High Resolution Copy Number Analysis Using Droplet Digital PCR

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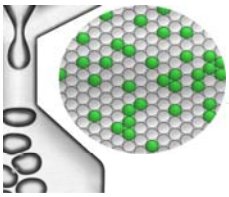
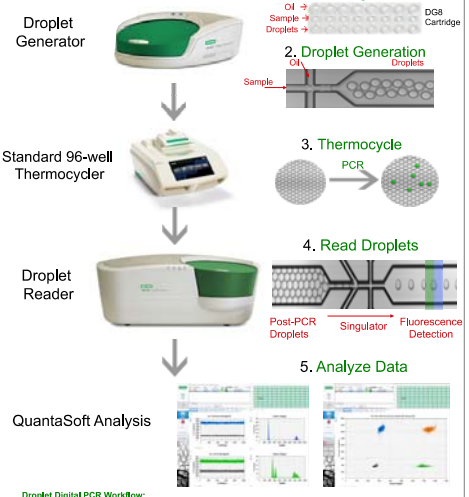


I. Abstract

Copy number variations (CNVs) from single-gene to segmental duplications are critically dynamic features of the human genome. Altered copy number status is associated with several conditions, including autism spectrum disorder, schizophrenia, and multiple cancers. SNP-based microarrays, aCGH, and qPCR typically lack the sensitivity and fine quantitative discrimination required for resolution of higher copy number states. This is particularly true for heterogeneous samples, like somatic mosaicism or tumor biopsies, where only a small fraction of cells might have copy number alterations. In addition, better tools to quickly and cost-effectively validate copy number alterations discovered by next-generation sequencing are in demand.

Droplet digital PCR (ddPCR) enables accurate and reproducible copy number determination using a simple, cost-effective workflow amenable to high throughput. Using single-well ddPCR, consecutive copy number states can be distinguished between samples of 5 and 6 copy number at 95% confidence levels. Here we use validated Bio-Rad ddPCR CNV assays to discriminate copy number status of multiple genes with low to high copy number states. The evaluation of higher-order oncogene amplifications, such as MYC, MET, and FGFR2, are demonstrated. The ability to resolve a 5% difference (1.05-fold) in copy number will be demonstrated using mosaic or admixed samples heterogeneous for copy number status. This has immediate implications for researchers interested in reproducibly resolving somatic mosaicism, tumor heterogeneity, or other applications where fine quantitative discrimination is essential.

II. QX200 ddPCR Workflow



Droplet Digital PCR Workflow:

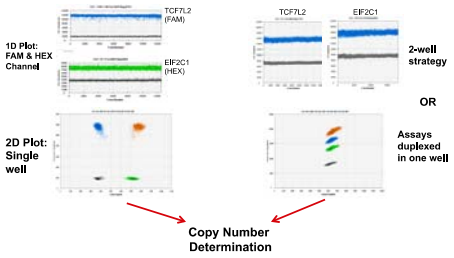
1. Samples and oil are loaded into an 8-channel droplet generator cartridge.
2. In ~2 min, 8 samples are converted into 8 sets of 20,000 droplets.
3. Amplification to end-point (~40 cycles) occurs in a conventional thermal cycler.
4. The Droplet Reader sips droplets from each well and streams them single-file past a two-color detector at the rate of ~1000 per second. 32 wells can be read in one hour.
5. Droplets are assigned as positive or negative based on their fluorescence amplitude. The fraction of positive droplets in each channel is used to calculate the concentration of the target and reference DNA sequences.

III. Methods and Tools for CNV ddPCR

- Template DNA is digested with restriction enzymes (10 U enzyme/ug human genomic DNA) prior to ddPCR to separate tandem copies and improve template accessibility
- CNV ddPCR uses duplexed target (FAM) and reference (HEX) Taqman assays in a 20uL reaction containing ddPCR master mix (ddPCR Supermix for Probes or Droplet PCR Supermix).
- Bio-Rad now offers 385 fully-validated CNV ddPCR target assays for digital assessment of important cancer and neurological targets. 4 reference assays (TERT, RPP30, EIF2C1, & AP3B1) are available.

