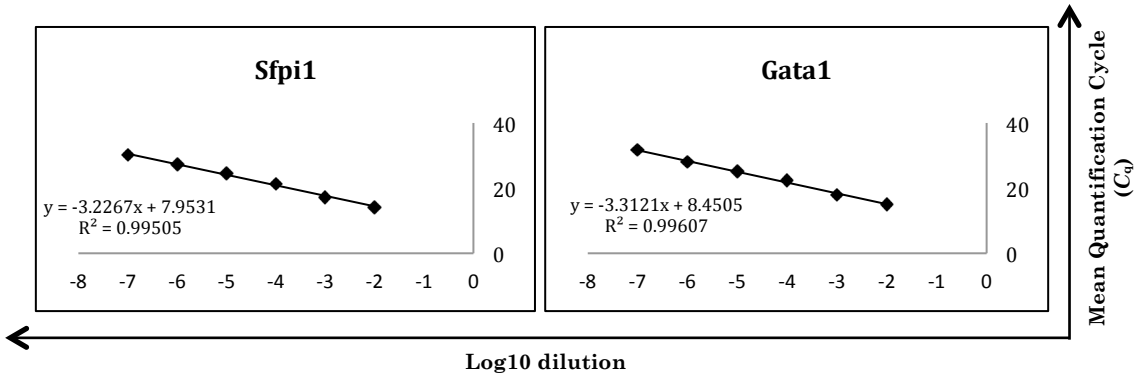


Figure S6: Quantification cycles on digital PCR arrays. GATA1 is assessed in columns 1-48 (left) and PU1 in columns 49-96 (right) in all three arrays: progenitor (EML) cells (**a**), erythroid (ERY) cells (**b**), and myeloid (MYL) cells (**c**). Color bars indicate reported C_q-values of each reaction.

