Supporting information for research article

Title of article:

Evaluation of a Droplet Digital Polymerase Chain Reaction Format for DNA Copy Number Quantification

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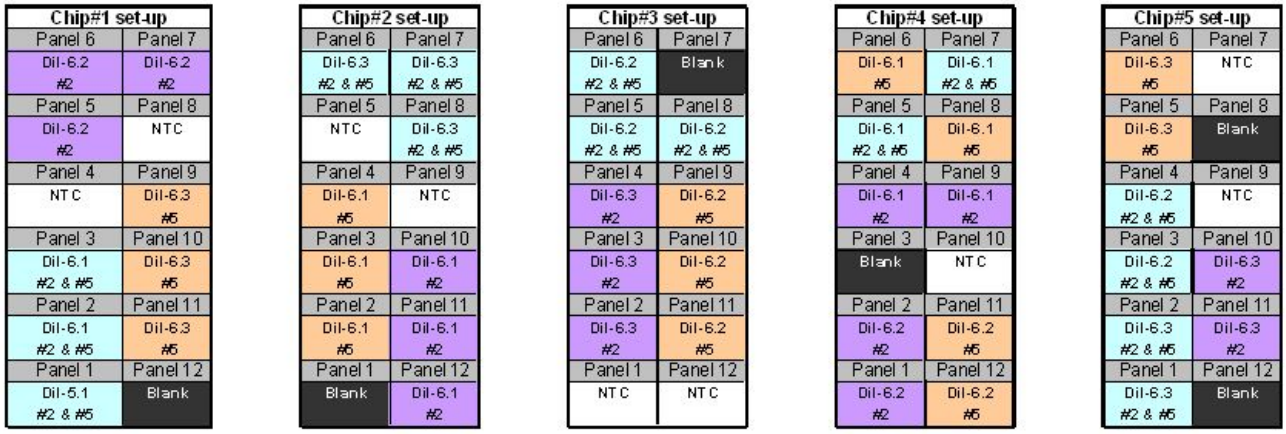
Supporting information S-1. Gravimetric protocol

The sample tube (Lambda DNA) stored at 4°C was retrieved and placed in the thermomixer and heated to 60°C at 800 rpm for 2 min. The tube was allowed to cool to room temperature and then spot centrifuged and placed in a separate rack until further use. Based on the NaOH denatured A_{260nm} value of stock lambda DNA (Bhat et al, 2010), three independent gravimetric dilutions were prepared in polypropylene microcentrifuge tubes (Axygen Scientific, California) with 1x $TE_{0.1}$ (10 mM Tris, 0.1 mM EDTA, pH 8.0) as diluent using a calibrated Mettler Toledo XP-205 five figure balance and in-house calibrated pipettes. A nine step dilution was carried out for each of the three independent dilutions. All three sets of gravimetric dilution tubes were stored at 4°C until further use.

For gravimetrically combining the respective sample and diluents or pre- sample mix combinations, the following steps were performed.

1. The balance was tared to subtract the weight of the stand and the value printed.
2. An empty Axygen tube was placed into the balance and the mass (g) of the empty vessel plus the stand was printed.
3. The required volume of diluent (1x $TE_{0.1}$) or pre-sample mix was added and the mass (g) of the diluent or pre-sample mix was printed.
4. Then the required volume of Lambda DNA from the stock tube or the diluted tube was added and the mass (g) of the DNA was printed.
5. The tube was then removed and the unloaded mass (g) reading on the balance was then printed.
6. This process (steps 1-5) was repeated for all other dilutions.
7. In between each dilution step, the tubes were mixed at room temperature on the thermomixer at 800 rpm for 1 min and spot centrifuged.

8. When preparing sample mix for both ddPCR and cdPCR, the respective sample mixes were mixed by flicking the tube and spot centrifuged prior to loading onto the digital array or droplet generator.



Chip#1 set-up	
Panel 6	Panel 7
Dil-6.2 #2	Dil-6.2 #2
Panel 5	Panel 8
Dil-6.2 #2	NTC
Panel 4	Panel 9
NTC	Dil-6.3 #5
Panel 3	Panel 10
Dil-6.1 #2 & #5	Dil-6.3 #5
Panel 2	Panel 11
Dil-6.1 #2 & #5	Dil-6.3 #5
Panel 1	Panel 12
Dil-5.1 #2 & #5	Blank

Chip#2 set-up	
Panel 6	Panel 7
Dil-6.3 #2 & #5	Dil-6.3 #2 & #5
Panel 5	Panel 8
NTC	Dil-6.3 #2 & #5
Panel 4	Panel 9
Dil-6.1 #5	NTC
Panel 3	Panel 10
Dil-6.1 #5	Dil-6.1 #2
Panel 2	Panel 11
Dil-6.1 #5	Dil-6.1 #2
Panel 1	Panel 12
Blank	Dil-6.1 #2

