Improving the sensitivity of somatic mutation detection in serum and plasma-derived circulating free DNA (cfDNA) in metastatic breast cancer patients.

Introduction

Analysis of plasma circulating free DNA (cfDNA) has been shown to accurately predict the mutational status of oncogenes in metastatic colon cancer [1], lung cancer [2] and breast cancer [3]. Analysis of tumour mutational status in plasma cfDNA from patients with metastatic cancer has been used to monitor disease response and outcome [1], and to detect the emergence of therapy resistant clones [3].

Several studies have also shown that serum cfDNA can be used to monitor tumour dynamics in patients with various solid cancers [1-4] and to identify mutations associated with acquired drug resistance in advanced cancers.

Lee et al in 2001 [5] showed serum contains a higher amount of cfDNA than plasma and most cfDNA in serum samples is generated during the process of clotting in the original collection tube most likely due to white cell blood (WBC) lysis. Although serum samples, due to their high cfDNA content, are not ideal to monitor the mutational status by cfDNA analysis, they can still be used if a highly sensitive technique, such as digital PCR (dPCR) is used for this purpose.

Droplet Digital PCR (dPCR) is a method for performing digital PCR based on water-oil emulsion droplet technology. A sample is fractionated into 20,000 droplets, and PCR amplification of the template molecules occurs in each individual droplet providing high sensitivity.

The higher cfDNA concentration in serum is due to high molecular weight genomic DNA (gDNA) contamination and the ability to isolate low molecular weight cfDNA would be useful in order to improve the sensitivity to detect somatic mutation in serum-derived cfDNA.

Aims

The aim of this study is to set up a reliable and reproducible method to isolate low molecular weight cfDNA from serum in order to improve the sensitivity of detection of somatic mutations in metastatic Breast Cancer (MBC) Patients.
A secondary objective of this study was to compare automated versus manual cfDNA extraction using two different systems, the circulating nucleic acid (cna) kit from Qiagen and the MagMax cfDNA extraction kit on an automated system from Thermo.

Material and Methods

Study Cohort

13 paired plasma and serum samples from MBC patients from the Royal Marsden Hospital sponsored trial "*Plasma DNA as a surrogate for breast cancer phenotype study*".

Processing of plasma and serum

Blood collected in EDTA K2 tubes was processed within two hours of sample collection and centrifuged at 1600 rpm for 20 min, with plasma stored at -80°C until DNA extraction. Serum samples were allowed to clot at room temperature for approximately 45 minutes. Subsequently and within 4 hours of collection, they were centrifuged for 15 minutes at 1600 g to separate the serum from the clot. Serum was stored at -80°C until DNA extraction.

Manual extraction of circulating DNA from plasma and serum

DNA was extracted from 2 ml of plasma or serum using the QIAamp circulating nucleic acid (cna) kit (Qiagen) according to the manufacturer's instructions. The DNA was eluted into buffer AVE in two independent 50µl aliquots (elution 1 and elution 2) and stored at -20°C.

Automated extraction of circulating DNA from plasma

DNA was extracted from 2ml of plasma using the automated KingFisher Flex purification system (Thermo) using the MagMax cfDNA extraction kit (Thermo) as per manufacturer instructions. DNA was eluted either on 50µl or 100µl elution buffer and stored at -20°C.

cfDNA quantification from serum and plasma

DNA isolated from plasma or serum was quantified on a Bio-Rad QX-200 droplet ddPCR using RNase P as the reference gene. One µl of eluate was added to a digital PCR reaction containing 10 µl ddPCR Supermix for probes (Bio-Rad) and 1 µl of TaqMan Copy Number Reference Assay, human, RNase P (Life Technologies) on a total volume of 20 µl. The reaction was partitioned into ~14,000 droplets per sample in a QX-200 droplet generator according to manufacturer's instructions. Emulsified PCR reactions were run on a 96 well plate on a G-Storm GS4 thermal

cycler incubating the plates at 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec, followed by 10 min incubation at 98°C. The temperature ramp increment was 2.5°C/sec for all steps. Plates were read on a Bio-Rad QX-200 droplet reader using QuantaSoft v1.7.4 software from Bio-Rad. At least two negative control wells with no DNA were included in every run. The amount of amplifiable RNase P DNA was calculated using the Poisson distribution in QuantaSoft.

Size analysis of cfDNA extracted from plasma and serum

1 µl of each eluate of plasma or serum extracted cfDNA was analysed using Agilent High Sensitivity DNA Kit as per manufacturer instructions on a 2100 Bioanalyzer.

Results and Discussion

cfDNA content of plasma versus serum

cfDNA from elution 1 and elution 2 of plasma and serum were quantified using ddPCR. DNA concentration was significantly higher in serum as compared to plasma DNA for both elutions (Table 1), confirming the data already reported in literature. We recovered considerable amounts of cfDNA from the second serum elution, particularly for samples 7018, 1161, 1198 and 1207. On the other hand, the amount of recovered cfDNA from the second elution of the plasma samples was negligible (Table 1).

Sample	Total RNase P copies / ul	ng / ul	Total ng	Sample	Total RNase P copies / ul	ng / ul	Total ng	Sample	Total RNase P copies / ul	ng / ul	Total ng	Sample	Total RNase P copies / ul	ng / ul	Total ng
7013 SR 1	2580	8.514	425.7	7013 SR 2	2580	8.514	425.7	7013 P1	32	0.1056	5.28	7013 P2	14.6	0.04818	2.409
7015 SR 1	1408	4.6464	232.32	7015 SR 2	1720	5.676	283.8	7015 P1	60	0.198	9.9	7015 P2	14	0.0462	2.31
7016 SR 1	3200	10.56	528	7016 SR 2	2520	8.316	415.8	7016 P1	208	0.6864	34.32	7016 P2	56	0.1848	9.24
7018 SR 1	406	1.3398	66.99	7018 SR 2	1016	3.3528	167.64	7018 P1	68	0.2244	11.22	7018 P2	10.4	0.03432	1.716
7017 SR 1	2160	7.128	356.4	7017 SR 2	2440	8.052	402.6	7017 P1	74	0.2442	12.21	7017 P2	32	0.1056	5.28
1162 SR 1	866	2.8578	142.89	1162 SR 2	1494	4.9302	246.51	1162 P1	120	0.396	19.8	1162 P2	13.8	0.04554	2.277
7009 SR 1	570	1.881	94.05	7009 SR 2	1178	3.8874	194.37	7009 P1	50	0.165	8.25	7009 P2	8.2	0.02706	1.353
1161 SR 1	2180	7.194	359.7	1161 SR 2	5760	19.008	950.4	1161 P1	152	0.5016	25.08	1161 P2	40	0.132	6.6
1208 SR 1	1168	3.8544	192.72	1208 SR 2	1350	4.455	222.75	1208 P1	36	0.1188	5.94	1208 P2	24	0.0792	3.96
1198 SR 1	1480	4.884	244.2	1198 SR 2	3200	10.56	528	1198 P1	54	0.1782	8.91	1198 P2	11.2	0.03696	1.848
7024 SR 1	638	2.1054	105.27	7024 SR 2	1620	5.346	267.3	7024 P1	18	0.0594	2.97	7024 P2	3.2	0.01056	0.528
1207 SR 1	1152	3.8016	190.08	1207 SR 2	4080	13.464	673.2	1207 P1	132	0.4356	21.78	1207 P2	56	0.1848	9.24
7030 SR1	1176	3.8808	194.04	7030 SR2	45.6	0.15048	7.524	7030 P1	15	0.0495	2.475	7030 P2	12.8	0.04224	2.112

Table 1 – SR1: serum sample first elution; SR2: SR2 serum sample second elution; P1: plasma sample first elution (25µl +25µl); P2 plasma sample second elution (25µl +25µl).

