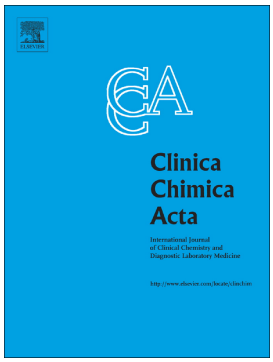

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Quick assessment of cell-free DNA in seminal fluid and fragment size for early non-invasive prostate cancer diagnosis.

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Abstract

Background

Liquid biopsy consists in the quantification and qualification of circulating cell-free DNA (cfDNA) and tumor-derived DNA (ctDNA) for cancer recognition. Recently, the characterization of seminal cfDNA (scfDNA) has been reported as a possible biomarker for prostate cancer (PCa) diagnosis.

Methods

Thirty patients with histologically proven PCa, 33 with benign prostate hyperplasia (BPH) and 21 healthy controls were enrolled. cfDNA was extracted from seminal fluid samples. cfDNA quantification and analysis were performed using Qubit ssDNA Kit and Agilent 2100 Bioanalyzer. Statistical analysis included: Levene's test, Shapiro-Wilk, Kolmogorov-Smirnov and Kruskal Wallis tests.

Results

Median cfDNA was significantly higher in PCa patients 428.45 ng/mL (173.93-1159.62) compared to BPH patients 77.4 ng/mL (18.23-501) and healthy controls 25.4 ng/mL (15.37-76.62). scfDNA fragments longer than 1000 base-pairs were more common in patients with PCa compared to those with BPH and controls.

Conclusions

scfDNA concentration and fragment size differed significantly in the three groups of PCa, BPH and healthy controls. Both parameters are potential clinical biomarkers for PCa and can be used in both early diagnosis and follow-up. Using automated systems for high-throughput cfDNA quantification could improve the reproducibility of the method and facilitate the implementation of liquid biopsies in the clinical setting.

Keywords: seminal cell-free DNA, cfDNA fragment size distribution, non-invasive prostate cancer diagnosis, benign prostatic hyperplasia.

1.0 Introduction

Liquid biopsy consists in the quantification and qualification of circulating cell-free DNA (cfDNA) and tumor-derived DNA (ctDNA) in bloodstream and biological fluids. Previous studies reported higher plasma concentrations of cfDNA in PCa patients compared to healthy controls [1,2]. Significant differences in levels and size distribution of cfDNA were also found in seminal fluid of PCa and BPH patients, respectively [3–5].

cfDNA and ctDNA originate through different cellular processes: necrosis, phagocytosis, oncosis and even active cellular secretion. Not only cancer cells, but also healthy cells and the tumour microenvironment are considered the main sources of cfDNA in cancer patients. Each above mentioned compartment releases different forms of cfDNA into the bloodstream or other biological fluids [6,7]. Previous studies associated high cfDNA concentrations with specific size-distribution patterns to cancer diagnosis [8,9]. However, levels of cfDNA/ctDNA in blood samples vary depending on cancer type. ctDNA was detected in over 75% of patients with advanced pancreatic, ovarian, colorectal, bladder, gastroesophageal, breast and hepatocellular cancer, as well as in melanomas and head and neck tumours. In the case of prostate cancer however, significantly lower levels of plasma cfDNA/ctDNA were found [10]; this is probably due to diverse influencing factors, such as tumor clonality and/or the involvement of different compartments in the release of cfDNA, mainly originating from tumour microenvironment [11].

The extraction and characterization of ctDNA from seminal fluid (scfDNA) is probably a major step forward in PCa diagnosis and follow-up compared to traditional diagnostic techniques involving prostate-specific antigen (PSA), digital rectal examination (DRE), transrectal ultrasound (TRUS) and invasive biopsy [12]. Recently, we reported that the quantification and the electrophoretic characterization of cfDNA in seminal plasma can differentiate between PCa patients, BPH patients, and age-matched healthy individuals; indeed, scfDNA concentrations are higher in PCa patients and present a distinct electrophoretic pattern [3,4]. scfDNA can be easily analysed through cost-

effective procedures such as fluorometry and electrophoresis. In this scenario, scfDNA may significantly change the diagnosis and management of PCa and other genitourinary tumours [5].

The aim of this study was to assess the levels and fragment size distribution of scfDNA in a cohort of PCa patients, BPH patients and healthy controls, in order to identify a novel biomolecular signature for PCa diagnosis and follow-up.