Chip#3 set-up	
Panel 6	Panel 7
Dil-6.2 #2 & #5	Blank
Panel 5	Panel 8
Dil-6.2 #2 & #5	Dil-6.2 #2 & #5
Panel 4	Panel 9
Dil-6.3 #2	Dil-6.2 #5
Panel 3	Panel 10
Dil-6.3 #2	Dil-6.2 #5
Panel 2	Panel 11
Dil-6.3 #2	Dil-6.2 #5
Panel 1	Panel 12
NTC	NTC

Chip#4 set-up	
Panel 6	Panel 7
Dil-6.1 #5	Dil-6.1 #2 & #5
Panel 5	Panel 8
Dil-6.1 #2 & #5	Dil-6.1 #5
Panel 4	Panel 9
Dil-6.1 #2	Dil-6.1 #2
Panel 3	Panel 10
Blank	NTC
Panel 2	Panel 11
Dil-6.2 #2	Dil-6.2 #5
Panel 1	Panel 12
Dil-6.2 #2	Dil-6.2 #5

Chip#5 set-up	
Panel 6	Panel 7
Dil-6.3 #5	NTC
Panel 5	Panel 8
Dil-6.3 #5	Blank
Panel 4	Panel 9
Dil-6.2 #2 & #5	NTC
Panel 3	Panel 10
Dil-6.2 #2 & #5	Dil-6.3 #2
Panel 2	Panel 11
Dil-6.3 #2 & #5	Dil-6.3 #2
Panel 1	Panel 12
Dil-6.3 #2 & #5	Blank

Figure S-1. Microfluidic cdPCR chip set up

The chip setup was randomised. No Template Controls (NTC) containing 1X $TE_{0.1}$ buffer in place of DNA or blank panels containing 1X $TE_{0.1}$ buffer in place of DNA and primer/probes were analysed in one or more alternate panels. Blank panels were used in order to accommodate the chip-setup.

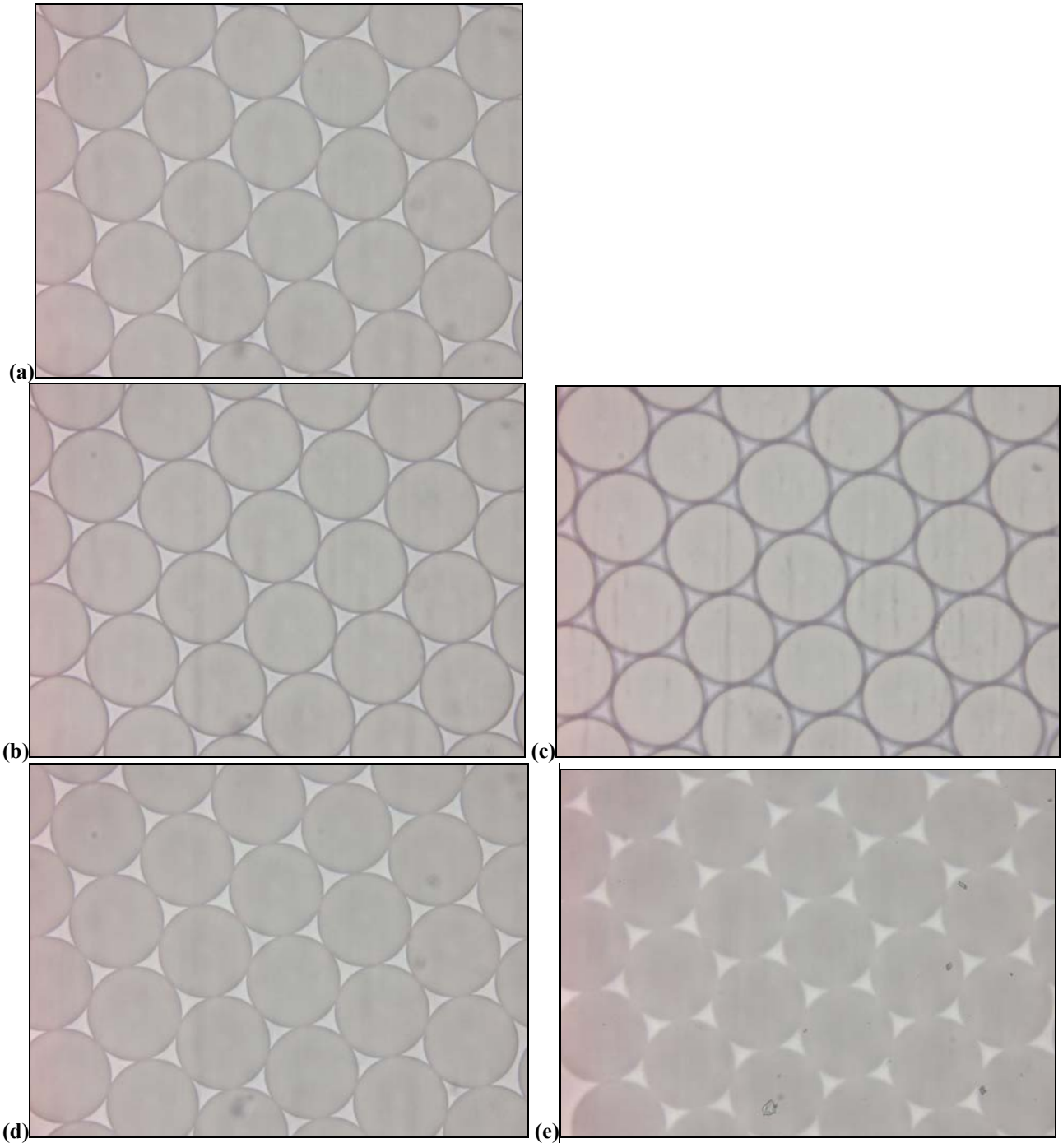


Figure S-2. Optical microscopy images of ddPCR droplets 200 x apparent magnification