We did not find any correlation between the amount of cfDNA recovered from the paired plasma and serum samples (Figure 1) either by comparing the total number of copies of RNase P as detected by ddPCR or the total amount of cfDNA

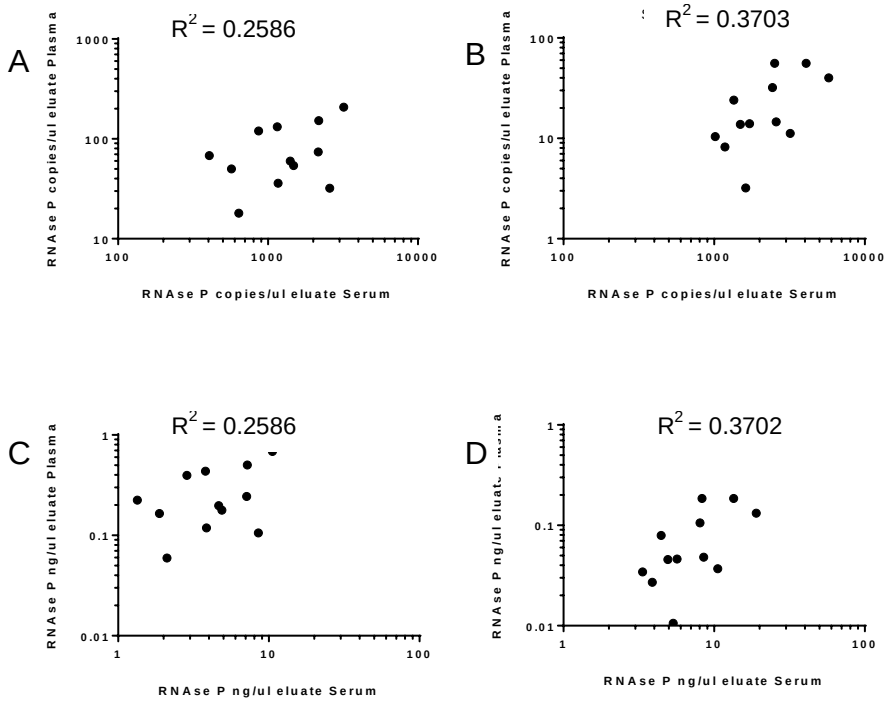


Figure 1: Correlation of RNAse P copies/µl between first eluate plasma vs serum (A); RNAse P copies/µl between second eluate plasma vs serum (B); RNAse P ng/µl between first eluate plasma vs serum (C); RNAse P ng/µl between second eluate plasma vs serum (D).

To further investigate the size profile of cfDNA fragments extracted from plasma and serum samples, we analysed them using an Agilent's 2100 Bioanalyzer. Figure 2 shows the Bionalyzer traces for the two elutions of two of the samples analysed.

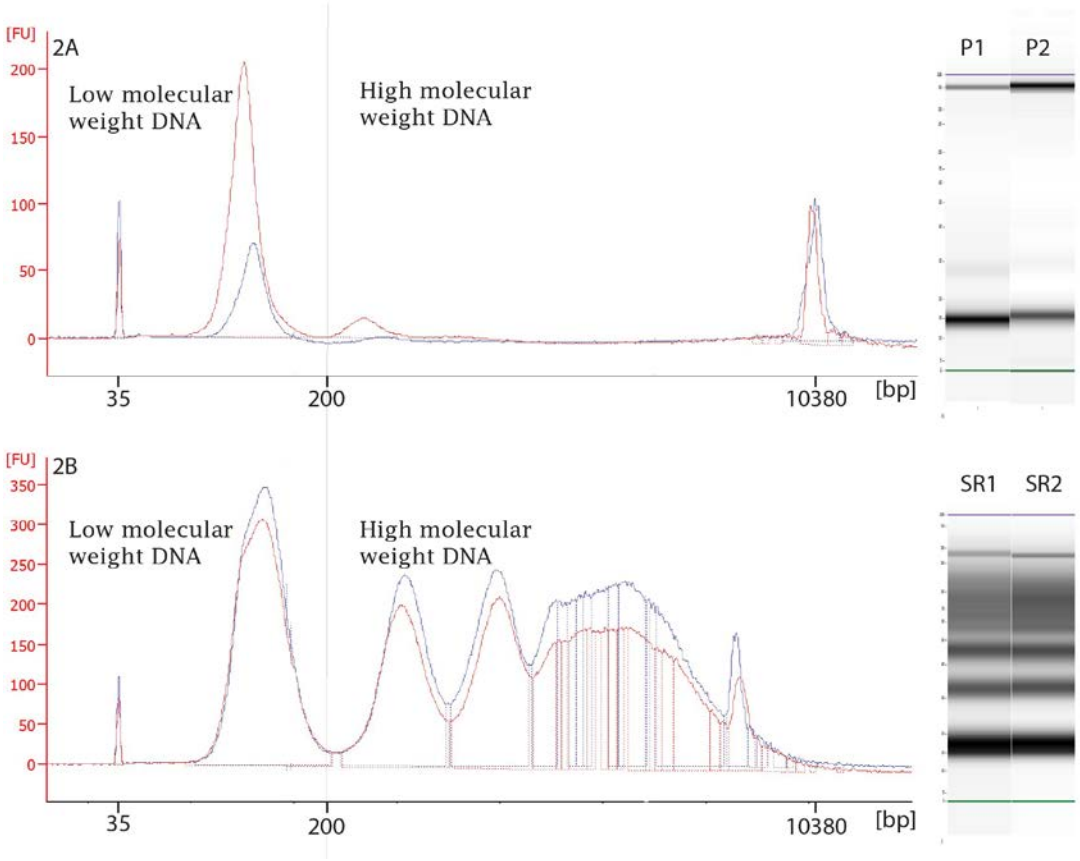


Figure 2: A) Analysis of plasma DNA first elution (red) vs second elution (blue). Both samples show a significant presence of low molecular weight DNA (<200bp) which would correspond to the expected size for cfDNA. **B)** Analysis of serum DNA first elution (red) vs second elution (blue). The electropherogram and the gel show the presence of high amount of DNA, both for the first and the second elution. The high molecular weight DNA concentration is significantly higher for the first elution in this sample.

