Highly Multiplexed SNP Discrimination Utilizing Digital PCR

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Introduction

Digital PCR (dPCR) enables highly sensitive detection of single-nucleotide polymorphism (SNPs) by splitting a single reaction into thousands of partitions. Here, we demonstrate the ability to detect SNPs and quantify minor allele fraction down to 4% from liquid biopsy in a highly multiplexed reaction using ChromaCode's High Definition PCR (HDPCR™) technology[1]. While conventional multiplexing limits the number of detectible targets by the number of available fluorescence detection channels on the instrument, HDPCR enables 10X the number of detectible targets per color channel in a single dPCR reaction[2]. This prototype assay serves as a proof of concept for the wide variety of applications in which HDPCR can highly multiplex SNPs on a dPCR instrument.

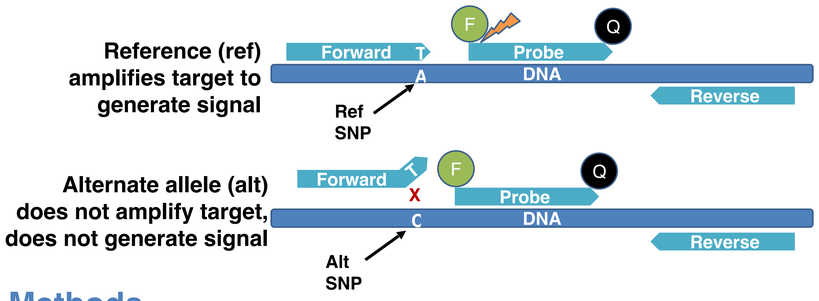

Figure 1 (left). ARMS[3] primer design to characterize SNPs. When reference allele is present, DNA is amplified, and the hydrolyzed probe (F) generates signal as it is released from the quencher (Q). Designs were screened for sensitivity and specificity for reference alleles (no amplification of alternate).

Methods

The assay leverages HDPCR on the Bio-Rad QXONE instrument. Cell line DNA with known genotypes from a variety of ethnicities were sheared to ~150 bp to simulate cell-free DNA from liquid biopsy. Contrived samples were made by spiking target sheared DNA (to simulate fetal fraction) into background sheared DNA (to simulate maternal fraction) with a known genotype to generate informative SNP combinations at a range of fractions from 0-100%. The multiplexed assay was run utilizing a minimum of seven replicates per SNP target and minor allele fraction. Data were analyzed using ChromaCode's proprietary cloud-based algorithm. Informative SNPs were called (Fig 3.) and fetal fraction for each SNP was calculated and benchmarked against the contrived percentage (Table 2 and Fig. 4). Correlations were calculated to determine the accuracy of the SNP calling assays.

SNP	REF	ALT	Channel	Level	Maternal example	Paternal example	Fetal example
1	G	T	FAM	*1*	0|0	0|0	0|0
2	A	G	FAM	*2*	1|1	1|1	1|1
3	A	G	HEX	*1*	0|1	0|1	0|0
4	T	C	HEX	*2*	0|1	0|1	0|1
5	G	A	Cy5	*2*	0|1	0|1	1|1
6	A	C	Cy5.5	*2*	1|1	0|1	1|1
7	T	C	Cy5.5	*3*	1|1	0|1	0|1

Genotype	Legend
0|0	Reference (REF)
0|1, 1|0	Heterozygous (HET)
1|1	Alternate (ALT)

Informative:	
Fetus (FET)	Maternal (MAT)
HET	ALT

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Table 1 (above). Example SNPs and legend. Liquid biopsy from a pregnant woman contains 3 haplotypes: Maternal transmitted, maternal non-transmitted, and paternal. A SNP is informative for fetal fraction when the fetus carries a copy of a paternal allele not shared by the mother (heterozygous fetus, homozygous mother).

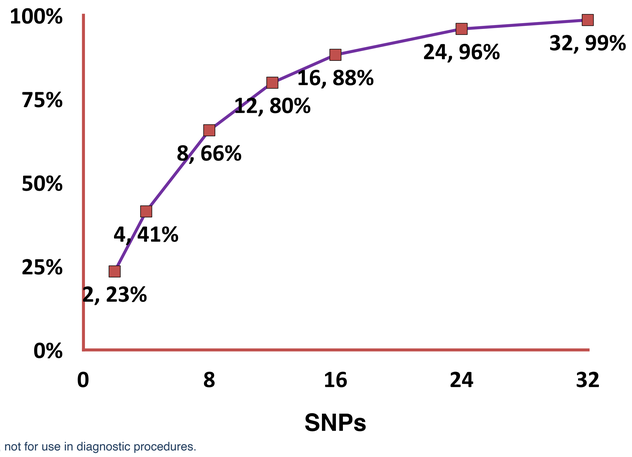

Figure 2. (right). Copies of Chromosome Y can be used to determine fetal fraction in male fetuses, but not female. The percentage of female fetuses for which fetal fraction can be determined is a function of the number of targets in the assay.