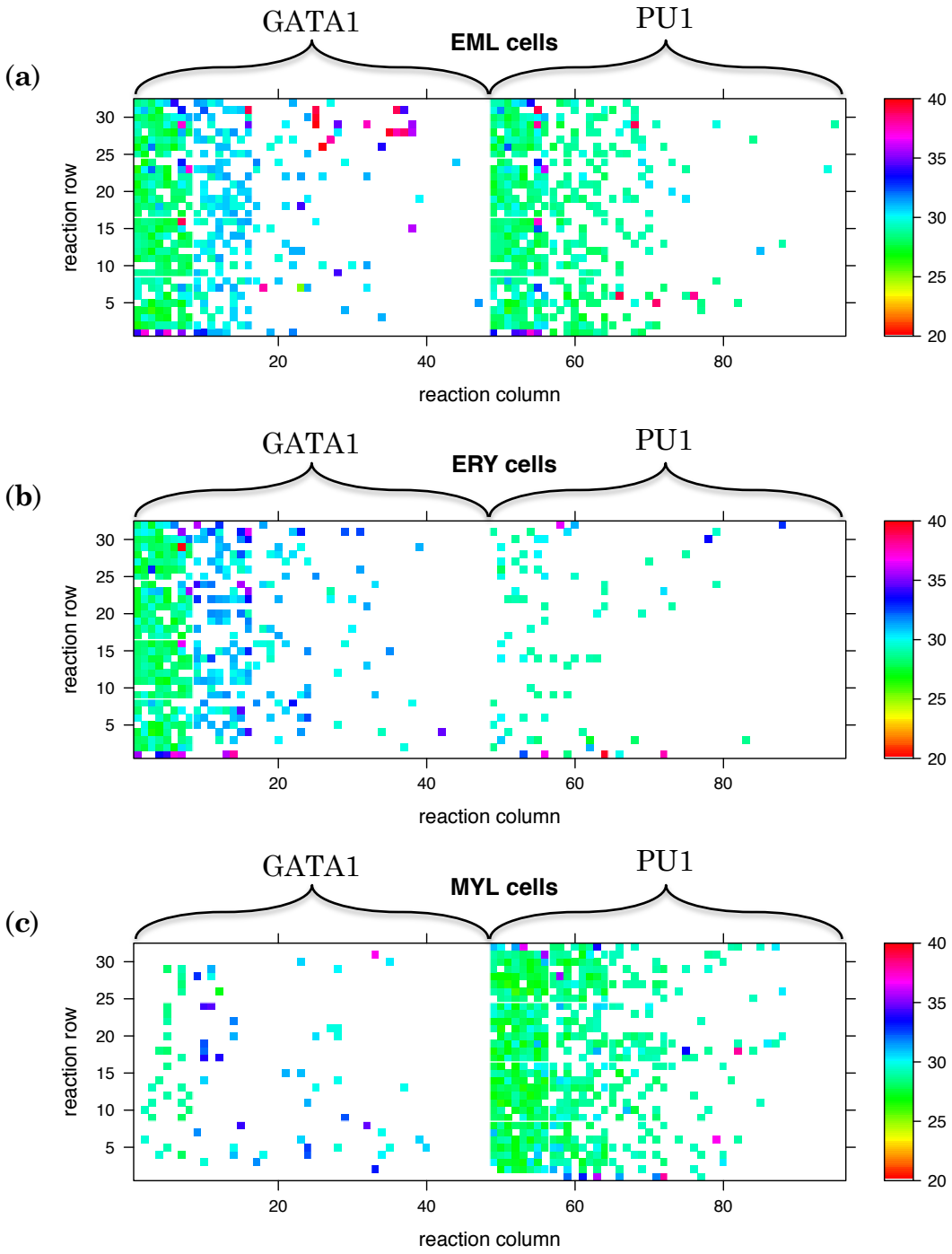


Figure S7: Selection of Sca-1 high- and low-expression cells by flow cytometry. Histograms of Sca-1 expression profiles in EML and MYL cells on day three of differentiation exhibit bimodality. The boxed regions around the modes were used to define gates from which the high and low Sca-1 expression cells for the pre-amplification assay were sorted.

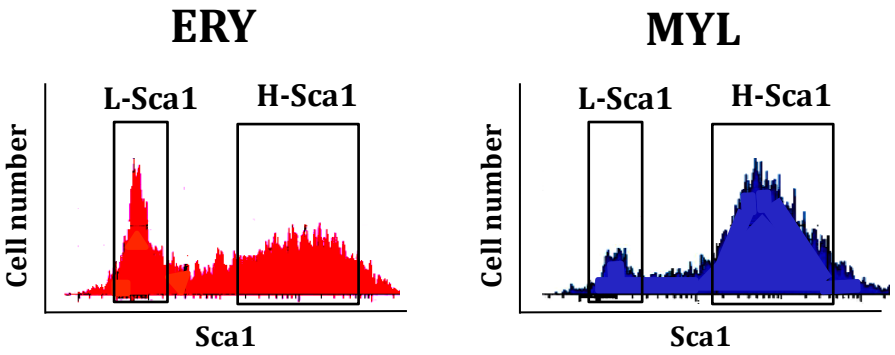


Figure S8: Quantification cycles of pre-amplified samples and array layout. Each subarray of 64 replica reactions was loaded with samples of myeloid (MYL) or erythroid (ERY) cells with high (H) or low (L) expression of SCA-1. Cells were assessed for their expression of GATA1 or PU1. The color bar indicates reported C_q-values of each reaction. Crossed out subarrays in the layout pane remained unused in the experiment. Neither these nor water (H_2O) nor no-template controls (NTC) gave rise to signal.