We further investigated the size distribution fragment of cfDNA extracted from plasma and serum by running a paired plasma and serum sample on the Agilent's 2100 BioAnalyzer (Figure 3).

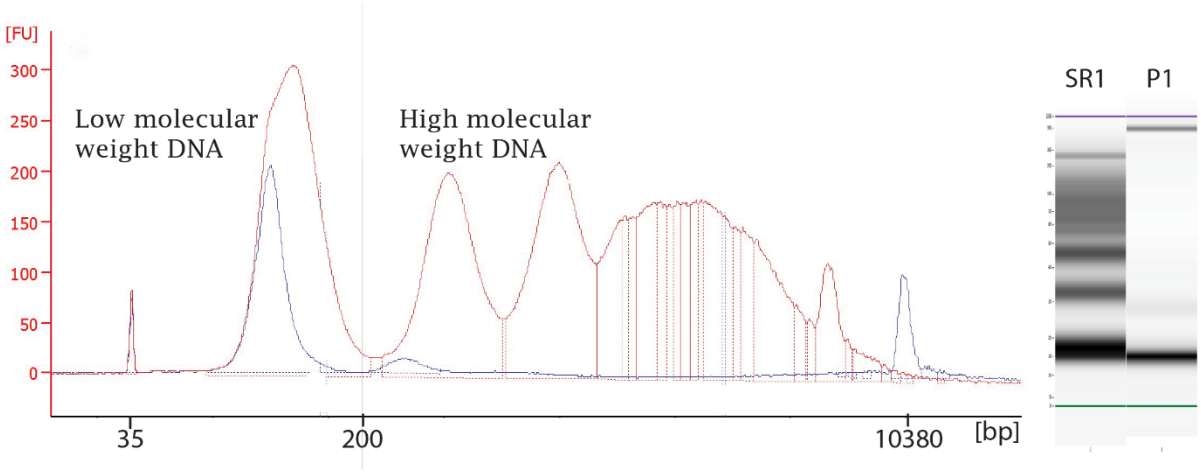


Figure 3: Analysis of serum DNA first elution (red) vs plasma DNA first elution (blue).

This analysis highlighted the differences between plasma and serum cfDNA fragment size and content. Serum samples showed an increased amount of high molecular weight DNA most probably due to contamination from high molecular gDNA released from WBC lysis during clotting. Serum cfDNA also showed a higher content of the low molecular weight fraction cfDNA highly likely due to further fragmentation of gDNA during the sample processing.

Mutational Analysis of cfDNA extracted from plasma and serum

In MBC patients a small proportion of the cfDNA in circulation is tumour released DNA, circulating tumour DNA (ctDNA) that contains genomic aberrations like mutations and copy number variations. In order to asses if there was any difference in the sensitivity of detection of ctDNA between plasma and serum we analysed mutations by means of multiplex dPCR (mdPCR).

We developed and used mdPCR assays to hotspot mutations in *PIK3CA* and *ESR1* (Table 2) in ctDNA extracted from paired plasma and serum samples.

PIK3CA	***ESR1***
PIK3CA c.1624 G>A; pE542K	*c.1138G>C; pE380Q*
PIK3CA c.1633 G>A; pE545K	*c.1387T>C; pS463P*
PIK3CA c.3140 A>T; pH1047L	*c.1607T>G; pL536R*
PIK3CA c.3140 A>G; pH1047R	*c.1610A>G; pY537C*
	c.1609T>A; pY537N
	c.1610A>C; pY537S
	c.1613A>G; pD538G

Table 2: *PIK3CA* and *ESR1* analysed mutations.

PIK3CA mutational status

We analysed 0.25, 0.5 or 1 ml of plasma or serum equivalent by mdPCR to test for the most commonly occurring *PIK3CA* hotspot mutations in 12 paired plasma and serum samples from our cohort (Table 3).

Sample	Fraction mutant	Fraction 95% CI low	Fraction 95%CI high	mutant copies per ml	Mutation
7013 P 1	0	0	0	0	
7015 P 1	0	0	0	0	
7016 P 1	0	0	0	0	
7018 P 1	0.0544482	-0.007176	0.1278096	18.4852565	c.1633G>A; p.E545K
7017 P 1	0	0	0	0	
1162 P 1	0	0	0	0	
7009 P 1	0	0	0	0	
1161 P 1	0	0	0	0	
1208 P 1	0	0	0	0	
1198 P 1	0	0	0	0	
7024 P 1	0	0	0	0	
1207 P 1	0	0	0	0	

0.25 mL equivalent (1 well)

Sample	Fraction mutant	Fraction 95% CI low	Fraction 95%CI high	mutant copies per ml	Mutation
7013 SR 1	0	0	0	0	
7015 SR 1	0	0	0	0	
7016 SR 1	0	0	0	0	
7018 SR 1	0	0	0	0	c.1633G>A; p.E545K
7017 SR 1	0	0	0	0	
1162 SR 1	0	0	0	0	
7009 SR 1	0	0	0	0	
1161 SR1	0	0	0	0	
1208 SR 1	0	0	0	0	
1198 SR 1	0	0	0	0	
7024 SR 1	0	0	0	0	
1207 SR 1	0	0	0	0	

0.25 mL equivalent (1 well)

A

Sample	Fraction mutant	Fraction 95% CI low	Fraction 95%CI high	mutant copies per ml	Mutation
7013 P 1	0	0	0	0	
7015 P 1	0	0	0	0	
7016 P 1	0	0	0	0	
7018 P 1	0.0111338	0.0034162	0.019015	21.3611313	c.1633G>A; p.E545K
7017 P 1	0	0	0	0	
1162 P 1	0	0	0	0	
7009 P 1	0	0	0	0	
1161 P 1	0	0	0	0	
1208 P 1	0	0	0	0	
1198 P 1	0	0	0	0	
7024 P 1	0	0	0	0	
1207 P 1	0	0	0	0	

0.5 mL equivalent (2 wells)

Sample	Fraction mutant	Fraction 95% CI low	Fraction 95%CI high	mutant copies per ml	Mutation
7013 SR 1	0	0	0	0	
7015 SR 1	0	0	0	0	
7016 SR 1	0	0	0	0	
7018 SR 1	0	0	0	0	c.1633G>A; p.E545K
7017 SR 1	0	0	0	0	
1162 SR 1	0	0	0	0	
7009 SR 1	0	0	0	0	
1161 SR 1	0	0	0	0	
1208 SR 1	0	0	0	0	
1198 SR 1	0	0	0	0	
7024 SR 1	0	0	0	0	
1207 SR 1	0	0	0	0	