2.0 Materials and Methods

2.1 Ethics statement

The Ethics Committee of the University of Modena and Reggio Emilia approved the study and informed consent was obtained from each recruited subject.

This study was conducted according to the principles of the Helsinki Declaration of 1975, amended in 1996 (http://www.wma.net/e/humanrights/policy_meetings.htm).

2.2 Sample Collection

Thirty patients with histologically proven PCa, 33 with benign prostate hyperplasia (BPH) and 21 healthy controls were enrolled at the Urology Department of the University of Modena and Reggio Emilia. Median age was: 70 (66-74.25) in the PCa cohort, 60 (58.5-67) in the BPH cohort and 62 (58.5-65.5) in the control cohort. Median PSA value was 6.15 ng/mL (5.89-10.96) in the PCa cohort and 4.49 ng/mL (4.22-6.11) in the BPH cohort. Gleason Score was between 6 and 8 in the PCa cohort (Table 1).

Patient eligibility criteria included: age between 50 and 78 years, seminal fluid samples collected at least one month after prostate biopsy, availability of clinical data and known PSA levels.

Patient seminal samples were collected prior to radical prostatectomy and/or chemotherapy or radiotherapy.

2.3 Extraction of scfDNA

As the samples ranged from 0.65 to 3.0 mL, a standardized sample volume of 0.65 mL was analysed. Following WHO's guidelines [13], to avoid sperm lysis, we processed samples within 2 hours from collection at low speed centrifugation (400 rcf) for 10 minutes at room temperature. Then we preserve the supernatant (seminal plasma) from the pellet containing the cells and sperms, and a second step of centrifugation at high speed (16000 rcf) for 5 minutes at room temperature was done to eliminate debris and eventually remaining cells. Seminal plasma samples were then stored at -80 ° C till scfDNA extraction.

scfDNA was extracted with the QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany) and eluted in 120 µL buffer AVE according to the manufacturer's instructions. Aliquots of 120 µL scfDNA were cryopreserved at -20°C until quantification.

2.4 Quantification of scfDNA.

Following recent evidence stating that scfDNA is both single and double strand [14], the concentration levels were measured using the Qubit ssDNA Assay Kit to assess the total amount of scfDNA.

2.5 Fragment size evaluation of seminal cell-free DNA

Microfluidic electrophoresis using the Agylent 2100 Bioanalyzer and high sensitivity DNA chips (Agilent technologies Inc., Palo Alto, CA, USA) was performed to determine DNA fragment length. In order to analyse the same standard concentration of double strand DNA with automated electrophoresis, we quantified the samples with Qubit dsDNA Assay Kit (Life Technologies, Carlsbad, California, US) and made dilutions in buffer AVE to have 2 ng/µL. According to the instructions provided by the manufacturer, 1 µL of each sample were analysed. Following separation, the nucleic acids were normalized, and two DNA markers were represented as a virtual

band. The software automatically calculated the size of each band, representing it as an electropherogram.

2.6 Statistical analysis

STATA program was used to perform statistical analysis. Levene's test was used to assess equality of variances for the groups, and Shapiro-Wilk and Kolmogorov-Smirnov tests were used for exploring the normality of distributions. The results are presented as the Median and IQR for continuous variables. The Kruskal-Wallis test was used to compare numerical data between the groups. The Kruskal Wallis test is used when you have one independent variable with two or more levels and an ordinal dependent variable. In other words, it is the nonparametric version of ANOVA. P less than 0.05 was considered statistically significant.

3.0 Results

3.1 scfDNA concentration is increased in PCa patients

Median cfDNA was significantly ($p<0.001$) higher in PCa patients 428.45 ng/mL (173.93-1159.62) compared to BPH patients 77.4 ng/mL (18.23-501) and healthy controls 25.4 ng/mL (15.37-76.62) (Table 1, Figure 1).

3.2 PCa is characterized by distinctive scfDNA fragment size distribution pattern

scfDNA fragments were divided into five groups: <100 bp, between 101 and 146 bp, between 147 and 190 bp, between 191 and 999 bp, and >1000 bp.

Longer scfDNA fragments were more common in patients with PCa compared to those with BPH and controls (Figure 2, 3 and 4). The Kruskal Wallis analysis of long scfDNA (≥1000 bp) demonstrated a significantly higher median concentrations in the PCa cohort 34.29 pg/µL (19.79-191.81) compared to BPH cohort 24.13 pg/µL (15.54-40.94) and to healthy controls 13.48 pg/µL (8.29-25.96) ($p<0.0001$). The median fragments length was 2858 bp (1714-5045) in healthy

subjects, 2614.5 bp (1680.25-5094.75) in BPH patients and 2825.5 bp (1775.25-5116.25) in PCa patients.

