High-precision Copy Number Variation (CNV) measurement achieved on Droplet Digital PCR with a high multiplexing approach

Richard Dannebaum, Nyaradzo Dzvova, Raymond-John Abayan, Maria Gencoglu, Olga Mikhaylichenko, Anthony Henriquez, Severine Margeridon, Monica Herrera

Bio-Rad Laboratories, Inc. – Clinical Diagnostics Group (Pleasanton, CA)

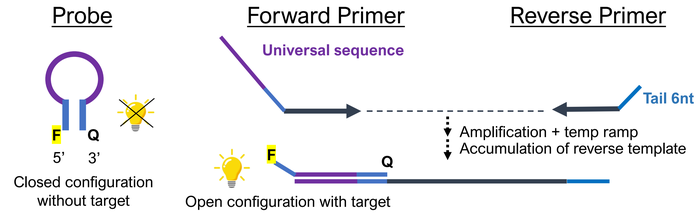
Abstract

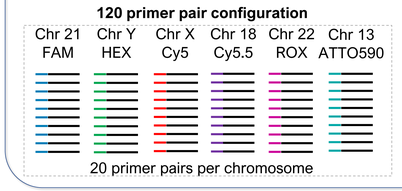
The ability to test circulating cell-free DNA in the bloodstream has been a revolution for the development of liquid biopsies for clinical applications. Likewise, digital PCR (dPCR) offers a fast and cost-effective means to target and accurately count DNA molecules of interest. However, due to the limited amount of material available in a single tube of blood, high levels of precision are difficult to achieve without sufficiently large numbers of multiplexed PCR assays. In this study, we demonstrate the ability to achieve high levels of precision and accuracy for CNV detection in dPCR by combining highly multiplex assays with a universal probe approach.

Introduction

This study focused on the use of universal dPCR probes, which enable several target molecules to be combined in the same digital channel. In contrast to hydrolysis probes (e.g. Taqman), which require a probe to be defined for each PCR target, universal probes are hybridization probes that have a reduced cost and decreased manufacturing complexity while also maintaining high specificity in the assay. Highly multiplexed dPCR assays were tested on the QX600 of Droplet Digital PCR (ddPCR) instrument. Contrived and clinical samples were tested to evaluate the performance of the multiplexed primers. Furthermore, an in-silico model was constructed to evaluate the performance of multiplexed assays for CNV detection applications.

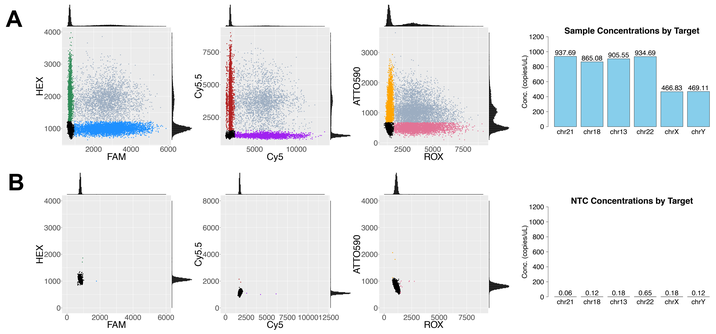
High Multiplexing and Universal Probe selection Beacons





- Multiplex primer pairs targeting the same chromosome are combined in a single fluorescent channel using a single universal probe
- Universal probes, combined with a universal sequence on the forward primer, are opened after the PCR reaction. Increased concentration of the reverse primer accumulates template complementary to the probe
- 20 primer pairs were combined in each channel, reaching a 120-plex ddPCR reaction mixture

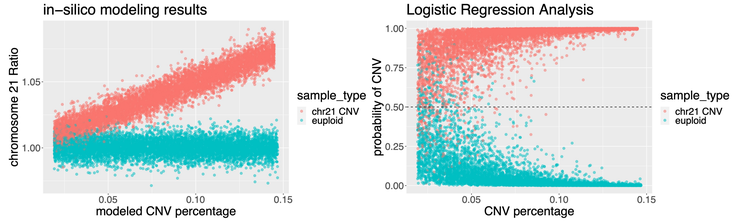
120-Plex Proof of Concept on QX600



A) Male cfDNA was analyzed on the QX600 ddPCR instrument using a 120-plex with universal probes across 6 fluorescence channels. Positive and negative droplets are well separated for most channels and concentrations match the expectation for a male sample.

B) A No Template Control (NTC) well was also run and as negative control and demonstrates minimal cross-reactivity between the 120-plex primers.

In-silico model for CNV performance

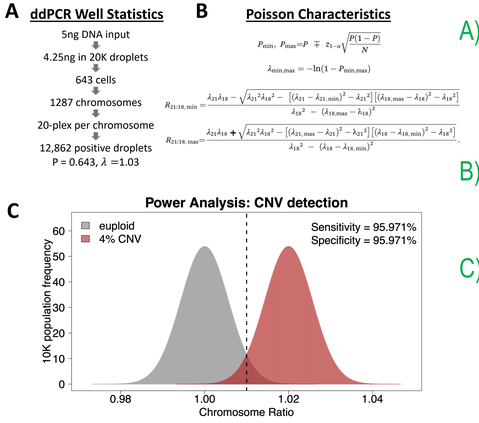


In-silico Model Cumulative Performance

CNV cut-off	TP	TN	FP	FN	sensitivity	specificity
>= 4%	5832	5861	35	35	99.40%	99.41%
>= 6%	4766	4797	5	9	99.81%	99.90%
>= 8%	3668	3707	2	3	99.92%	99.95%

- 14,000 samples were modeled at various DNA input amounts (3-7 ng) and at various sample mixtures (2-15%). Each sample was modeled using 3 replicate wells, with 6-channels per well.
- A logistic regression model was derived to differentiate samples based on CNV status. Using this model, >99% sensitivity and specificity was achieved for samples with at least 4% CNV, and > 99.9% for samples with at least 8% CNV

Mathematical Modeling to optimize CNV performance



A) Diagram of expected ddPCR statistics using 5ng of input per well. On average, 85% of sample input is captured into droplets. Using a 120-plex, 64% of total droplets are expected to fluoresce in each channel

B) Poisson sampling equations used to model confidence intervals, capturing the upper and lower limits expected between two channels (Reference 1).

C) Power Analysis for CNV limit of detection in ddpcr. Utilizing 3 replicate wells with 5ng of DNA input and combining all 6 channels together, it is possible to achieve a 95% LOD for CNV detection for CNV mixtures as low as 4%.

Conclusions

- Seventy-eight universal digital PCR probe combinations were analyzed, (13 universal probes combined with 6 dyes). Out of these probes, 6 were selected as best performers based on probe-probe interactions, ddPCR fluorescence amplitude, lack of ddPCR positivity in no template controls, and ddPCR concentration accuracy.
- Using these universal probes, 120 PCR assays were combined in a single reaction achieving precise measurements of wild-type copies and low positivity in no template controls.
- Using an in-silico model, we demonstrate the potential for digital PCR applications to detect CNV in sample mixtures as low as 4% with greater than 95% sensitivity. initial data sets with contrived and clinical samples support this modeling. additional testing is ongoing.

References

1) Dube S, Qin J, Ramakrishnan R (2008) Mathematical Analysis of Copy Number Variation in a DNA Sample Using Digital PCR on a Nanofluidic Device. PLoS ONE 3(8): e2876. doi:10.1371/journal.pone.0002876