Results

A total of 20 SNPs were multiplexed across a total of four fluorescence channels. Nine SNPs were found to be highly sensitive and specific to the allele of interest. Correlation to spike in percentage were high for seven SNPs, with the majority greater than 95%. For each assay, accurate quantification of the fetal fraction down to 4% was achieved.

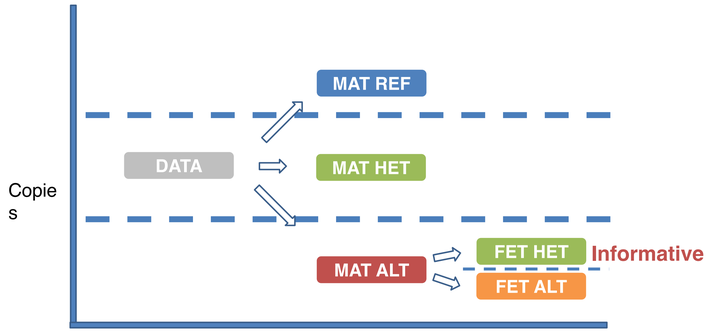

Figure 3 (above). Data flow for SNP calling. Positive partition count is dominated by the maternal genotype, which is called first for each SNP. Those SNPs with maternal alternate genotype are then called as either fetal heterozygous (informative for fetal fraction) or fetal alternate (non-informative) based on empirically determined thresholds.

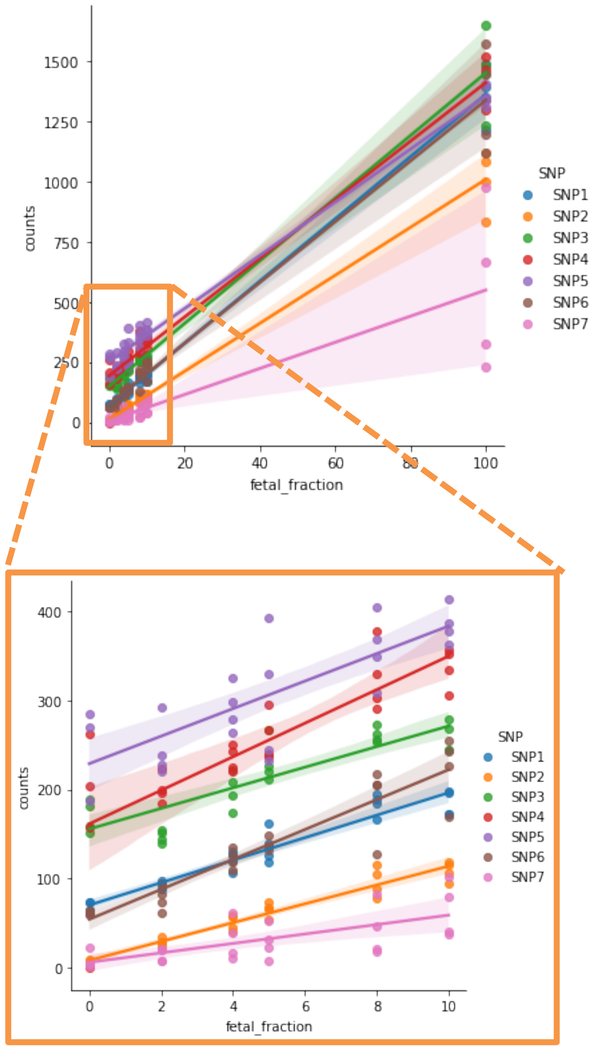

Figure 4 (right). Spike-in series for each SNP demonstrate correlation between positive partition counts and contrived fetal fraction.

SNP	channel	level	R-squared
SNP1	1	1i	0.992
SNP2	1	2i	0.984
SNP3	2	1i	0.981
SNP4	2	2i	0.988
SNP5	3	2i	0.972
SNP6	4	2i	0.971
SNP7	4	3i	0.721

Table 2. Partition counts for 6 out of 7 SNPs demonstrated high correlation with fetal fraction in contrived samples.

Conclusion

The ability to accurately detect SNPs in a highly multiplexed manner utilizing dPCR is demonstrated herein. The applications of this technology include fetal fraction determination for NIPT, single nucleotide, and other variants for oncology, and forensics. Highly multiplexed dPCR assays for these applications will enable reduced cost and increased throughput over NGS based methods.

References

1. Jacky, Lucien, et al. "Robust Multichannel Encoding for Highly Multiplexed Quantitative PCR." Analytical Chemistry 93.9 (2021): 4208-4216.
2. Jacky, Lucien, et al. "Virtual-Partition Digital PCR for High-Precision Chromosomal Counting Applications." Analytical Chemistry (2021).
3. Little, Stephen. "Amplification-refractory mutation system (ARMS) analysis of point mutations." Current Protocols in Human Genetics (1995): 9.8.1-9.8.12