Single molecule digital PCR approach offers fundamental breakthroughs in multiplexing and sensitivity

Q. Zhong[a], S. Bhattacharya[a], S. Kotsopoulos[a], J. Olson[a], V. Taly[b], A.D. Griffiths[b], D.R. Link[a], S. Kellett[a] and J.W. Larson[a]

RainDance™ Technologies

Introduction

Digital PCR (dPCR) is an attractive alternative to qPCR due to its superior sensitivity and absolute quantification of target molecules. The RainDance RainStorm™ droplet-based microfluidics method utilizes up to 10 million reactions to enable detection of rare mutations in a background of wild-type at levels better than 1 in 200,000.[1] The combination of superior sensitivity, unprecedented multiplexing, and flexibility in experiment design provide a powerful genomic analysis platform for new research in cancer including rare variant detection, absolute quantification of biomarkers, tumor profiling, and the ability to monitor residual disease.

What is Digital PCR?

Digital PCR is an absolute method of quantifying DNA. Template DNA is diluted to single molecule occupancy per reaction. Using millions of droplets as reaction chambers and end-point amplification, positive PCR reactions are counted as a direct measurement of the number of DNA molecules originally present. Fluorogenic probes are commonly used to discriminate PCR(+) and (-) reactions, and Poisson statistics account for the possibility of multiple molecules occupying the same reaction. Unlike qPCR, standard curves are not necessary.

Figure 1

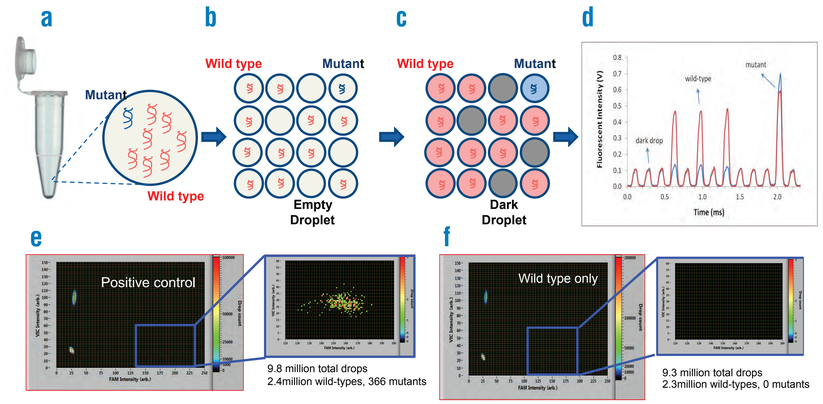

Figure 1
a. Genomic sample contains low level of mutant in high background of wild type. **b.** Sample diluted to single molecule per droplet and partitioned into millions of reaction droplets. **c.** End point PCR produces positive and negative reactions. **d.** Droplets flow past laser; two color fluorescent detection yields positive (wild type & mutant) and negative (dark) droplets. **e & f.** 2-D histograms show relative concentrations of mutant and wild type.

Superior Sensitivity for Detecting Rare Mutations

Superior sensitivity is potentially enabling for non-invasive diagnostic applications including use of cell-free mutant DNA in blood samples.

EGFR c.2573T>G SNP Detection Assay (L858R)

Mutations of the human epidermal growth factor receptor (EGFR) are strong indicators of patient response to cancer treatment with tyrosine kinase inhibitors (TKIs) in non-small cell lung carcinoma: T to G single base pair mutation at position 2573 is found in 43% of EGFR mutant tumors[2], and is associated with increased sensitivity to TKIs.

Figure 2

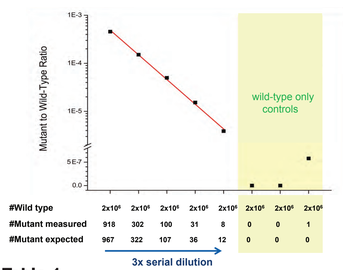

Figure 2
To assess the lower limit of detection (LLOD) of EGFR mutation, varying concentrations of mutant were diluted into genomic wild-type DNA. Mixtures were emulsified into ~10^7 droplets using the process previously described. The number of PCR(+) droplets at the reaction end point was compared between mutant and wild-type as a direct measurement of the ratio of concentrations. The assay delivered a linear response (R^2 = 0.998) down to 1 mutant amongst 250,000 wild-type molecules, with a LLOD of 1 in more than 1,000,000 as defined by the average of the wild-type only controls plus 3x the standard deviation. Results of other assays are shown below in **Table 1**.