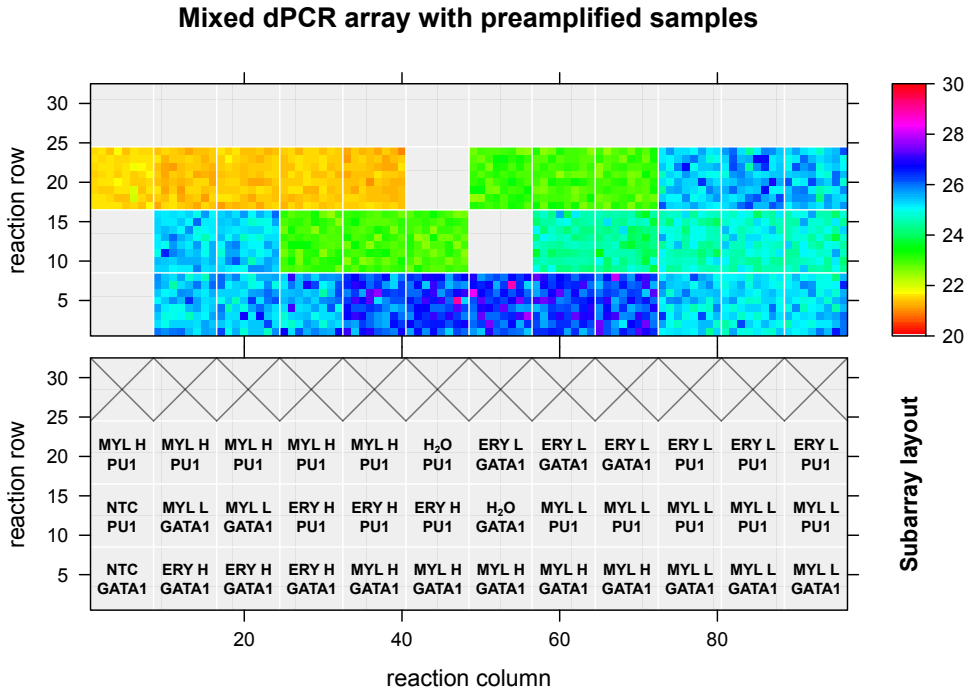


Figure S9: Distributions of reported C_q-values in pre-amplified samples. Replica reactions shown in Figure S8 give rise to different C_q distributions, which can be used to infer template concentrations using the retroflex method. In each plot the number of underlying data (N) is indicated. The shown distributions were computed using a Gaussian kernel with 0.2 as bandwidth.

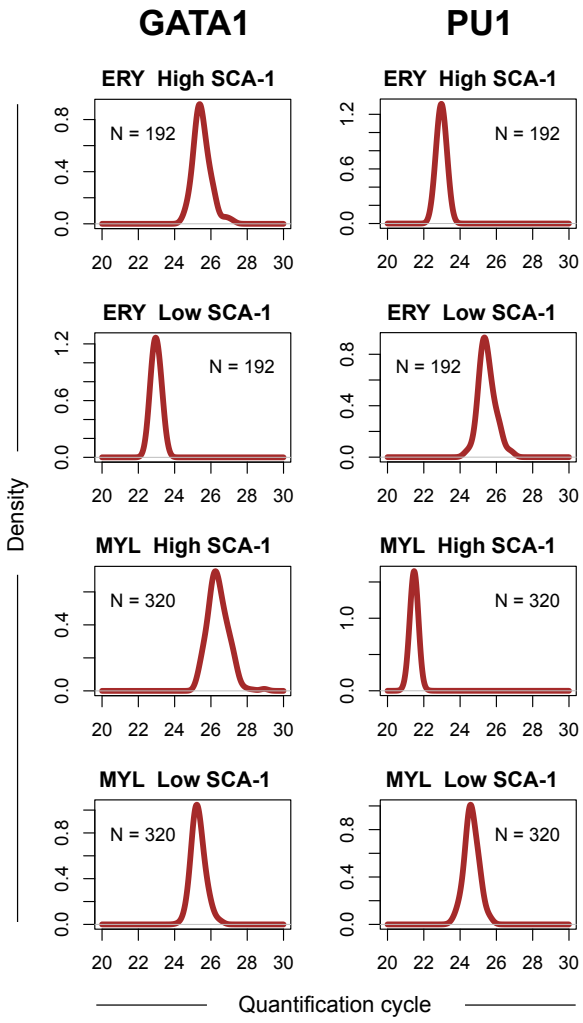


Figure S10: Relationship between coefficient of variation for positive calls/subarray and nominal template copy number for GATA1 plasmid serial dilution. Each point corresponds to triplicate experiments at indicated nominal copy number of GATA1 assayed in 704 reactions/replicate. Error bars represent sample standard deviation over the triplicates.