

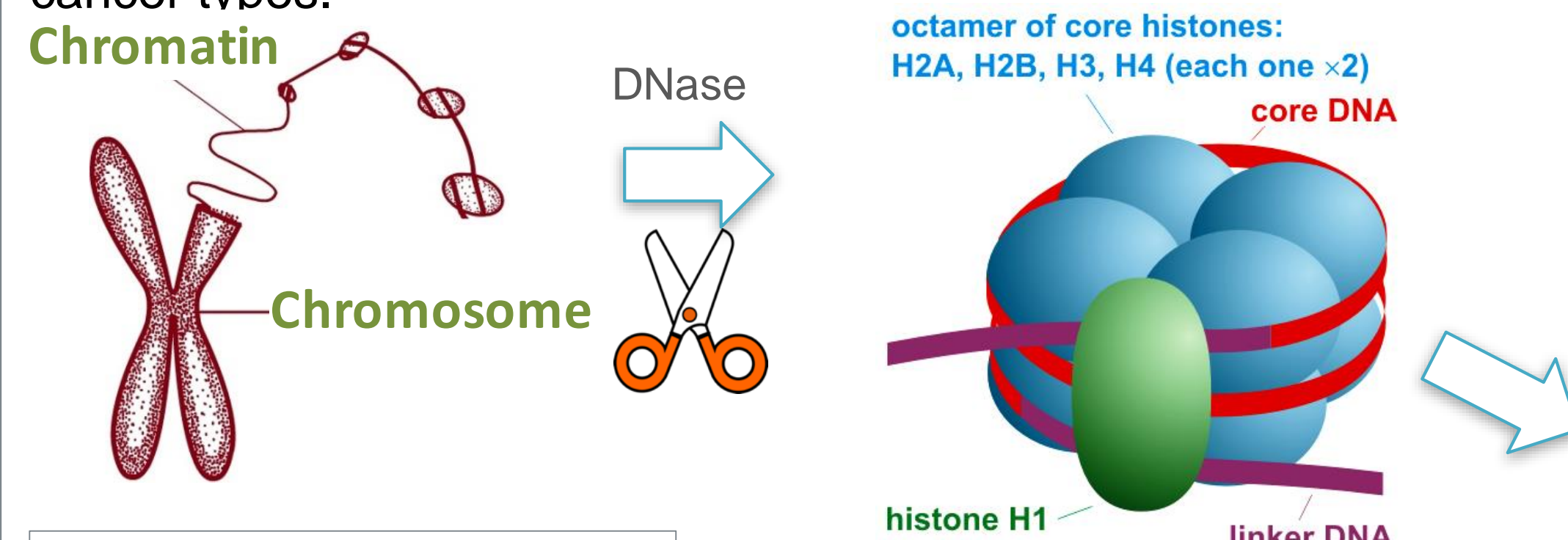


# Comparison between mutation profiles of paired whole blood and cfDNA samples

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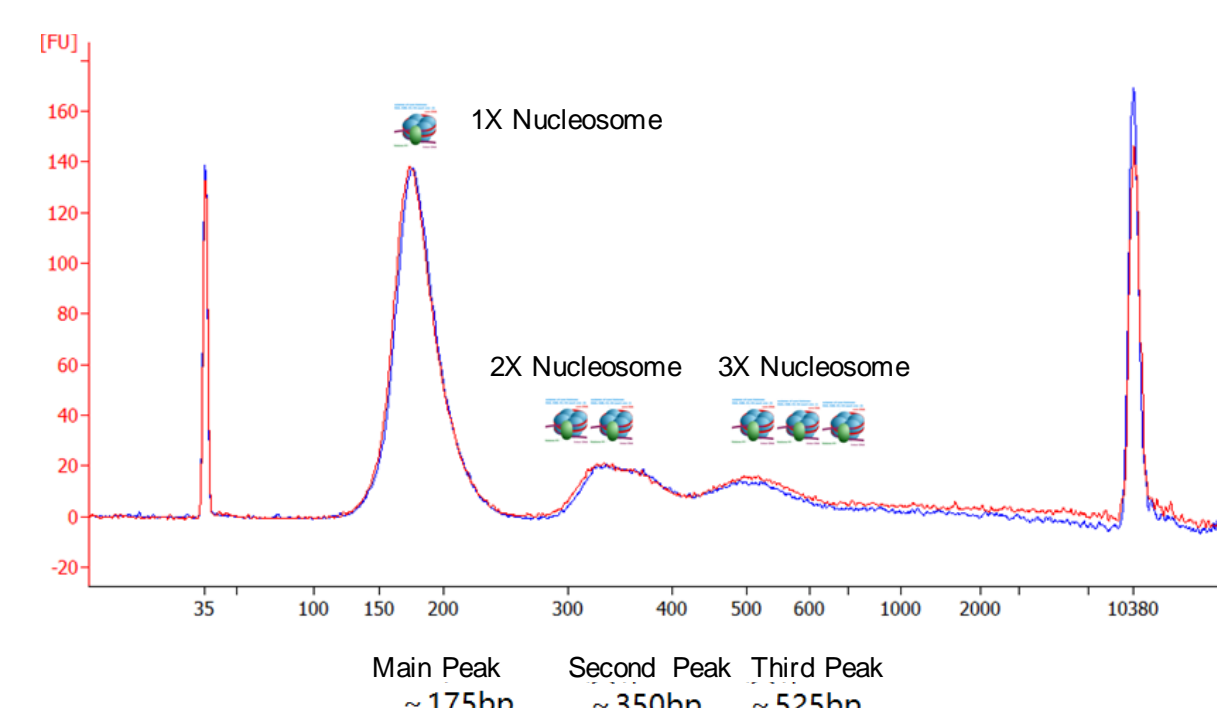
## Introduction

Liquid biopsies are increasingly becoming a tool of choice for researching cancer detection and monitoring. Cell-free DNA or cfDNA is simply small fragments of DNA circulating in bodily fluids. It is also known as circulating cell-free DNA (ccfDNA), circulating tumor DNA (ctDNA) and cell free-fetal DNA (cffDNA). Next-Gen Sequencing of cfDNA is coming into maturity as a non-invasive method to identify mutational profiles in many cancer types.



Apoptotic or necrotic cell death results in near-complete digestion of native chromatin from normal cell, tumor or fetus.

Each 160-175 bp DNA is wrapped ~1.67 times around one nucleosome. These protein-bound DNA fragments preferentially survive digestion and are released into the circulation, and can be recovered from peripheral blood plasma as cfDNA.



Typical cfDNA peaks characterized by Agilent 2100 Bioanalyzer, with a main peak at 175 bp, second and third peaks at 350 and 525 bp.

A big question is how do you separate a germ line variant from a tumor variant. Understanding the difference in a patient sample can give a more thorough understanding of a variant that can be used to study a cancer type. An easy solution is to compare germ line variants from whole blood genomic DNA (gDNA). This would couple easily with plasma sample collection as plasma can be directly separated from a single sample point.

Here we describe a simple method to isolate both gDNA and cfDNA from a donor blood sample and discuss the automation of both extractions. We show the efficacy of cfDNA as reliable biomarker analysis tool by comparing mutations in cfDNA vs whole blood. The study determines if the difference between tumor and germ line mutations can be established and the limitations.