Table 1

Assay	Mutation Type	Total Runs	LLOD
EGFR_c.2573TG	T>G	5	>1/1,000,000
Assay B	C>G	3	>1/1,000,000
Assay C	C>A	6	>1/500,000
Assay D	C>G	4	>1/500,000
Assay E	A>G	3	>1/200,000

Unprecedented Multiplexing Capability With Droplet-Based dPCR

Multiplexing enables development of assays for biomarker panels at reduced cost and sample consumption, increased throughput and the potential for built-in assay controls. Conventional qPCR has limited multiplexing capability due to spectral overlap of fluorescent probes. A new method for differentiating targets on the basis of fluorescence intensity was developed by varying the concentration of the fluorescent probes.[3] To demonstrate, results of a 5-plex TaqMan® dPCR copy number assay for spinal muscular atrophy (SMA) were measured simultaneously with just VIC and FAM fluorophores. This approach can be expanded to higher plex levels.

Figure 3

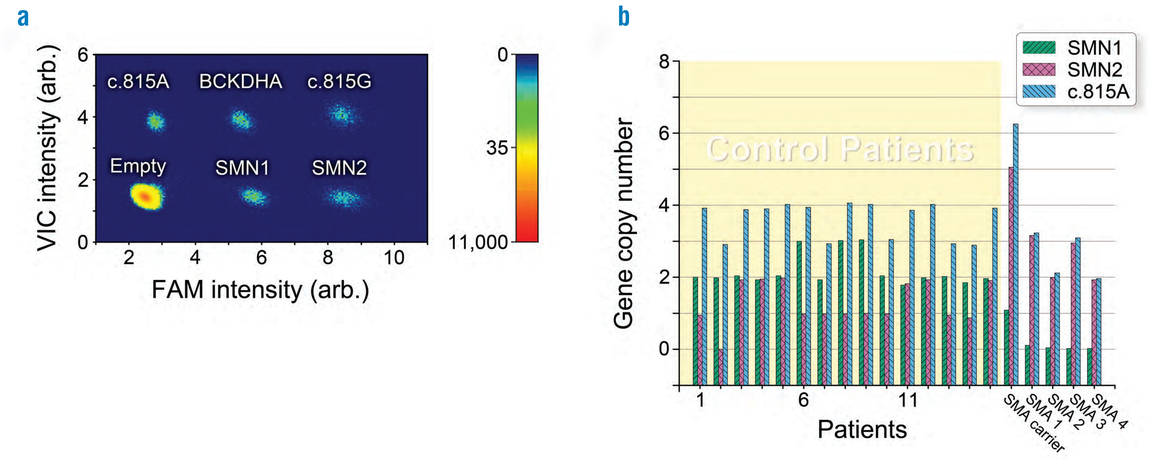

Figure 3
a. Histogram (heat map) of droplet fluorescence intensities, for the 5-plex assay against a synthetic model. Standard techniques were used to compensate for spectral overlap of FAM and VIC signals. The six droplet populations correspond to the five individual assays plus the empty droplets. **b.** Results of the SMA pilot study on 20 different patient samples from the Coriell cell repositories: 4 afflicted with SMA, 1 SMA carrier, and 15 negative controls. The measured genotypes of the different patients were consistent with their disease conditions (unafflicted, carrier, or afflicted). The patients afflicted with SMA each had zero copies of SMN1 (numbers SMA 1–4 in the figure), the carrier had just one copy, and the negative controls all had two or three copies (patients 1–15).

Summary

The RainDance RainStorm droplet-based digital PCR, with up to 10 million reactions per sample, demonstrates superior sensitivity and unprecedented multiplexing capability. This powerful genomic analysis platform should enable new research and the ability to detect and monitor diseases where identification and quantification of rare events is critical.

References

[1] D. Pekin, Y. Skhiri, J. Baret, D. Le Corre, L. Mazutis, C. Ben Salem, F. Millot, A. El Harrak, J. B. Hutchison, J.W. Larson, D.R. Link, P. Laurent-Puig, A.D. Griffiths and V. Taly, Lab Chip, 2011, 11, 2156-2166.

[2] T. Mitsudomi and Y. Yatabe, FEBS J., 2010, 277(2), 301-8.

[3] Q. Zhong, S. Bhattacharya, S. Kotsopoulos, J. Olson, V. Taly, A.D. Griffiths, D.R. Link and J.W. Larson, Lab on a Chip, 2011, 11, 2167-2174.

[a] RainDance Technologies, Inc., Lexington, MA, USA

[b] Institut de Science et d'Ingenierie Supramoleculaires (ISIS), Universite de Strasbourg, France