Shorter (<100 bp) fragments' concentration was different between three groups ($p<0.05$): 36.59 pg/µL (17.58-88.24) in healthy controls, 26.4 pg/µL (9.59-59.68) in BPH patients and 15.84 pg/µL (5.83-50.62); median fragment length was 71 bp (63-96) in healthy controls, 74.5 bp (62.75-87.25) in BPH patients and 84.5 bp (73.75-95) in PCa patients. Also, fragments sized between 101 and 146 bp differed in the three groups ($p<0.05$): 58.98 pg/µL (16.6-83.76) in healthy controls, 26.9 pg/µL (15.78-52.49) in BPH patients and 13.44 pg/µL (6.94-28.24) in PCa patients. The median fragments length was 106.5 bp (103.75-113) in healthy controls, 109.5 bp (103-118.25) in BPH patients and 105 bp (103-110) in PCa patients. Fragments sized between 147 and 190, and between 191 and 999 demonstrated similar concentrations among the three groups. The median fragments length was respectively: 163 bp (157-166.5) and 447 bp (324-627) in healthy controls, 166 bp (160-177) and 434 bp (329-604) in BPH patients, 165 bp (157-171) and 458.5 bp (334.5-607.5) in PCa patients.

4.0 Discussion

Seminal cfDNA levels are notably (approximately 100 times) higher than blood cfDNA in PCa patients, as reported in the literature (19.62–21.25 ng/µL)[1,2]. Recently, we could demonstrate that human seminal fluid can be a valuable source of cfDNA for the identification of novel oncological markers. In this study, we confirm that scfDNA levels and size distribution are potential clinical biomarkers in the non-invasive diagnosis of PCa and its differentiation from BPH. In our cohorts, mean concentration of scfDNA was significantly higher in PCa patients compared to BPH patients and healthy controls (Table 1, Figure 1).

In addition, we suggest that the electrophoresis of seminal plasma cfDNA enables the discrimination between PCa patients and BPH or age-matched healthy individuals, because of a distinct electrophoretic pattern.

The scfDNA analysis through automated microfluidic electrophoretic systems (i.e. Bioanalyzer) showed distinct DNA fragmentation patterns, characterized by high molecular weight DNA (longer than 1000 bp) compared to BPH patients and healthy individuals. These long DNA fragments in PCa patients could derive from the prominent necrotic processes characterizing PCa growth and differ from the typical apoptotic DNA fragments (147-190 bp and multimeric). From our experience we didn't found over-estimation of the total cfDNA concentration due to fragment length by using Qubit. We had some of PCa' samples with less long fragments but high cfDNA concentration, or some controls' and BPH's samples with long fragments but low cfDNA concentration. Moreover, we analysed some samples using Bioanalyzer for the total amount of cfDNA (data not reported) and there was correlation with the Qubit's measurements. cfDNA fragment size was associated with a more advanced stage at the diagnosis, representing a potential prognostic factor for PCa patients.

The above-mentioned concept has already been discussed in the literature with regard to cfDNA in bloodstream. It is known that higher levels of cfDNA assessed in cancer patients compared to healthy individuals are the result of the release of cfDNA either by malignant or non-malignant cells [6,7]. The assessment of total cfDNA level in the bloodstream can depict tumor dynamics and clonal heterogeneity over time. Thierry AR et *al.* showed that total cfDNA and mutant cfDNA were strongly correlated with decrease of overall survival as well as high levels of mutation load and fragmentation [6,15]. The fragment size distribution analysis is an important factor, which is associated to tumor load, response to treatment and overall survival of cancer patients.

The determination of cfDNA fragment size and cfDNA concentration is a non-invasive, simple method for PCa diagnosis and for PCa staging. Fluorometric and electrophoretic assessments allow a reliable quantification and qualification of scfDNA, that could be routinely adopted for PCa screening programs.