0.5 mL equivalent (2 wells)

B

Sample	Fraction mutant	Fraction 95% CI low	Fraction 95%CI high	mutant copies per ml	Mutation
7013 P 1	0	0	0	0	
7015 P 1	0	0	0	0	
7016 P 1	0	0	0	0	
7017 P 1	0	0	0	0	
7018 P 2	0.0126859	0.0055033	0.0200258	21.9461177	c.1633G>A; p.E545K
1162 P 1	0	0	0	0	
7009 P 1	0	0	0	0	
1161 P 1	0	0	0	0	
1208 P 1	0	0	0	0	
1198 P 1	0	0	0	0	
7024 P 1	0	0	0	0	
1207 P 1	0	0	0	0	

1 mL equivalent (4 wells)

Sample	Fraction mutant	Fraction 95% CI low	Fraction 95%CI high	mutant copies per ml	Mutation
7013 P 1	0	0	0	0	
7015 P 1	0	0	0	0	
7016 P 1	0	0	0	0	
7017 P 1	0	0	0	0	
7018 SR 2	0.00056819	0.00011356	0.00102333	12.5740428	c.1633G>A; p.E545K
1162 P 1	0	0	0	0	
7009 P 1	0	0	0	0	
1161 P 1	0	0	0	0	
1208 P 1	0	0	0	0	
1198 P 1	0	0	0	0	
7024 P 1	0	0	0	0	
1207 P 1	0	0	0	0	

1 mL equivalent (4 wells)

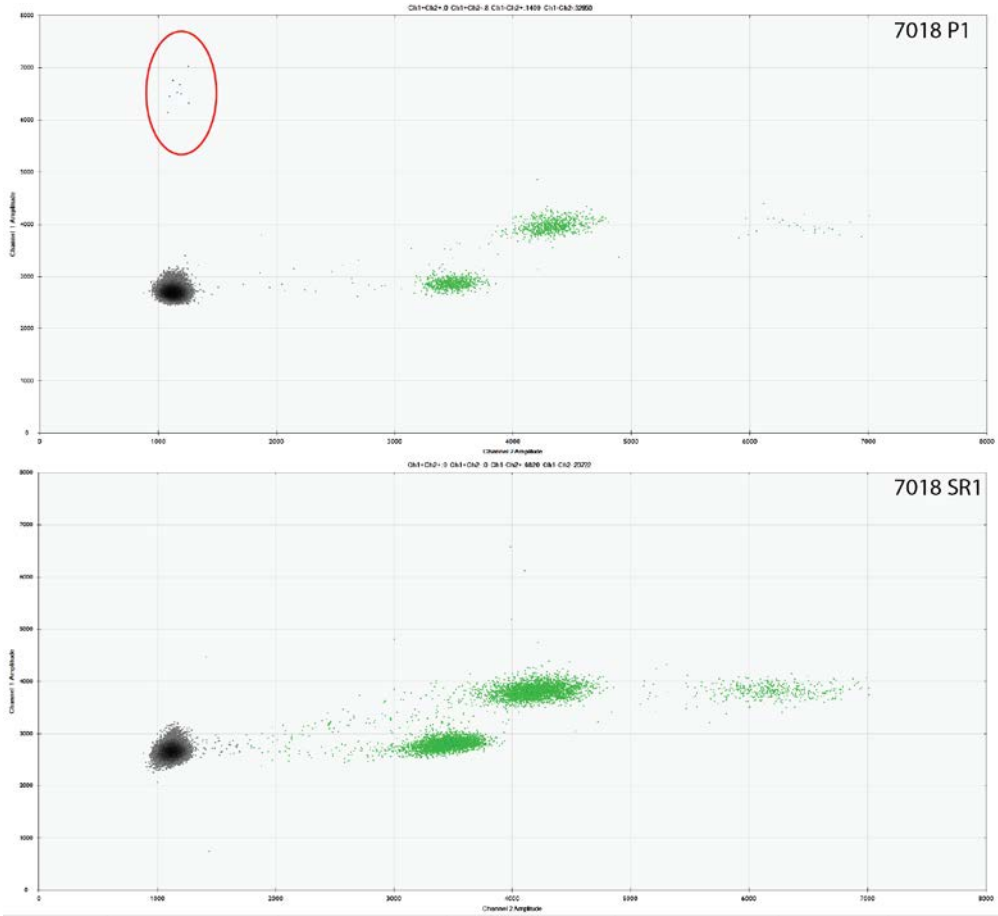
C

Table 3: *PIK3CA* mutation analysis using 0.25ml plasma or serum equivalent (A); using 0.5ml plasma or serum equivalent (B); using 1ml plasma or serum equivalent (C).

We only found 1 sample, 7018, harbouring a *PIK3CA* mutation on exon 9, *PIK3CA c.1633 G>A; pE545K*. We were able to detect this mutation in as low as 0.25 ml plasma equivalent at roughly the same Allele Frequency (AF) or mutant copies per ml in all the plasma volumes analysed.

However, in order to detect the same mutation in serum extracted ctDNA we had to use a higher volume of serum equivalent. We were able to detect the same mutation, albeit at a lower AF and mutant copies per ml in 1 ml serum equivalent. This result would point to the hypothesis that serum ctDNA might be more difficult to detect due to gDNA contamination from WBC.

When we analysed the dPCR plots (Figure 3) we found that plasma-derived ctDNA containing the *PIK3CA* mutation was clearly observed, while in serum-derived ctDNA the mutation was not detected.



Sample	SumFAM+ VIC+	Sum FAM+ VIC-	Sum FAM- VIC+	Sum FAM- VIC-	FAM conc	DNA conc
7018 P1	0	8	703	32953	0.0002	0.0213
7018 SR1	0	0	3402	23737	0	0.1339

Figure 4: The dPCR plots showing the mutant allele as blue droplets (FAM channel), while the green droplets represent wild type allele (VIC channel). Black droplets represent those droplets devoid of any DNA. Top panel, plasma, bottom panel serum.