(a) on focus, (b) 6 µm over focus resulting in ~0.12% droplet volume variation compared to image on focus, (c) 24 µm over focus, image so poorly focused that wouldn't be selected for analysis, (d) 6 µm under focus resulting in ~1.32% droplet volume variation compared to image on focus, (e) 24 µm under focus, image poorly focused and wouldn't be selected for analysis

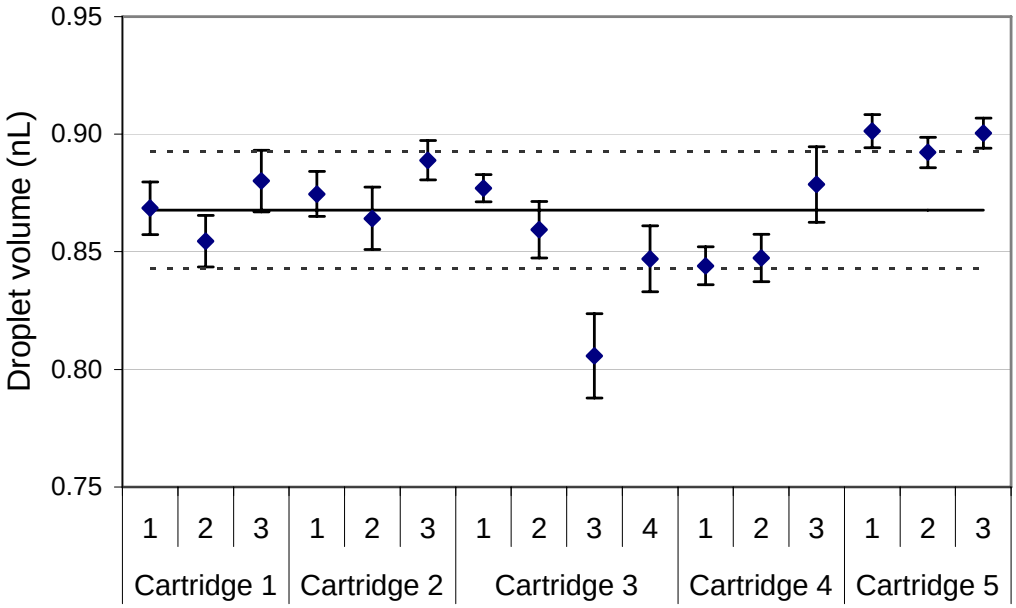


Figure S-3. Droplet volume variation between wells.

Each symbol denotes the average droplet volume (nL) for droplets generated from a single well. Either three or four wells were randomly selected from each of five different droplet generator cartridges. Five separate images containing between 11 and 17 complete droplets were captured for each well and the droplet volume estimated as described in the Experimental Section. Between 66 and 77 droplets were measured from each well. Error bars represent the standard deviation of the data generated from the five images. The Grand Mean well droplet volume was 0.868 nL (solid horizontal line) with a relative standard deviation of the mean droplet volume of 2.8% (dotted lines show standard deviation of the mean).

Table S-1. Primers and Probes used in the study

Assay	Primer / probe	Sequence 5′ to 3′	Concentration (nM)	Product length (bp)	Source
#2	Forward	TGCGCTGTATGCCGGTATG	500	188	Bhat et al, 2010
	Reverse	GTTGTTCGGGTCAATCCAGTTC	500		
	Probe	5-HEX-CCTCAACGGCATTATGGCGGTCCTT -BHQ1	300		
#5	Forward	CCCAGCAACAGCACAACCC	500	76	Bhat et al, 2010
	Reverse	GCCGCAGCGTAACTATTACTAATG	500		
	Probe	6-FAM-ACTGAGCCGTAGCCACTGTCTGTCCT -BHQ1	40		

The entire lambda genome used for this study is a forward sequence. (F) forward primer, (R) reverse primer, (P) probe, [6-FAM] 6-carboxyfluorescein, BHQ-1 Black Hole Quencher 1, [5-HEX] hexachloro-6-carboxyfluorescein

As stated in Bhat et al, 2010[1], the PCR assays were optimised using gradient end-point PCR to first verify the optimal annealing/extension temperature for each assay. The concentrations of the primer and probe sequences were then optimized by real-time PCR on the Stratagene Mx3005P prior to use in either 765- cdPCR or 20,000-ddPCR.

References

(1) Bhat, S.; Curach, N.; Mostyn, T.; Bains, G. S.; Griffiths, K. R.; Emslie, K. R. *Analytical Chemistry* **2010**, *82*, 7185-7192.