Seminal fluid can be easily collected from patients, and the quantification of cfDNA in seminal fluid does not add another layer of complexity compared to other substrates as are blood or urine [2,16]. Over and above that, isolating cfDNA from seminal fluid grants more efficiency: all

conditions being like other isolation methods, it allows the extraction of a greater quantity of cfDNA. The high concentration of scfDNA allow a more accurate and precise primary early diagnosis of localized PCa. This makes scfDNA an attractive clinical marker for non-invasive cancer diagnosis and longitudinal monitoring of response to treatments. The demand on "liquid" biopsies is likely to grow. Automated systems for high-throughput cfDNA quantification and analysis could improve the reproducibility of the process and facilitate its implementation in the clinical setting.

A quick, cost-effective and simple test offers important advantages. cfDNA analytic methods vary in quantitative range, sensitivity, costs and workflow simplicity. Our Agilent 2100 Bioanalyzer technique is much simpler and faster than NGS and qPCR, it provides accuracy and reproducibility, and has the potential to allow any laboratory to perform molecular analysis, without the need for specialized electrophoresis equipment. The Bioanalyzer characterizes DNA fragments for their integrity, purity, concentration, and size distribution; it is faster and more sensitive compared to standard agarose gel electrophoresis in isolating and displaying the differences in genes expression, providing at the same time accurate quantitative information for each DNA sample fragment [17,18].

To sum up, our data suggest that the level and fragment size distribution of seminal cfDNA are potential non-invasive and easily collectable biomarkers for PCa diagnosis and follow-up.

Summary points

Liquid biopsy consists in the quantification and qualification of circulating cell-free DNA (cfDNA) and tumor-derived DNA (ctDNA) for cancer recognition.

The characterization of seminal cfDNA (scfDNA) is a potential biomarker for prostate cancer (PCa) diagnosis.

30 PCa, 33 BPH patients and 21 healthy individuals were enrolled. Median cfDNA from PCa patients 428.45 ng/mL were significantly higher than those from BPH patients 77.4 ng/mL and healthy controls 25.4 ng/mL.

A higher concentration of long scfDNA fragments (>1000bp) was observed in PCa patients in respect to BPH patients and healthy controls, probably associated to tumour necrosis.

scfDNA can be adopted in early diagnosis of PCa and clinical surveillance, avoiding unnecessary biopsies.

Application of automated systems for high-throughput cfDNA quantification and analysis could facilitate the implementation of liquid biopsy in the uro-oncological clinical setting.

Conflict of Interest Statement: None declared.

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Figure1 Box-plot representation of scfDNA concentrations in Prostate Cancer (PCa) patients, Benign Prostatic Hyperplasia patients (BPH) and Control (Cntrl) groups (*p<0.05, **p<0.01, ***p<0.001).

Figure 2 Bioanalyzer gel-like representation of scfDNA fragment distribution in controls (c14, c15, c11), BPH (13i, 14i, 22i) and PCa patients (15, 16, 17).

Figure 3 Bioanalyzer electropherogram representation of scfDNA fragment distribution in a control (blue), a BPH patient (red) and a PCa patient (green).

Figure 4 Bar-chart representation of scfDNA fragments concentrations separated in 5 fragment size class: a) ≤100 bp, b) between 101 and 146 bp, c) between 147 and 190 bp, d) between 191 and 999 and e) ≥1000 bp in Prostate Cancer patients (PCa), Benign Prostatic Hyperplasia patients (BPH) and Controls (Cntrl) groups. (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001)

Table 1 Clinical and epidemiological features along with scfDNA concentrations of patients affected by Prostate Cancer (PCa), Benign Prostatic Hyperplasia (BPH) and healthy controls (Cntrl).

