Supplemental Information

The Self-Digitization Microfluidic Chip for the Absolute Quantification of mRNA in Single Cells

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This supplemental information provides additional details relating to the PCR assays and microfluidic device design.

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PCR primers

ABL:

5’-CAG GCA TCA ACA CTG CTT CTG-3’

5’-TCG GCC AGG GTG TTG AA-3’

probe: CalFluor Gold 540-5’-TGG CAA GCT CTA CGT CTC CTC CGA GA-3’-Black Hole Quencher

BCR-ABL:

5’-CAT TCC GCT GAC CAT CAA TAA-3’

5’-AAC GAG CGG CTT CAC TCA GA-3’

probe: FAM-5’-AGC GGC CAG TAG CAT CTG ACT TTG AGC-3’-Black Hole Quencher

GAPDH RNA standards:

5’-GAT GAT GTT CTG GAG AG-3’

5’-TAA TAC GAC TCA CTA TAG GGA TTT GGT CGT ATT GG-3’

Oligomers were purchased as standard, desalted DNA from Integrated DNA Technologies.

GAPDH and TFRC were purchased from a catalog of hydrolysis probe assays available from Integrated DNA Technologies. GAPDH assay ID# Hs.PT.42.1164609 and TFRC assay ID# Hs.PT.56a.3164874.

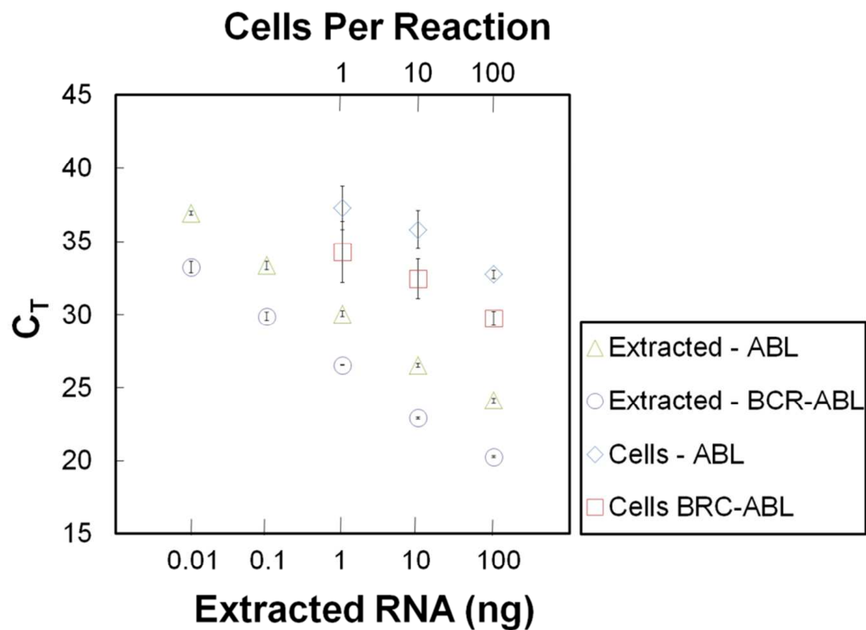

Figure S-1. Results of qPCR for BCR-ABL or wild type ABL performed on individual, 10 or 100 K562 cells alongside a titration of extracted total RNA from K562 cells. While the duplex qPCR assays demonstrated linearity and accuracy down to inputs of total extracted RNA below the levels found in single cells (R2 of 0.9959, slope of -3.24 for ABL and R2 of 0.9979, slope of -3.29 for BCR-ABL), the variability seen in single cells was far more than expected due to technical variability alone.