ESR1 mutational status

For *ESR1* mutational analysis, we assayed 0.5 ml of plasma or serum equivalent on a mdPCR to test for the most commonly occurring hotspot mutations in 12 paired plasma and serum samples from our cohort (Table 4)

Sample	Fraction mutant	Fraction 95% CI low	Fraction 95%CI high	mutant copies per ml	Mutation
7013 P1	0	0	0	0	
7015 P1	0	0	0	0	
7016 P1	0.0138	0.0093	0.0183	126.7398	c.1613A>G; p.D538G
7017 P1	0	0	0	0	
7018 P1	0	0	0	0	
1162 P1	0.0157	0.0084	0.0232	55.7789	c.1613A>G; p.D538G
7009 P1	0	0	0	0	
1161 P1	0	0	0	0	
1208 P1	0	0	0	0	
1198 P1	0	0	0	0	
7024 P1	0	0	0	0	
1207 P1	0	0	0	0	

0.5ml equivalent (2 wells)

Sample	Fraction mutant	Fraction 95% CI low	Fraction 95%CI high	mutant copies per ml	Mutation
7013 SR 1	0	0	0	0	
7015 SR 1	0	0	0	0	
7016 SR 1	0.0004	0.0001	0.0007	32.7370	c.1613A>G; p.D538G
7017 SR 1	0	0	0	0	
7018 SR 1	0	0	0	0	
1162 SR 1	0.0012	0.0003	0.0021	28.0886	c.1613A>G; p.D538G
7009 SR 1	0	0	0	0	
1161 SR 1	0	0	0	0	
1208 SR 1	0	0	0	0	
1198 SR 1	0	0	0	0	
7024 SR 1	0	0	0	0	
1207 SR 1	0	0	0	0	

0.5ml equivalent (2 wells)

Sample	Fraction mutant	Fraction 95% CI low	Fraction 95%CI high	mutant copies per ml	Mutation
7013 SR 2	0	0	0	0	
7015 SR 2	0	0	0	0	
7016 SR 2	0.0005	0.00026045	0.00084	51.51154	c.1613A>G; p.D538G
7017 SR 2	0	0	0	0	
7018 SR 2	0	0	0	0	
1162 SR 2	0.000454	5.7295E-05	0.000871	17.4076	c.1613A>G; p.D538G
7009 SR 2	0	0	0	0	
1161 SR 2	0	0	0	0	
1208 SR 2	0	0	0	0	
1198 SR 2	0	0	0	0	
7024 SR 2	0	0	0	0	
1207 SR 2	0	0	0	0	

0.5ml equivalent (2 wells)

Sample	Fraction mutant	Fraction 95% CI low	Fraction 95% CI high	mutant copies per ml	Mutation
7013 SR 1	0	0	0	0	
7015 SR 1	0	0	0	0	
7016 P2	0.007708623	0.003151709	0.012326034	49.56334971	c.1610A>C, p.Y537S
7017 SR 1	0	0	0	0	
7018 SR 1	0	0	0	0	
1162 SR 1	0	0	0	0	
7009 SR 1	0	0	0	0	
1161 SR 1	0	0	0	0	
1208 SR 1	0	0	0	0	
1198 SR 1	0	0	0	0	
7024 SR 1	0	0	0	0	
1207 SR 1	0	0	0	0	

0.5ml equivalent (2 wells)

Sample	Fraction mutant	Fraction 95% CI low	Fraction 95% CI high	mutant copies per ml	Mutation
7013 SR 1	0	0	0	0	
7015 SR 1	0	0	0	0	
7016 SR2	0.00030	0.00001	0.00059	23.02696	c.1610A>C, p.Y537S
7017 SR 1	0	0	0	0	
7018 SR 1	0	0	0	0	
1162 SR 1	0	0	0	0	
7009 SR 1	0	0	0	0	
1161 SR 1	0	0	0	0	
1208 SR 1	0	0	0	0	
1198 SR 1	0	0	0	0	
7024 SR 1	0	0	0	0	
1207 SR 1	0	0	0	0	

0.5ml equivalent (3 wells)

Table 4: *ESR1* mutation analysis using 0.5ml plasma or serum equivalent.

We found two samples with *ESR1* mutations, 7016 and 1162. Sample 7016 was a polyclonal sample with two different ligand binding domain (LBD) mutations, *c.1613A>G, p.D538G* and *c.1610A>C, p.Y537S*, while sample 1162 was a monoclonal sample with one LBD mutation *c.1613A>G, p.D538G*.

The AF and mutant copies per ml of all the mutations detected were higher on the plasma samples as compared to the serum samples which will suggest less gDNA contamination on plasma as compared to serum.

Removal of high molecular gDNA contamination from serum samples

In order to improve the detection of mutant alleles and eliminate the burden of germline DNA from serum samples we set out to test two different methods to accomplish this. Firstly we tried a yet unreleased sizing kit from Analityk Jena and a fast spin of the serum before processing for cfDNA extraction.

Removing high molecular weight DNA by sizing columns

We carried out a size fragmentation on four different serum samples using a beta kit from Analytik Jena according to manufacturer instructions, selecting 200 bp fragments as the high molecular weight cut off (Figure 4)

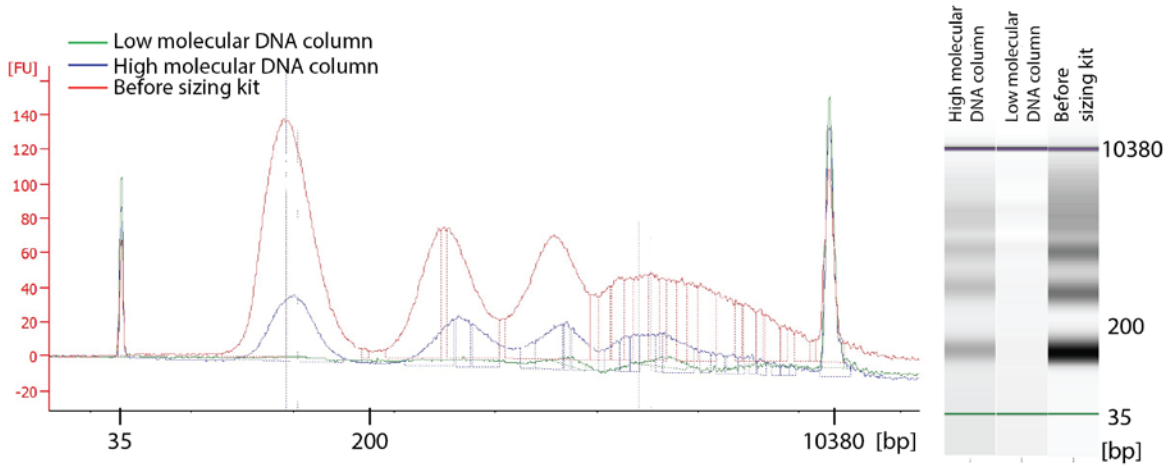


Figure 5: The fragmentation analysis was performed on a 2100 Bioanalyzer after the sizing kit process: the red line shows the DNA before being processed, the blue line the DNA after being processed through the column that allows only the high molecular weight DNA to be eluted, the green line shows the DNA after being processed through the column that allows only the low molecular weight DNA to be eluted**.**

Unfortunately on our hands, there was no size fragmentation using the kit and both elutions showed the same profile. Besides the completely lack of fragment selection, the column caused a significant loss of DNA (Figure 4, red line vs blue line).

Removing high molecular weight DNA by fast spin of serum

The total separation of high molecular weight DNA in serum samples remains a challenge. In order to remove high molecular weight DNA from serum cfDNA samples, 4 samples were spun down for 10 min 4ºC and 14000 rpm before cfDNA extraction. 100µl of elution buffer was used for both the first and the second elution. The pellet obtained, was also used to extract DNA to assess how much DNA was pelleted along the process.