Cntrl	Age	scfDNA (ng/mL)	BPH	Age	PSA (ng/mL)	scfDNA (ng/mL)	PCa	Age	PSA (ng/mL)	Gleason Score	scfDNA (ng/mL)
1	54	15.2	**1**	68	4.49	202.6	**1**	68	8.7	7(4+3)	4738.5
2	62	9.01	**2**	66	7.12	1294.9	**2**	62	7.05	6(3+3)	44
3	55	5.14	**3**	69	5.75	583.4	**3**	66	6.2	7(3+4)	3430.8
4	63	89.5	**4**	56	4.9	5.77	**4**	75	4.02	6(3+3)	440
5	66	126.31	**5**	53	7.52	28.77	**5**	77	4.37	7(4+3)	1535.4
6	58	80.15	**6**	67	9.5	10.96	**6**	64	4.73	7(3+4)	2242.8
7	57	16	**7**	65	7.37	89.1	**7**	72	12.66	6(3+3)	592.3
8	65	18.61	**8**	64	4.13	4.98	**8**	59	14	7(4+3)	378.5
9	61	64.9	**9**	68	6	177.42	**9**	65	6.06	6(3+3)	34.3
10	68	184.6	**10**	52	5.77	116.9	**10**	67	12.12	7(4+3)	455.4
11	63	25.4	**11**	61	3.7	25.8	**11**	72	6.14	8(5+3)	340
12	59	28.77	**12**	58	4.14	8.86	**12**	72	4.73	6(3+3)	500
13	64	3.98	**13**	66	4.55	47.8	**13**	75	5.37	6(3+3)	409.2
14	66	17.61	**14**	62	4.42	16.46	**14**	69	18.44	6(3+3)	46.9
15	60	15.54	**15**	59	4.02	27.7	**15**	60	6.89	6(3+3)	372.31
16	67	16.31	**16**	61	4.52	8.81	**16**	58	18	7(3+4)	689.2
17	56	169.2	**17**	56	4.77	1538.5	**17**	70	6.59	6(3+3)	423.1
18	64	40.6	**18**	54	3.89	77.4	**18**	78	10.44	7(3+4)	1223.1
19	62	27.2	**19**	62	4.8	24.9	**19**	75	10.58	6(3+3)	433.8
20	62	73.1	**20**	68	4.88	9.51	**20**	75	2.69	7(3+4)	175.5
21	68	10.35	**21**	68	3	12.41	**21**	72	10	6(3+3)	203.7
			22	56	4.25	20	**22**	66	4.8	7(4+3)	88.5
			23	60	5.4	1293.8	**23**	68	31	7(4+3)	169.23
			24	68	5	1692.3	**24**	75	6.7	6(3+3)	153.84
			25	69	2.1	64.31	**25**	71	7.65	6(3+3)	3142.9
			26	62	4.75	578.9	**26**	69	6.93	6(3+3)	1309.1
			27	62	6.22	101.54	**27**	74	6.18	6(3+3)	901.5
			28	67	6.7	2430.77	**28**	70	6.5	6(3+3)	46.15
			29	59	5.3	118.6	**29**	69	22	7(4+3)	241.11
			30	65	6.87	2076.9	**30**	73	6.99	6(3+3)	1138.46
			31	63	5	65.69					
			32	65	6.37	169.2					
			33	50	4.19	423.1					
Median	**62**	**25.4**		**60**	**4.49**	**77.4**		**70**	**6.15**		**428.45**
IQR	**58.5 65.5**	**15.37 76.62**		**58.5 67**	**4.22 6.11**	**18.23 501**		**66 74.25**	**5.89 10.96**		**173.93 1159.62**

Highlights

- Liquid biopsy consists in the quantification and qualification of circulating cell-free DNA (cfDNA) and tumor-derived DNA (ctDNA) for cancer recognition.
- The characterization of seminal cfDNA (scfDNA) is a potential biomarker for prostate cancer (PCa) diagnosis.
- 30 PCa, 33 BPH patients and 21 healthy individuals were enrolled. Median cfDNA from PCa 863.32 ng/mL patients were significantly higher than those from BPH patients 404.49 ng/mL and healthy controls 49.40 ng/mL.
- A higher concentration of long scfDNA fragments (>1000bp) was observed in PCa patients in respect to BPH patients and healthy controls, probably associated to tumour necrosis.
- scfDNA can be adopted in early diagnosis of PCa and clinical surveillance, avoiding unnecessary biopsies.
- Application of automated systems for high-throughput cfDNA quantification and analysis could facilitate the implementation of liquid biopsy in the uro-oncological clinical setting.