Due to larger volumes necessary to extract sufficient concentrations of cfDNA, automation can assist in the extraction. The Apostle MiniMax™ High Efficiency Cell-Free DNA (cfDNA) extraction kit was automated on the Biomek i-Series. It provides equal recovery of cfDNA as a manual extraction with much less hands on-time. The kit used in the study to extract whole blood, GenFind V3, has also been automated on the Biomek i-Series; allowing for reduced hands with the same quality results as a manual extraction



## Methods

### Sample Preparation

Blood was collected from 3 donors in EDTA tubes. After the blood was delivered to the site 10ng of Horizon Multiplex I cfDNA Reference Standard Set was added to 1/2 of the blood collected from each donor. The whole blood was then centrifuged twice, for 2,000xg for 10 minutes and supernatant was moved to a fresh tube and for centrifuged a second time at 6,000xg for 30 minutes. The second supernatant was used for cfDNA extractions; and the lower phase was used for genomic DNA extractions. Both phases were then stored at -80°C. The plasma was thawed at 37°C. Half of plasma from each donor that did not have 10ng of Horizon Multiplex I cfDNA Reference Standard Set had 200ng of Horizon Multiplex I cfDNA Reference Standard Set added to it as a positive control. The plasma samples were then processed using Apostle MiniMax™. Due to the lag in separating the whole blood from the plasma the cfDNA was size-selected using SPRIselect for 200bp size. The blood was thawed at room temperature. The genomic DNA was extracted from blood using GenFind V3.

Sample Name	Sample Type	Treatment
cfDNA_508plus	Plasma	10 ng of Horizon Multiplex I cfDNA Reference Standard Set added prior to plasma isolation
cfDNA_509plus		
cfDNA_510plus		
Blood_508plus	Lower Phase of Blood	10 ng of Horizon Multiplex I cfDNA Reference Standard Set added prior to plasma isolation
Blood_509plus		
Blood_510plus		
cfDNA_508C	Plasma	200 ng of Horizon Multiplex I cfDNA Reference Standard Set added after plasma isolation
cfDNA_509C		
cfDNA_510C		
Blood_508C	Lower Phase of Blood	Nothing added
Blood_509C		
Blood_510C		

### Library Preparation

Following extraction of gDNA and cfDNA, the DNA was quantified using Quant-it Picogreen Assay for the gDNA and using Kappa hgQuant kit for the cfDNA. Library construction was done using 100 ng of DNA with the Swift Biosciences Accel-NGS 2S Hyb DNA Library Kit, following the library construction genes were enriched prior to sequencing using the Swift Biosciences Pan-Cancer Hyb Panel. The libraries were sequenced on a NextSeq 550.

### Sequencing Analysis

The sequencing was analyzed using Illumina BaseSpace; the reads were aligned to the genes enriched in the Swift Biosciences Pan-Cancer Hyb Panel by using the BWA enrichment application.

## Similar coverage of reads for cfDNA and gDNA

Sample Name+	Total Aligned Reads	Percent Aligned Reads	Targeted Aligned Reads	Read Enrichment	Padded Target Aligned Reads	Padded Read Enrichment
Blood_508C	58,813,981	100%	33333485	57%	35026658	60%
Blood_508plus	22,512,598	100%	13011447	58%	13655481	61%
Blood_509C	17,747,434	100%	11593225	65%	12237570	69%
Blood_509plus	18,678,850	100%	12752101	68%	13427976	72%
Blood_510C	20,504,009	100%	12370374	60%	12973986	63%
Blood_510plus	16,742,634	100%	11320236	68%	11894055	71%
cfDNA_508C	26,162,565	100%	17505630	67%	17734578	68%
cfDNA_508plus	30,039,905	100%	18265618	61%	18567893	62%
cfDNA_509C	33,127,967	100%	18915218	57%	19157799	58%
cfDNA_509plus	8,382,099	100%	5973231	71%	6154497	73%
cfDNA_510C	32,309,659	100%	19874851	62%	20109025	62%
cfDNA_510plus	12,908,033	100%	8773743	68%	8907368	69%

The table above shows the number of reads that were aligned and the number of reads that were aligned to the targeted genes. The samples containing gDNA had on average 25 million reads and the cfDNA had on average 24 million reads.