Performing a fast spin allows almost complete DNA recovery in the first elution. Indeed, a negligible DNA amount was obtained from the second elution, although no significant differences were observed in the DNA concentration from fast spun serum and serum (Table 4).

Sample	Total RNase P copies/ul	ng/ul	Total ng	Sample	Total RNase P copies/ul	ng/ul	Total ng
1162 SR1	1162	3.8346	191.73	1162 SR2	98	0.3234	16.17
7013 SR1	2460	8.118	405.9	7013 SR2	132	0.4356	21.78
7015 SR1	2620	8.646	432.3	7015 SR2	136	0.4488	22.44
7030 SR1	294	0.9702	48.51	7030 SR2	11.4	0.03762	1.881
1162 fSR1	1312	4.3296	216.48	1162 fSR2	34	0.1122	5.61
7013 fSR1	2820	9.306	465.3	7013 fSR2	30	0.099	4.95
7015 fSR1	2260	7.458	372.9	7015 fSR2	98	0.3234	16.17
7030 fSR1	298	0.9834	49.17	7030 fSR2	4	0.0132	0.66
1162 pellet	50	0.165	8.25				
7013 pellet	44	0.1452	7.26				
7015 pellet	264	0.8712	43.56				
7030 pellet	52	0.1716	8.58				

Table 5: SR1: serum sample first elution; SR2: SR2 serum sample second elution; fSR1: fast spin serum first elution; fSR2: fast spin serum sample second elution.

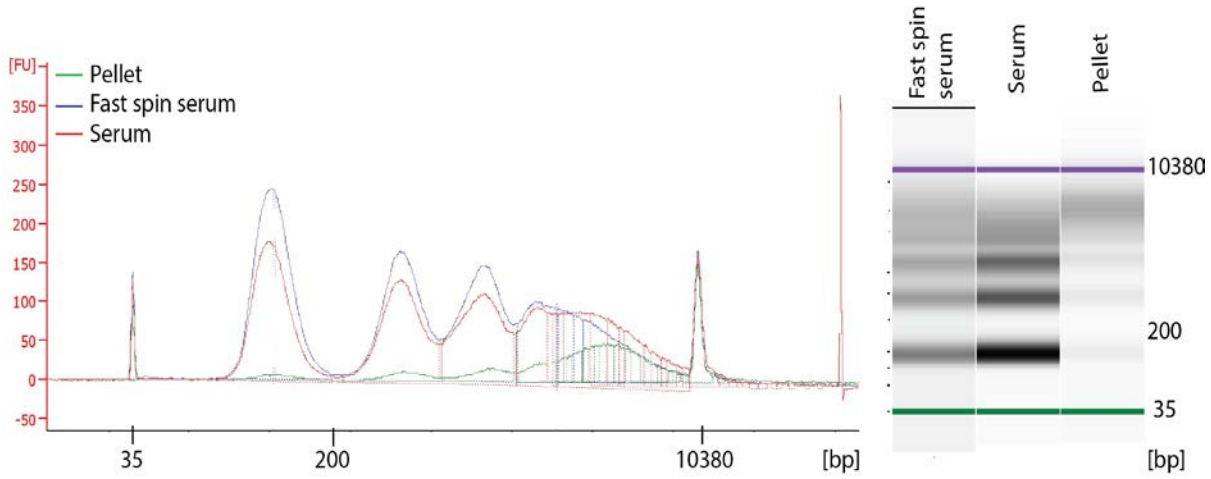


Figure 6: Electrophenogram showing the analysis of DNA extracted from serum (red line), fast spin serum (blue line) and pellet (green line).

The decrease of high molecular weight DNA observed in fast spun serum sample was almost negligible as compared to the untreated serum sample. This decrease does correspond to the germline DNA recovered from the pellet (Figure 6, green line).

Mutational analysis of fast spin serum

One of the serum samples treated with a fast spin, 1162, had a monoclonal *ESR1* mutation on the LBD, *c.1613A>G, p.D538G*. We set to asses if by treating this

sample to a fast spin we could improve both the AF of the mutant allele and the number of copies per ml.

Mutant copies per ml in the fast spun serum sample ctDNA were higher when compared to the mutant copies per ml in serum ctDNA (41.85 vs 28.08 respectively). This value was more comparable to the mutant copies per ml found in the plasma DNA sample where a lower contamination with high molecular gDNA was expected. This would indicate a significant improvement in the detection of mutations in fast spun serum samples as compared to untreated serum. No mutant alleles were detected in the pellet DNA which would strongly indicate that is mostly gDNA.

Sample	Mutation	Fraction mutant	Fraction 95% CI low	Fraction 95% CI low	Mutant copies per ml
1162 P1	c.1613A>G, p.D538G	0,01571	0,00844	0,02316	55,7788
1162 SR1	c.1613A>G, p.D538G	0,00118	0,0003	0,00205	28,0886
1162 SR2	c.1613A>G, p.D538G	0,00046	0,00005	0,00087	17,407
1162 fSR1	c.1613A>G, p.D538G	0,00016	0,00005	0,00027	41,855
1162 pellet	c.1613A>G, p.D538G	0	0	0	0

Table 6: Allele Fraction (AF) and mutant copies/ml in plasma, serum, fast spin serum and pellet DNA for sample 1162.

Automated extraction of cfDNA from plasma using an automated platform

In the last few years new automated platforms have been developed for the extraction of circulating nucleic acids in order to improve the recovery of cfDNA. With magnet heads, volume up to 5mL of plasma or serum can be processed in a single run, resulting in increased yield of the purified product.

In this study we set out to investigate one of these technologies, the KingFisher Flex platform using a beads-based kit, the MagMax cfDNA extraction kit. cfDNA was extracted from 2 ml of plasma either with the QIAmp circulating nucleic acid (cna) kit from Qiagen or with the MagMax kit (Thermo) on a KingFisher Flex automated platform.

DNA concentration for the differently extracted cfDNA was assessed by dPCR using RNase P (Table 7).