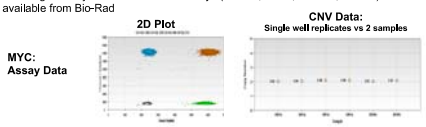
CNV ddPCR by Probe or EvaGreen Assays

- CNV ddPCR can be performed by either using duplexed fluorescent probe target and reference assays, or by visualizing primer pair amplified products using EvaGreen
- In this example, copy number of TCF7L2, a transcription factor implicated in cancer and diabetes, is assessed using either probe or EvaGreen-based strategies. EIF2C1 reference assay is used.



385 Fully-Validated PrimePCR CNV ddPCR Assays

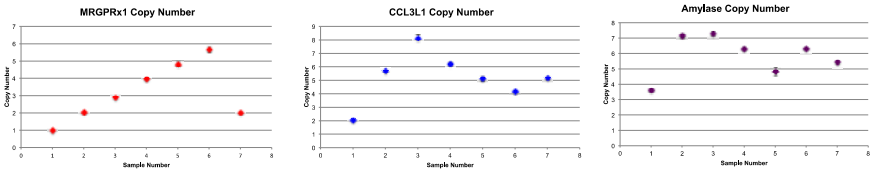
- Example wet-lab validation data for PrimePCR ddPCR CNV Assays
- 385 Target Assays and 4 Reference Assays (RPP30, TERT, EIF2C1, AP3B1) now available from Bio-Rad



IV. Results:

The data below illustrates the precision and diversity of CNV analysis enabled by the QX200

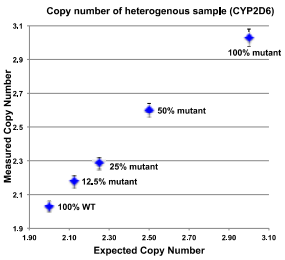
A. DISCRIMINATION BETWEEN CONSECUTIVE COPY NUMBER STATES: HOMOGENEOUS SAMPLES

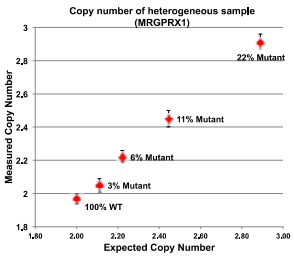


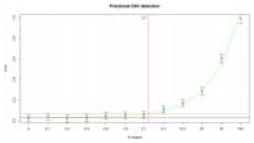
Discrimination of consecutive copy number states of three multi-copy loci in a panel of 7 human genomic (Coriell) DNA samples. A 20% difference in copy number (5 vs 6) is easily detected. Target assays (FAM) were duplexed to distinct reference assays (HEX) (RPP30, ultraconserved Ch5 region, and ultraconserved Ch1 region, respectively). 95% confidence intervals are shown. Each data point represents three merged technical replicates.

B. COPY NUMBER VARIATION IN HETEROGENEOUS SAMPLES

- Detection of copy number changes in heterogeneous samples is important for detecting tumor heterogeneity, somatic mosaicism, and prenatal diagnostics
- Admixed samples were created by titrating high CN sample into wild-type (CN 2) sample. CYP2D6 or MRGPRX1 copy number is assayed.
- As low as a 4% difference in copy number is easily detectable.
- Demonstrated resolution near the instrument's theoretical limit of detection







- Red dotted line: Predicted LoD for CNV
- Red solid line: predicted minimum dilution factor
- Plot represents CYP2D6 data

CYP2D6: Heterogeneous sample copy number detection

Percent Mutant	Expected CN	Measured CN	95% CI	Resolution Achieved
0.00%	2.00	2.03	(2.00-2.06)	
12.5%	2.13	2.18	(2.14-2.21)	6.5%
25%	2.25	2.29	(2.25-2.32)	12.5%
50%	2.50	2.6	(2.56-2.64)	25%
100%	3.0	3.03	(2.98-3.08)	50%