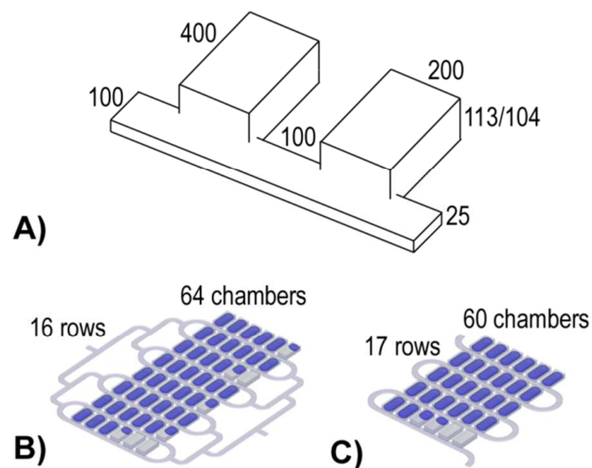

Figure S-2. SD Chip dimensions. A) Chamber dimensions are given in units of µm. Serpentine device for single-cell experiments had measured chamber height of 104 ± 3 µm, while bifurcated design used for total RNA dilution experiments had measured chamber height of 113 ± 5 µm. Both devices had measured main channel height of 25 ± 1 µm. B) Bifurcated device schematic. Bifurcated channel devices used for total RNA dilution experiments used 16 rows with 64 chambers per row, for 1024 total chambers. C) Serpentine device schematic. Serpentine channel devices used for single cell experiments used 17 rows with 60 chambers per row, for 1020 total chambers.

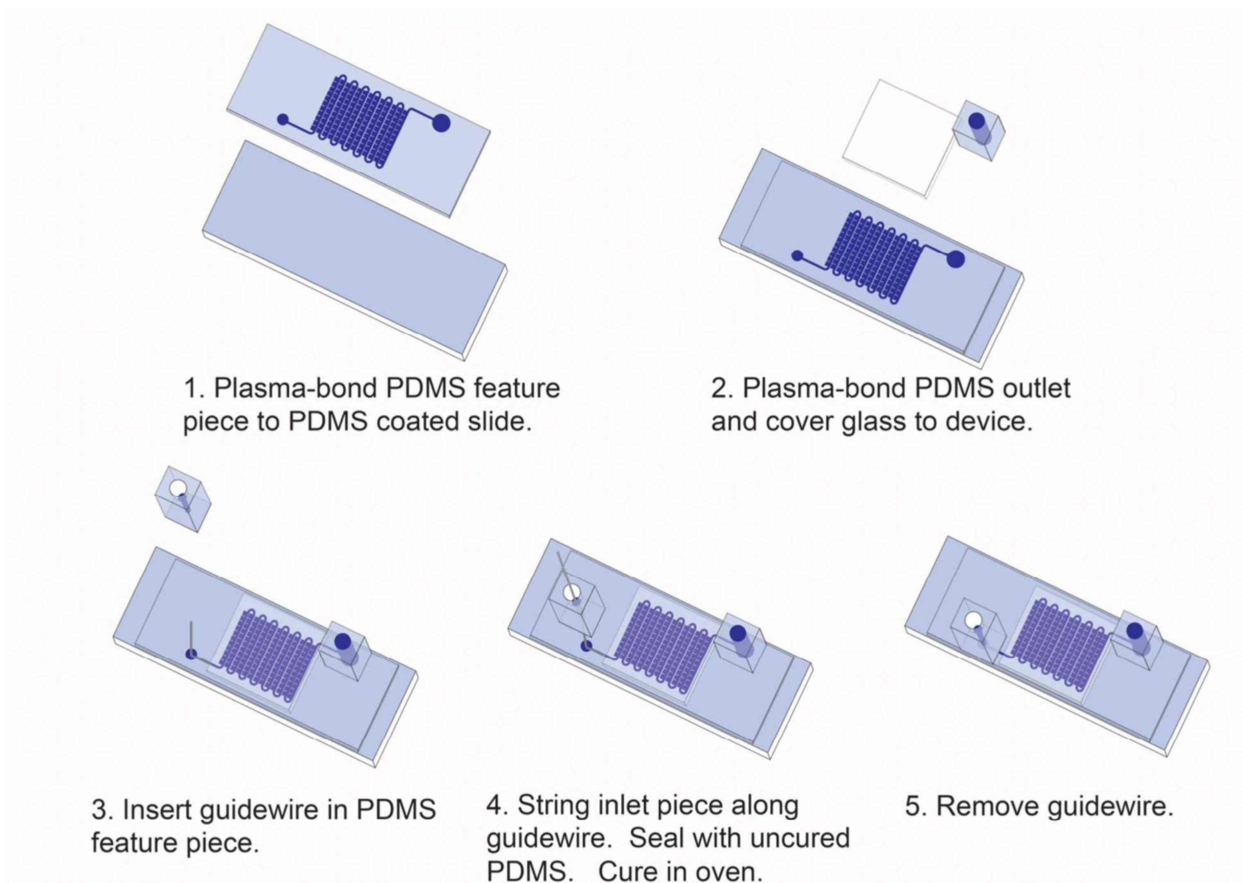

Figure S-3. Steps in SD Chip assembly. 1) Spin-coat PDMS on microscope slide and cure at 70°C for 3 hours, 2) plasma-bond PDMS feature piece to microscope slide, 3) plasma-bond cylindrical outlet piece and cover glass to feature piece, 4) insert guidewire, place a small amount of uncured PDMS over wire, and place PDMS inlet piece onto uncured PDMS. The devices were cured overnight at 115°C and stored at room temperature before oil priming.