Sample	Total RNase P copies/ul	ng/ul	Total ng	Sample	Total RNase P copies/ul	ng/ul	Total ng	Sample	Total RNase P copies/ul	ng/ul	Total ng	Sample	Total RNase P copies/ul	ng/ul	Total ng
7013 P1	26	0,0858	4,29	**7013 P2**	9,2	0,0304	1,518	**7013 Pkf50**	30	0,099	4,95	**7013 Pkf100**	34	0,1122	5,61
7015 P1	34	0,1122	5,61	**7015 P2**	20	0,066	3,3	**7015 Pkf50**	26	0,0858	4,29	**7015 Pkf100**	32	0,1056	5,28
7016 P1	262	0,8646	43,23	**7016 P2**	68	0,2244	11,22	**7016 Pkf50**	242	0,7986	39,93	**7016 Pkf100**	250	0,825	41,25
7017 P1	40	0,132	6,6	**7017 P2**	50	0,165	8,25	**7017 Pkf50**	60	0,198	9,9	**7017 Pkf100**	56	0,1848	9,24
7018 P1	56	0,1848	9,24	**7018 P2**	13,6	0,04488	2,244	**7018 Pkf50**	82	0,2706	13,53	**7018 Pkf100**	48	0,1584	7,92
1162 P1	34	0,1122	5,61	**1162 P2**	68	0,2244	11,22	**1162Pkf50**	76	0,2508	12,54	**1162 Pkf100**	116	0,3828	19,14
7009 P1	70	0,231	11,55	**7009 P2**	50	0,165	8,25	**7009 Pkf50**	56	0,1848	9,24	**7009 Pkf100**	56	0,1848	9,24
1208 P1	26	0,0858	4,29	**1208 P2**	34	0,1122	5,61	**1208 Pkf50**	26	0,0858	4,29	**1208 PKf100**	38	0,1254	6,27
1198 P1	48	0,1584	7,92	**1198 P2**	22	0,0726	3,63	**1198 Pkf50**	54	0,1782	8,91	**1198 Pkf100**	20	0,066	3,3
7024 P1	12,6	0,0416	2,079	**7024 P2**	9,6	0,03168	1,584	**7024 Pkf50**	9,4	0,03102	1,551				
1207 P1	110	0,363	18,15	**1207 P2**	62	0,2046	10,23	**1207 Pkf50**	70	0,231	11,55				
7030 P1	50	0,165	8,25	**7030 P2**	44	0,1452	7,26	**7030 Pkf50**	52	0,1716	8,58	**7030 Pkf100**	32	0,1056	5,28

Table 7: P1: plasma sample first elution; P2: plasma sample second elution using Qiagen cna kit; Pkf50: plasma sample KingFisher Flex 50µl elution; Pkf100: plasma sample KingFisher Flex 100µl elution.

When using columns, the second elution still recovered significant amounts of cfDNA as compared to the first elution. The KingFisher managed to recover higher amounts of cfDNA, that were comparable to the first elution using columns, if a larger volume of eluate was used.

Overall, we concluded that the automated method was as efficient as the manual method at recovering cfDNA if only one elution was performed on the column. We hypothesised that the second elution could have been mostly high molecular weight DNA that took longer to elute due to size.

In order to assess this, we set out to investigate if the first elution had more ctDNA, postulated to be of smaller size as compared to the two different volumes of elution on the automated system.

Mutational Analysis of ctDNA recovered from the automated and manual systems

Of the 12 samples extracted on the manual and automated systems, 3 samples had previously detected mutations. Sample 7018 had a *PIK3CA* mutation, *c.1633G>A, E545K*, sample 1162 had an *ESR1* LBD monoclonal mutation, *c.1613A>G; D538G* and sample 7016 had polyclonal LBD mutations, *c.1613A>G; D538G and c.1610A>C; p.Y537S*.We analysed 0.5 ml plasma equivalent of these samples with their corresponding mutational assay by dPCR.

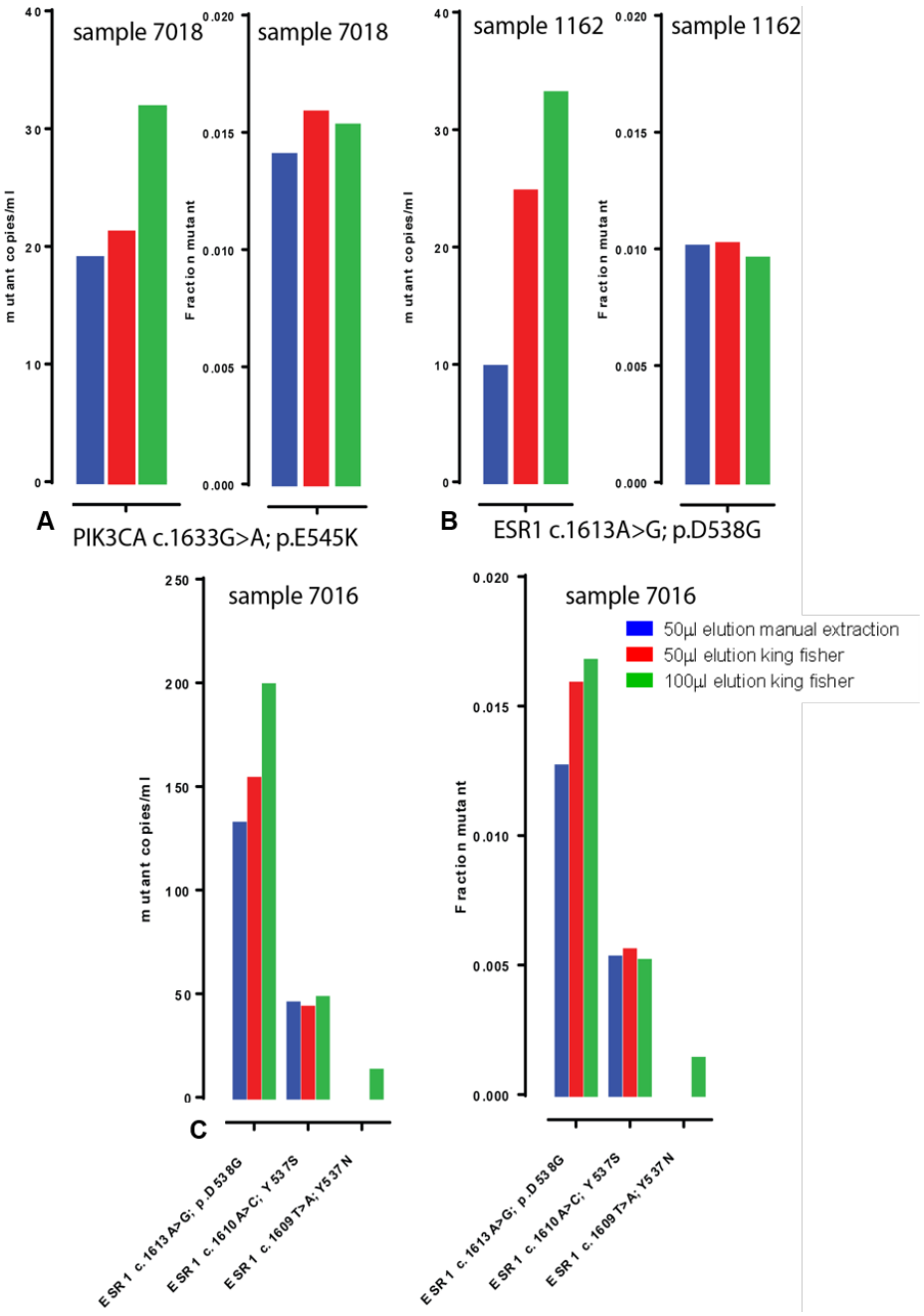


Figure 7: A) Analysis of sample 7018 *PIK3CA c.1633G>A, p.E545K* mutant copies per ml and fraction mutation. **B)** Analysis of sample 1162 *ESR1 c.1613A>G, p.D538G* mutant copies per ml and fraction mutation. **C)** Analysis of sample 7016 *ESR1 c.1613A>G, p.D538G; c.1610A>C, p.Y537S; c.1609T>A, p.Y537N* mutant copies per ml and fraction mutation