Sample Name	Mean Region Coverage Depth	Uniformity of Coverage (Pct > 0.2 mean)	Target Coverage at 1X	Target Coverage at 10X	Target Coverage at 20X	Target Coverage at 50X
Blood_508C	4489.2	99.70%	100.00%	100.00%	100.00%	100.00%
Blood_508plus	1739.2	90.40%	100.00%	100.00%	100.00%	99.90%
Blood_509C	1550.5	88.80%	100.00%	100.00%	100.00%	99.80%
Blood_509plus	1703.6	86.50%	100.00%	100.00%	100.00%	99.80%
Blood_510C	1651.4	91.10%	100.00%	100.00%	100.00%	99.80%
Blood_510plus	1513	87.40%	100.00%	100.00%	100.00%	99.80%
cfDNA_508C	2315.1	92.40%	100.00%	100.00%	100.00%	99.90%
cfDNA_508plus	2432.1	90.50%	100.00%	100.00%	100.00%	99.90%
cfDNA_509C	2523.4	99.70%	100.00%	100.00%	100.00%	100.00%
cfDNA_509plus	801.6	85.10%	100.00%	100.00%	99.90%	99.20%
cfDNA_510C	2634.5	93.70%	100.00%	100.00%	100.00%	100.00%
cfDNA_510plus	1166.7	88.90%	100.00%	100.00%	99.90%	99.70%

The table above shows the mean coverage depth for each sample. The average coverage for both gDNA and cfDNA was ~91%. At least 99% of the genes had coverage at 50x. This indicates that the variants can be called with high confidence.

## Variant differences are observed between cfDNA and gDNA

Three of 8 the variants were found in the spiked Horizon Multiplex I cfDNA Reference Standard Set. This most likely is due to the low amounts of horizon cfDNA sample compared to the overall DNA extracted. None of the Horizon cfDNA was detected in the gDNA sequences. The sequences could have not been detected due either to low amounts and insufficient sequencing coverage or the sequences were not present in the lower phase of blood after centrifugation.

Reference Standard Gene	Reference Standard Variant	Reference Standard Coordinate	Reference Standard mutation Type	cfDNA_508C	cfDNA_510C
KRAS	G12D	25398284	C>T	cfDNA_508C	cfDNA_510C
NRAS	Q61K	1.15E+08	G>T	cfDNA_509C	
NRAS	A59T	1.15E+08	C>T	cfDNA_509plus	