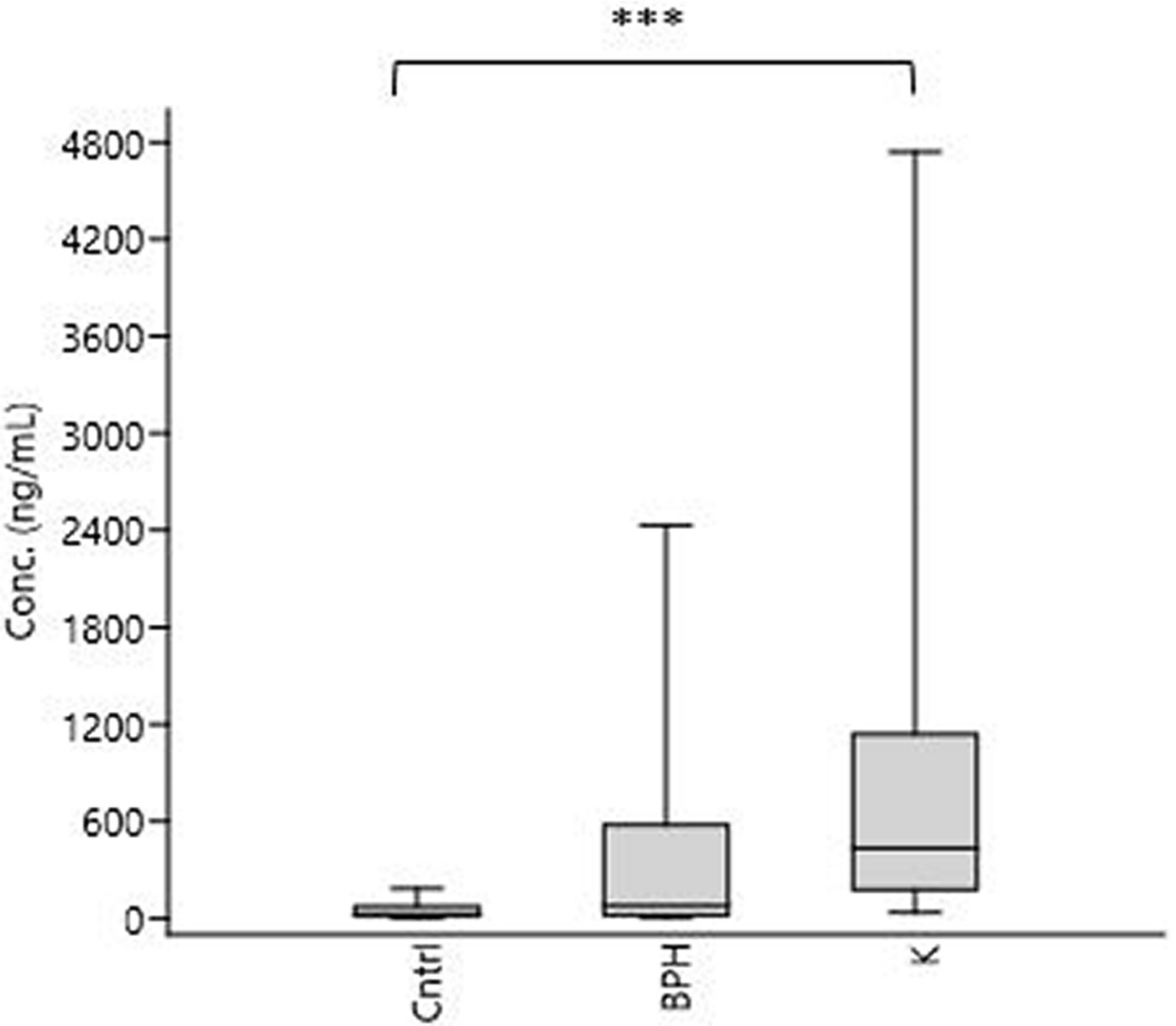
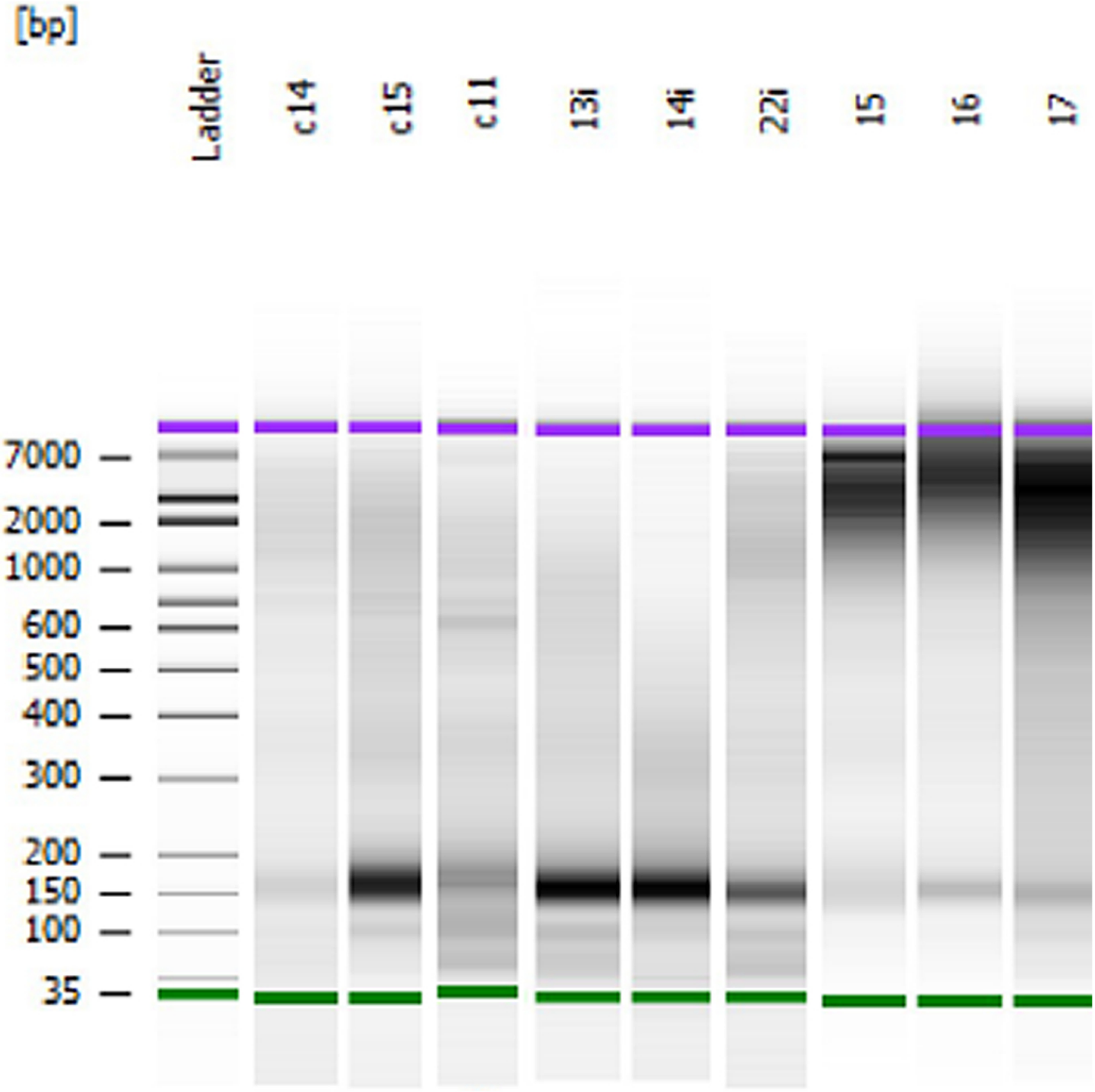


