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

Mitra Mojtahedi¹, Aymeric Fouquier d’Hérouël²,³ and Sui Huang¹,²

¹ Department of Biological Sciences, University of Calgary, Calgary, AB T2N 1N4, Canada
² Institute for Systems Biology, Seattle, WA 98109, USA
³ Luxembourg Centre for Systems Biomedicine, L-4362 Esch-sur-Alzette, Luxembourg
Supplementary Text

Derivation of model equation

Here we outline the reasoning that leads to the probabilistic model of quantification cycles \( \rho_T(y; \lambda, k, \varepsilon) \) 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:

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

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:

\[
\rho_X(x) = \partial_x P_X(x)
\]

\[
= \Gamma^{-2} \cdot (\partial_x \Gamma_\lambda \cdot \Gamma - \Gamma_\lambda \cdot \partial_x \Gamma_\lambda)
\]

\[
= \Gamma^{-2} \cdot \left( \int_0^\infty \log t \ e^{-t} t^{x-1} dt \cdot \Gamma - \Gamma_\lambda \cdot \int_0^\infty \log t \ e^{-t} t^{x-1} dt \right)
\]

\[
= \Gamma^{-2} \cdot \left( \int_0^\infty \log t \ e^{-t} t^{x-1} dt \int_0^\infty e^{-t} t^{x-1} dt - \int_\lambda^\infty e^{-t} t^{x-1} dt \int_0^\infty \log t \ e^{-t} t^{x-1} dt \right)
\]

\[
= \Gamma^{-2} \cdot \left( \int\int_{\lambda,0}^\infty \ e^{-s} s^{x-1} log t \ e^{-t} t^{x-1} dsdt - \int\int_{\lambda,0}^\infty e^{-t} t^{x-1} log s \ e^{-s} s^{x-1} dsdt \right)
\]

\[
= \Gamma^{-2} \cdot \left( \int\int_{\lambda,0}^\infty e^{-(s+t)} (st)^{x-1} log \frac{t}{s} dt ds \right).
\]
To find the probability distribution of amplification cycles, $\rho_x$ is transformed using the relation imposed by exponential amplification $x(y) = k_0 e^y$, with the quantification threshold $k_t = x(C_q)$. In terms of continuous variables the transformation reads:

$$
\rho_y(y) = \rho_x(x(y)) \cdot \left| \frac{\partial x}{\partial y} \right|
$$

$$
= \log \varepsilon \frac{k_t e^{-y}}{Y(k_t e^{-y})^2} \int_{\lambda}^{\infty} dr \int_{0}^{\lambda} ds e^{-(r+s)} (r s)^k \varepsilon^{-y-1} \log \frac{r}{s}
$$

$$
=: \rho_y(y; \lambda, k_t \varepsilon).$$

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 plasmid 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

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Table S1: Standard calculation for dPCR analysis

| Each dPCR array                                                                 | Unit | Note                                      |
|--------------------------------------------------------------------------------|------|-------------------------------------------|
| 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)

| 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)

| 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  | 1.9·10⁷                     |                            |
|                             |        | 1.9·10⁶                     |                            |
|                             |        | 1.9·10⁵                     |                            |
|                             |        | 1.9·10⁴                     |                            |
|                             |        | 1.9·10³                     |                            |
|                             |        | 1.9·10²                     |                            |
| Plasmid standard curve      | PU1    | 1.8·10⁷                     |                            |
|                             |        | 1.8·10⁶                     |                            |
|                             |        | 1.8·10⁵                     |                            |
|                             |        | 1.8·10⁴                     |                            |
|                             |        | 1.8·10³                     |                            |
|                             |        | 1.8·10²                     |                            |
| EML sample                  | GATA1 & PU1 |                         | 2000\n~1000\n~250\n~62\n~15\n~4\n~1 |
| ERY & MYL samples           | GATA1 & PU1 |                         | 2000\n~250\n~62\n~15\n~4\n~1 |
Table S4: Summary of plasmid dilution experiments

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

| Item                                                                 | Requirement | Submission |
|----------------------------------------------------------------------|-------------|------------|
| 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        |
| 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<sup>b</sup> | E | N/A |
|-------------------------------------------------------------|---|-----|
| RTPrimerDB Identification Number<sup>b</sup>               | 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 | ✓ |
|                                                   | Essential (E) | Desirable (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^4                    | 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).
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 \( \Delta C_q \) 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.
**Figure S5: qPCR primer efficiency plots.** Mean quantification cycle (Cₚ) 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.

\[
\begin{align*}
\text{Sfi1} & : y = -3.2267x + 7.9531 \\
R^2 & = 0.99505 \\
\text{Gata1} & : y = -3.3121x + 8.4505 \\
R^2 & = 0.99607
\end{align*}
\]
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.