PDMS elastomer (Sylgard 184 silicone elastomer kit, Dow Corning Corp., Midland, MI, USA) was mixed at a ratio of 5:1 for replication of microfluidic structures and for the spin-coating of glass microscope slides. The PDMS was mixed at a ratio of 10:1 for inlet and outlet reservoir features. Spin-coating of microscope glass and the microfluidic feature mold was performed at 4000 RPM and 300 RPM, respectively, for 1 minute each. PDMS was then cured at 70°C for 3 hours. Inlet features were cast against the outer bottom of a PCR tube, creating a tapered cylinder inlet with a convex bottom surface to direct the sample into the main channel. Outlet features were cut to fit from a slab of PDMS approximately 7 mm thick. An access hole was punched into the inlet with a 16-gauge punch and another was punched into the outlet with a 10-gauge punch to fit channel geometries. Devices were assembled in several steps. First, access holes were punched in the microfluidic feature piece using a 10-gauge punch. This feature piece along with a PDMS-coated glass slide was sealed on contact after exposure to oxygen plasma at medium level for 60 s (plasma cleaner/sterilizer, Harrick Plasma, Ithaca, NY). In a second plasma treatment step, the outlet feature and a No. 1 cover glass cut slightly larger than the array geometry were sealed to the device to create an access reservoir and vapor barrier over the array. Next, a 38-gauge Ni-Chrome guide wire was threaded part-way into the inlet channel. This wire will serve to cast a smooth entry channel from the inlet to the main channel. The inlet channel was sealed with a dab of PDMS cured at 70°C for 15 minutes. The inlet reservoir was threaded along the wire and sealed to the device with PDMS. The assembled device was incubated in a 115°C oven for 2 days to promote hydrophobic recovery of the PDMS surface. The guide wire was then removed from the inlet. This created a channel from the inlet funnel to the main channel, minimizing pockets where fluid might be retained. Prior to continuous phase priming, and a piece of double-sided Kapton® tape (DuPont, Wilmington, DE, USA), cut with access holes, was placed on the outlet feature.