MRGPRX1: Heterogeneous sample copy number detection

Percent Mutant	Expected CN	Measured CN	95% CI	Resolution Achieved
0.00%	2.00	1.97	(1.94-2.00)	
2.8%	2.11	2.05	(2.01-2.09)	4%
6%	2.22	2.22	(2.19-2.26)	13%
11%	2.44	2.45	(2.4-2.5)	24%
22%	2.9	2.91	(2.87-2.96)	48%
44%	3.78	3.87	(3.81-3.93)	96%
100%	6.0	5.94	(5.84-6.04)	202%

C. SCREENING SOMATIC COPY NUMBER ALTERATIONS IN CANCER

- Detecting somatic copy number alterations (SCNAs) is important for studying cancer etiology & making treatment decisions
- Validated CNV ddPCR assays were used to screen a panel of cancer cell lines for 8 commonly amplified oncogenes
- Example Data showing oncogene SCNA within and across samples. 25 ng sample used per well, duplicate wells.

Assays

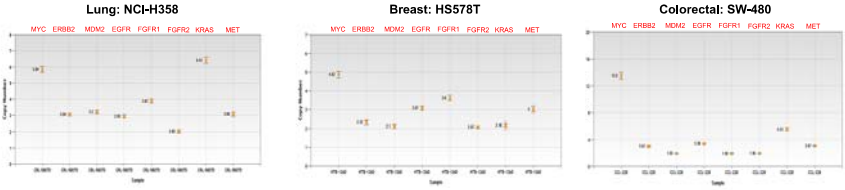
Target Assay	Location
MYC	8q24
ERBB2	17q12
MDM2	12q15
EGFR	7p11
FGFR1	8p11
FGFR2	10q26
KRAS	12p12
MET	7q31

Ref Assay	Location
EIF2C1	1p34
RPP30	10q23
AP3B1	5q14

Samples

Sample	ATCC	Cell Line	Type	Subtype
1	CRL-5807D	NCI-H358	Lung	Non-small cell
2	CRL-5802D	NCI-H1299	Lung	Non-small cell
3	CCL-256D	NCI-H126	Lung	Adenocarcinoma
4	CRL-5868D	NCI-H1395	Lung	Adenocarcinoma
5	HTB-126D	HS578T	Breast	Carcinoma
6	HTB-22D	MCF7	Breast	Adenocarcinoma
7	HTB-38D	HT-29	Colorectal	Adenocarcinoma
8	CCL-228	SW-480	Colorectal	Adenocarcinoma
9	CCL-227	SW-620	Colorectal	Adenocarcinoma
10	HTB-10	SK-N-MC	Neuroepithelial	
11		Raji	Lymphoma	
12		18916	WT	

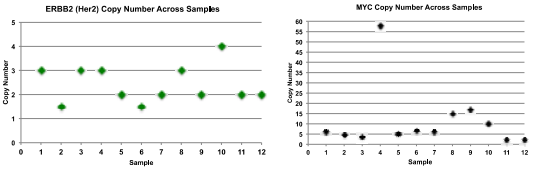
Oncogene CN Determination within Samples



WT: 18916

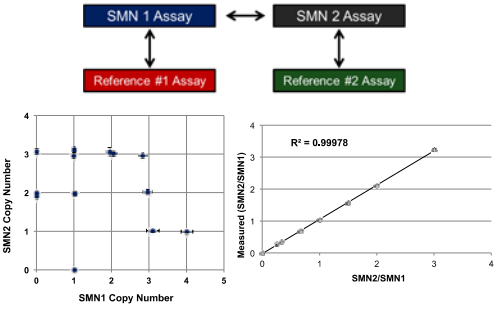
Oncogene CN Determination Across Samples

- Samples 12 is wild-type
- ERBB2 (Her2) & MYC amplification detected in multiple cancers



D. SMA: ALLELE-SPECIFIC COPY NUMBER MEASUREMENTS

- Spinal muscular atrophy (SMA) is an autosomal recessive disease caused by the loss of SMN1.
- The severity of the disease is determined by SMN2 copy number.
- SMN1 and SMN2 share 99% nucleotide identity.
- 13 samples screened
- Demonstrates specificity of single nucleotide allele-specific CNV assays in droplets.



V. Conclusions:

- **QX200 ddPCR enables precise copy number discrimination in a flexible, high throughput format**
- **Copy number analysis of diverse cancer, pathological, and research samples is possible**
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