Figure 1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c15
c11
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14i
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16
17
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600
500
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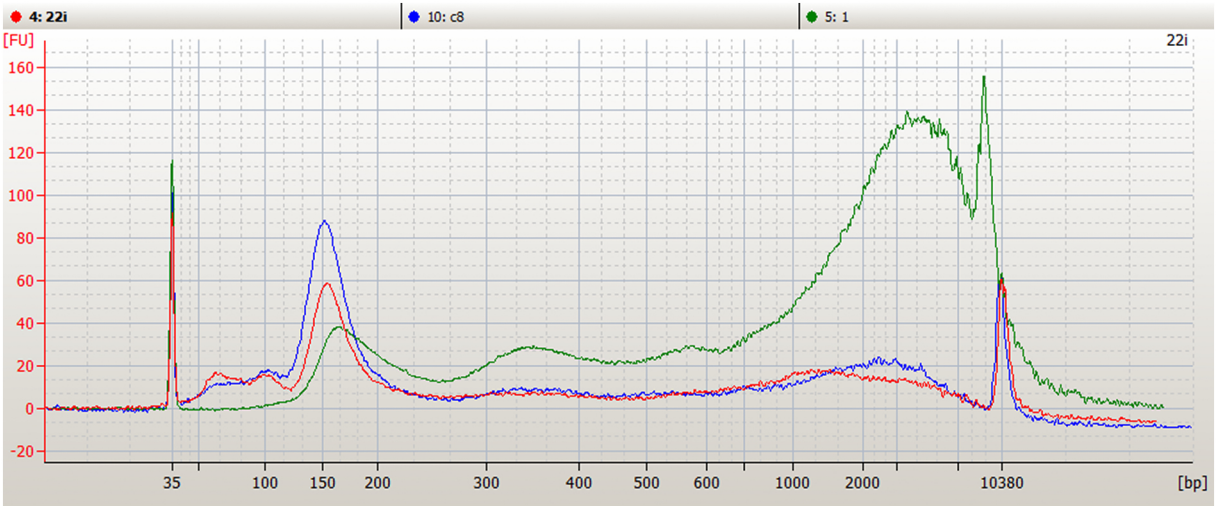
200
150
100
35