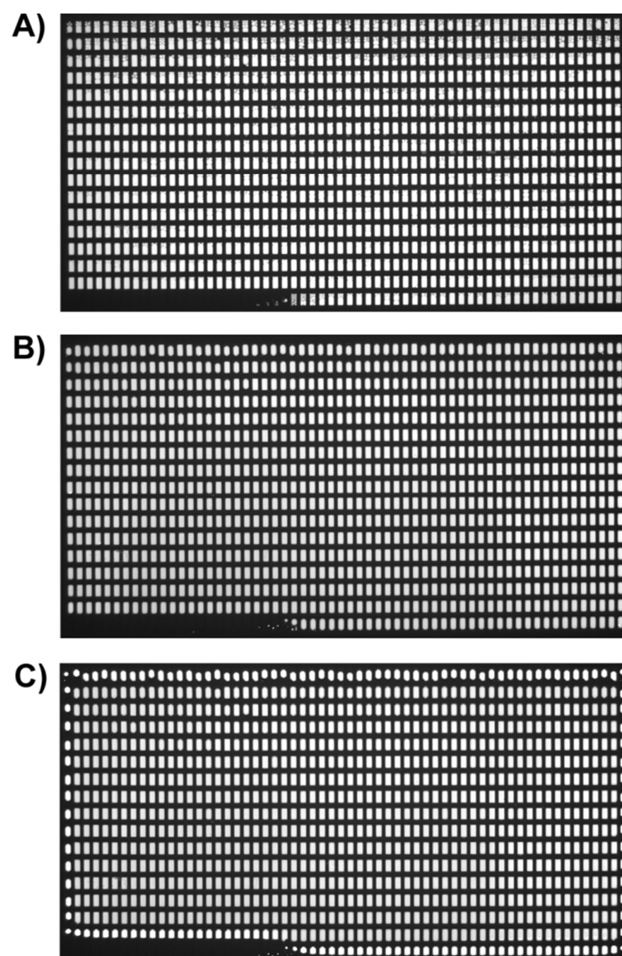

Figure S-4. Filled SD Chip serpentine array imaged at three stages of RT-PCR. A) Before heating, B) following reverse transcription and hot-start steps, C) following reverse transcription, hot start, and 40 cycles of PCR. Because chambers on the outermost rows and columns of the filled array lose volume during heating, these volumes were excluded from analyses.

Figure S-5. Images of filled chambers at ambient pressure following sample digitization. A) When using an air compressor to drive flow during digitization, some chambers overfill and sample is pushed into the main channel after pressure is released. The arrow indicates an area where sample exits the chamber. B) When vacuum pressure was used to digitize the sample this effect was not present.

Determination of mRNA copy number

Poisson statistics can be applied over the dynamic range of the device, from arrays containing at least one positive chamber and up to one negative chamber. At high concentrations, many chambers will contain more than one template. Because positive chambers contain unknown quantities of template, concentration is determined by the number of negative chambers. Briefly, note the Poisson distribution function:

$$f(k;\lambda) = \Pr(X = k) = \frac{\lambda^k e^{-\lambda}}{k!}$$

Here, the probability that a chamber will contain a number of copies k is a function of λ, the average concentration in copies per chamber. PCR negative chambers must have exactly k = 0. Defining p as the probability of observing a PCR negative chamber,

$$\Pr(k = 0) = p = e^{-\lambda}$$

In our analyses, we use the number of observed successful reactions, S, divided by the total number of chambers, C, to obtain an estimate of p. We can therefore obtain a theoretical value for λ, target copies per chamber, from our observation of S/C, as

$$\lambda = -\ln\left(1 - \frac{S}{C}\right)$$

Work by presented by Wilson (Wilson, E. *J. Am. Stat. Assoc.* **1927**, *22*, 209–212) and later evaluated by Newcombe et al. (Newcombe, R. G. *Stat. Med.* **1998**, *17*, 857–872) has shown that the accuracy of this approach fails at the extreme low and high ends of concentration and that the following equation gives a confidence interval that performs well at these extremes[1,2]:

$$(w^-, w^+) = \left(p + \frac{z^2}{2C} \pm z\sqrt{\frac{p(1-p)}{C} + \frac{z^2}{4C^2}}\right) \Big/ \left(1 + \frac{z^2}{C}\right)$$

With the relocated center estimate being

$$p' = \left(p + \frac{z^2}{2C}\right) \Big/ \left(1 + \frac{z^2}{C}\right)$$

This method was then used to calculate the target molecule concentration the 95 percent confidence intervals about this estimation (Shen, F.; Du, W.; Kreutz, J. E.; Fok, A.; Ismagilov, R. F. *Lab Chip* **2010**, *10*, 2666–2672). The concentration λ in copies per chamber can be divided by the volume per chamber to obtain a concentration of target molecules per volume. This concentration was multiplied by the loaded sample volume (8.1 μL for single cells and 10 μL for total RNA dilution) to obtain copy number per cell or copy number per μL RNA diluent.

Determination of theoretical dynamic range

The theoretical range of the device was calculated assuming all wells are filled and analyzed, excluding the outermost chambers. This analyzed volume would be 870 chambers of 8.32 nL volume, or 7.29 μL. Given a volume with a concentration of 3 molecules per analyzed sample, the average sample would yield 3 positive chambers, and would yield at least one positive chamber for 95% of samples. Likewise, at the upper limit, the average sample would yield 3 negative chambers and would yield at least one negative chamber for 95% of samples. This concentration corresponds to a range from to 0.41 to 680 copies per μL, or 3.3 to 5500 copies per single cell.

Determination of loading efficiency

The image obtained following the digital PCR reaction can be analyzed to determine the number of filled volumes (8.32 nL) and thus determine the total volume of sample digitized. For the single cell experiments (n=12), the average volume of the filled chambers was 7.02 ± 0.26 μL. As the input volume for these samples was 8.10 μL, the calculated efficiency is 86.7% ± 3.3.