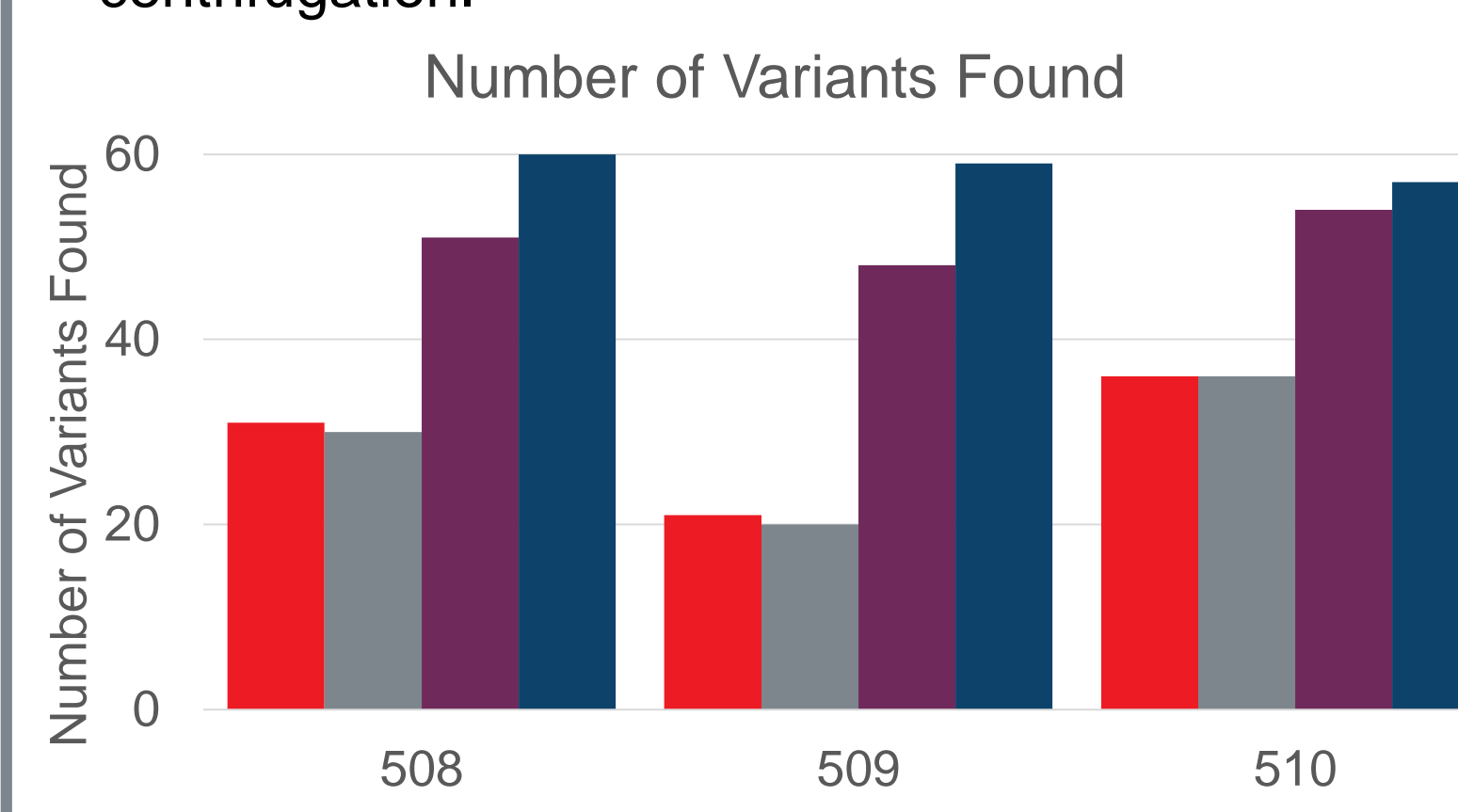


Figure 1

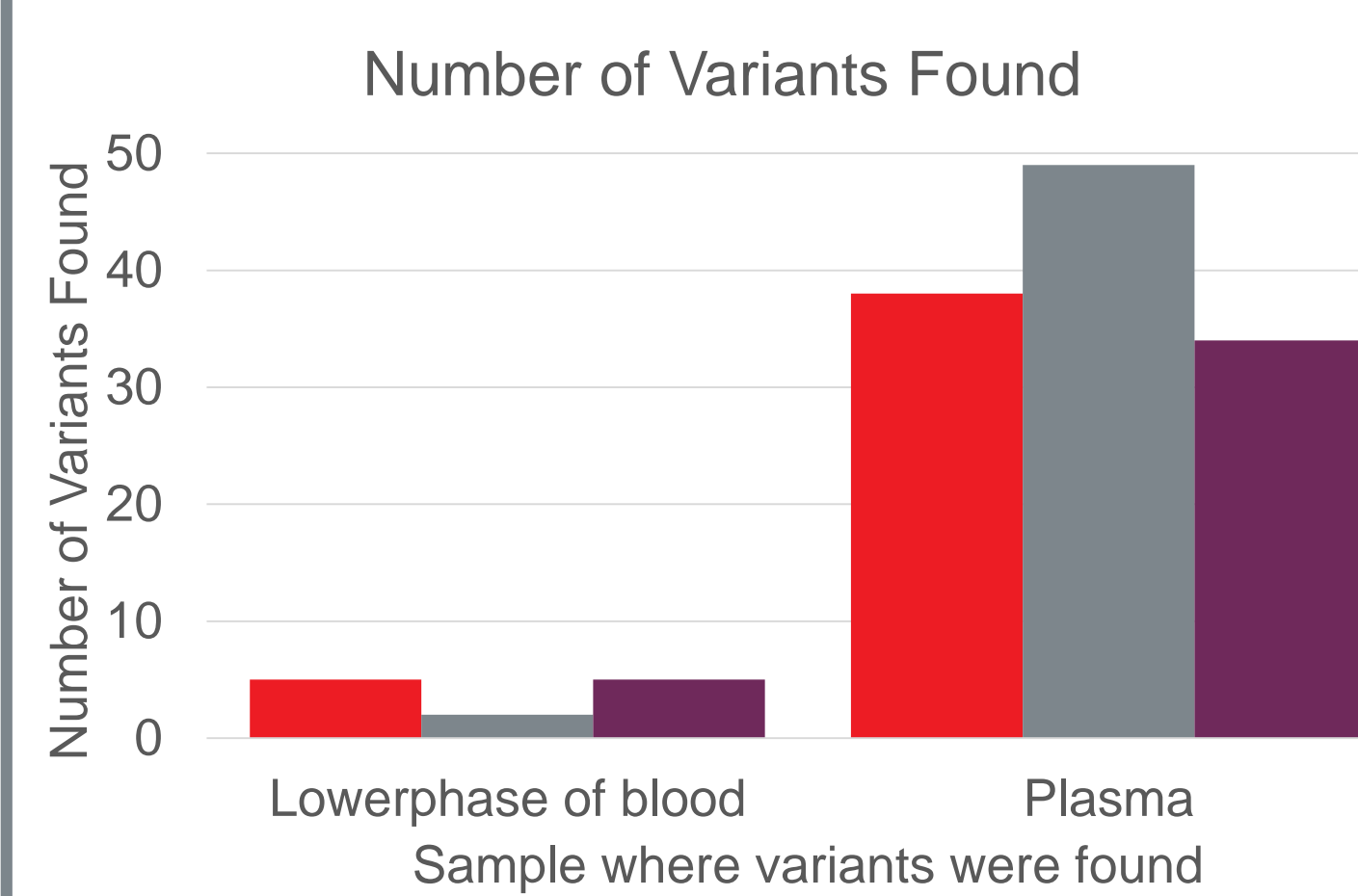


Figure 2

Sample Name	SNVs	SNV Het/Hom Ratio	SNV Ts/Tv Ratio	Indels	Indel Het/Hom Ratio
Blood_508C	500	1.4	2.2	117	3
Blood_508plus	500	1.4	2.2	181	6
Blood_509C	588	2.4	2.5	201	9.6
Blood_509plus	590	2.4	2.5	196	8.8
Blood_510C	501	1.7	2.3	197	8
Blood_510plus	502	1.8	2.2	183	7.3
cfDNA_508C	955	8.3	2.6	426	25.6
cfDNA_508plus	636	6.8	2.3	275	18.6
cfDNA_509C	963	8.2	2.6	434	26.1
cfDNA_509plus	717	8.3	2.5	304	32.8
cfDNA_510C	944	8.3	2.6	455	31.5
cfDNA_510plus	669	7.4	2.4	303	29.3

Because we were unable to detect all of the spiked cfDNA, we extended our analysis to all variants. The total number of SNVs and insertion and deletions can be seen in the table above. For all three of the donors the average total number of variants found in gDNA was 29 and for cfDNA was 55 (Figure 1). To further this analysis we examined the number of variants that were only found in cfDNA or only found in gDNA. The number of variants only found in gDNA was 10 fold less than the number of variants only found in cfDNA for all three donors (figure 2). Because the majority of the mutations were found in both, this could indicate that any variant found only in the cfDNA could be candidates for ctDNA analysis.

## Conclusions

- Variants found only in cfDNA could be used as an initial screen for ctDNA analysis
- Apostle MiniMax™ and GenFind V3 can be used together to get a picture of germ line variants and cfDNA, potential ctDNA, variants
- These results show that a holistic view of a cancer subject can be gained by using one sample source, whole blood