Figure 2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5: 1
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22i
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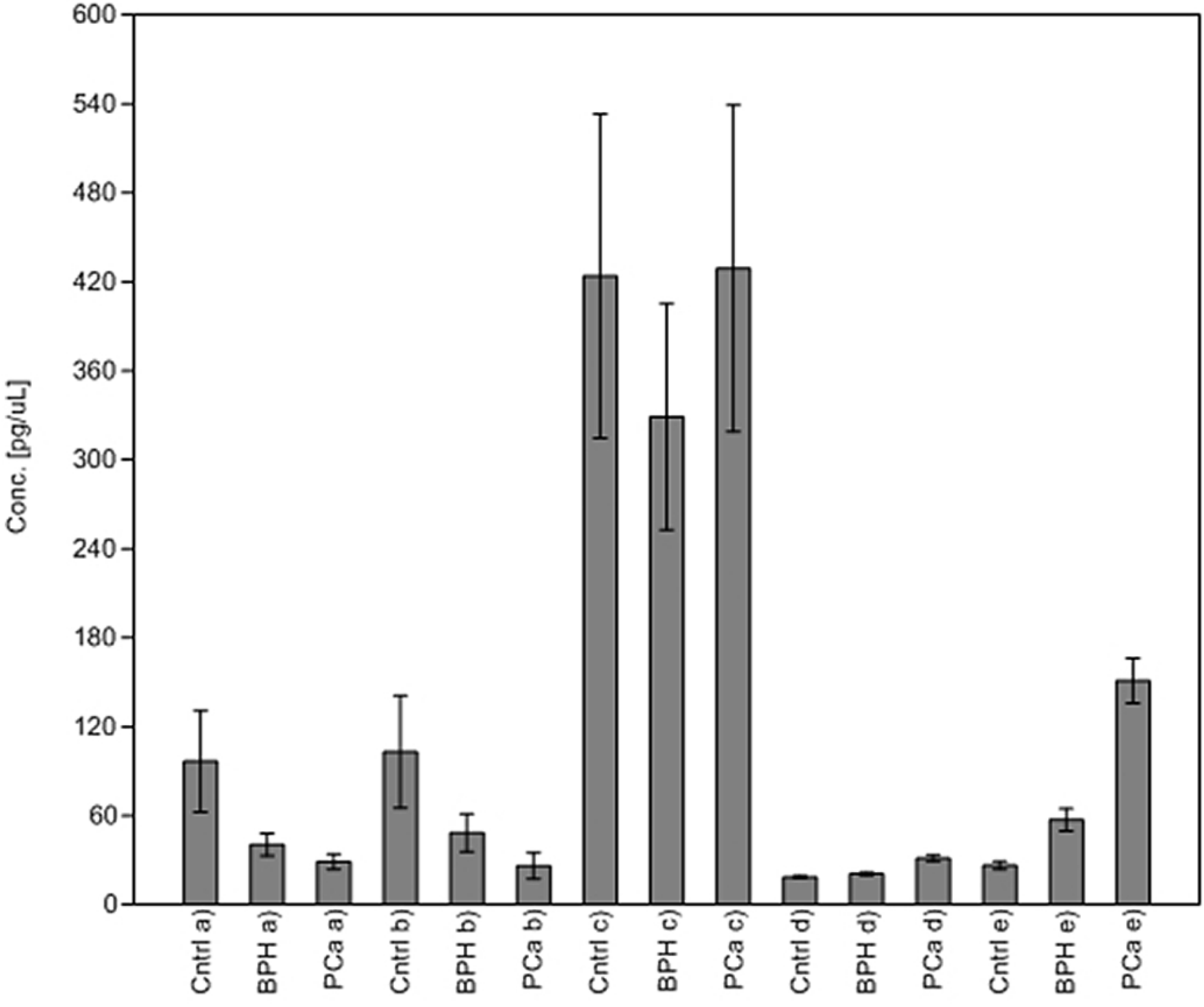
[bp]

Figure 3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Cntrl a)
BPH a)
PCa a)
Cntrl b)
BPH b)
PCa b)
Cntrl c)
BPH c)
PCa c)
Cntrl d)
BPH d)
PCa d)
Cntrl e)
BPH e)
PCa e)

Figure 4