We compared AF and mutant copies per ml for these three samples (Figure 7). Sample 7018 showed a higher mutant copies per ml when using the automated system on a higher elution volume (Figure 7A), while the AF remained the same for the three different extraction methods. Sample 1162 (Figure 7B) showed the same pattern of a higher allele frequency and similar AF, strongly suggesting the ability of

the automated system on a higher volume to greatly recover ctDNA fragment which would suggest that these system is better at separating lower molecular weight cfDNA species from high molecular weight DNA species.

Interestingly in sample 7016 we also observed the same trend, and we were able to pick a third mutation on the *ESR1* LBD, *c.1609T>A, Y537N* that we were not able to detect on ctDNA extracted manually (Figure 7C and Figure 8) that became apparent when the automated extraction system was used.

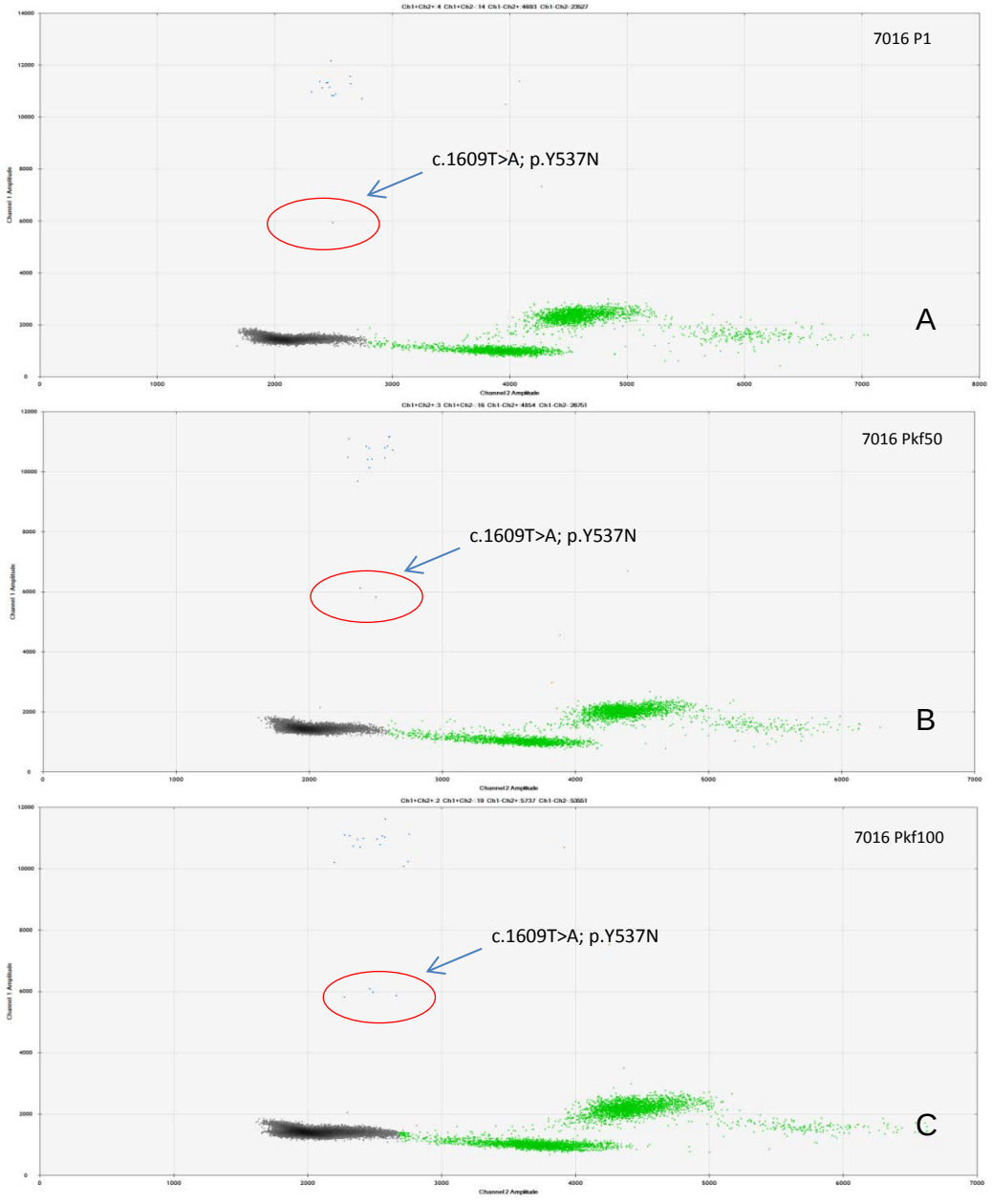


Figure 8: Analysis of *ESR1* mutations in sample 7016 on ctDNA extracted manually (A), DNA extracted on the automated system and low elution volume (B) and ctDNA extracted automatedly on a higher elution volume (C). The newly detected mutant allele can be seen circled in red. We only considered a sample positive for a mutation if >2 positive FAM droplets were observed.

Conclusion

The isolation of low molecular weight cfDNA from serum can be viewed as a strategy to improve the sensitivity of detection of somatic mutations in metastatic Breast Cancer (MBC) patients. Although the complete removal of high molecular weight DNA from serum samples remains a challenge, the fast spin (10min 4ºC) before cfDNA extraction led to significant improvements in the detection of mutations. Indeed, the detected mutant copies per ml in serum cfDNA were more comparable to those found in plasma DNA samples, where a lower contamination with high molecular gDNA was expected.

We observed similar recovery of cfDNA from plasma when using an automated extraction method as compared to manual extraction. When we analysed the ctDNA extracted by the two different methods we observed higher mutant copies per ml when using the automated system as compared to that observed on the manually extracted samples. Furthermore, we were able to detect mutant alleles at very low AF that were not detectable in the manually extracted samples.

Our study found that serum samples can be used to detect somatic mutation events in ctDNA in circulation, albeit with some limitations due to gDNA contamination and that by extraction of ctDNA from plasma or serum on an automated system it is possible to improve the level of detection of these events. The ability to combine these should allow the routine use of serum samples for rare event detection in samples from clinical trials were plasma is not available.

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