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Running head: cfDNA sizing by  $\mu$ LAS technology

# DNA sizing with pg/ $\mu$ L sensitivity for cell-free DNA analysis with the $\mu$ LAS technology

## 1 Summary

The  $\mu$ LAS technology enables in-line DNA concentration and separation in a microchannel. Here, we describe its operation to analyze the size profile of cell-free DNA (cfDNA) extracted from blood plasma. Operated on commercial systems for capillary electrophoresis, we provide the size distribution of healthy individuals or patients using an input of 10  $\mu$ L.

## 2 Key word

DNA, cfDNA, sizing, microfluidics, capillary, separation

## 3 Introduction

Cancer diagnostic and follow-up is essentially carried out with radiology techniques, tissue biopsy analysis, and body fluid analysis. While the first two methods can lead to several adverse effects (1,2), notably associated to surgical operations or exposure to irradiations, the detection of biomarkers in body fluids is minimally-invasive. Among these biomarkers, there is an upsurge in studies considering cell-free DNA in body fluids (cfDNA) to stratify patients, monitor the treatment response and predict disease progression (3). The characterization of cfDNA nevertheless remains a challenge due to its low concentration, that typically spans 1 to 100 ng/mL, and broad size distribution from 100 to 5000 bp. It can either be performed with target gene analysis, using enzymatic amplification to achieve detection (1), and/or by global size analysis using high sensitivity DNA separation technologies. The latter technologies generally include a concentration step so as to obtain a detectable signal by fluorescence microscopy. Among the different technological solutions (4,5,6), we describe the  $\mu$ LAS technology in microfluidic format and its version for capillary electrophoresis, termed the BIABooster, that performs

sizing and concentration of double stranded DNA with a sensitivity of 10 fg/ $\mu$ L and provides the size distribution of cfDNA analysis in minutes.

## 4 Materials

### 4.1 $\mu$ LAS using microfluidic chips

#### 4.1.1 microfluidic chips

$\mu$ LAS chips are fabricated by photolithography, reactive ion etching, thermal growth of a 300 nm layer of silicon oxide, and silicon-glass anodic bonding (see detailed protocol in (7)). The microchannel consists of a linear channel with a contraction midway through the inlet and outlet (see Figure A for dimensions). The channel height for the linear part and the constriction are 16 and 2  $\mu$ m, respectively. The constriction of the chip is designed with a linear head-to-head with a power-law geometry (Figure B). The user chooses the use of single- or double-channel chips (Figure C)).

#### 4.1.2 PVP-based working buffer:

Prepare solutions with ultrapure water (18 M $\Omega$ -cm at 25 °C) and store them at room temperature. Chemicals were supplied from Sigma-Aldrich, unless mentioned.

1. Dissolve neutral polymer polyvinylpyrrolidone (PVP; MW=1.3 MDa) at a 5% (w:v) concentration in electrolyte solution such as TBE 1X (note 1).
2. Perform vortex mixing of the solution for 2 min.
3. Leave the solution overnight at room temperature (RT) using a rotator (note 2).
4. Prior use, filter the working buffer with 0.2  $\mu$ m polyethersulfone (PES) filters.

#### 4.1.3 DNA ladders

1. Dilute the DNA ladder in the working buffer. DNA concentrations on the order of  $10^{-7}$  M ensure fast visualization of DNA bands.
2. Vortex the mixture for 2 min.

3. Leave the solution at room temperature for 1 hour before use.

## 4.2 $\mu$ LAS using the BIABooster device in a Capillary Electrophoresis Instrument

### 4.2.1 CE-LIF System

We use an Agilent Technologies Capillary Electrophoresis 7100 system equipped with an Adelis Zetalif LASER488 or a Zetalif LED480 fluorescence detector (see Notes 6, 7 and 8). The capillary is a BIABooster device (ref: 16-BB-DNA/11, Adelis, France). An external pressure system (compressed air or nitrogen) at 12 bar is necessary. The pressure and electric field are applied at the same time. We use the CEC mode, as selected in CE configuration in OpenLAB Chemstation (Agilent Technologies).

### 4.2.2 Liquid reagents and Solutions

- Hydrochloric acid solution at 0.1M: Mix 833  $\mu$ L of concentrated hydrochloric acid in 99.2 mL of water (molecular biology grade water) using a magnetic stirrer for 5 minutes.
- Capillary washing solution (ref: 16-BB-DNA1K/13, Adelis)
- Capillary conditioning solution (ref: 16-BB-DNA1K/09, Adelis)
- Running buffer: Running buffer DNA 1K solution (ref: 16-BB-DNA1K/10, Adelis). See Note 9 for home-made buffer.
- Dye: 16-BB-DNA1K/02, ADELIS, France. See Note 10 for alternative dyes.
- DNA ladder 1K (ref: 16-BB-DNA1K/08, Adelis)
- RNase1, 10 U/ $\mu$ L (ref: EN0601, ThermoFisher)

### 4.2.3 Preparation of the running buffer with dye

1. Pipette 2 mL of Running buffer DNA 1K solution in a tube
2. Add 20  $\mu$ L of fluorescent dye; Vortex for 10 s
3. Collect this solution with a 5 mL syringe (provided in the kit)
4. Place a syringe filter on the syringe (provided in the kit) and filter the solution in 2 polypropylene vials (Agilent Technologies, P/N: 5182-0567). See Note 11.

#### 4.2.4 Required number of vials

The liquid reagents and solutions are dispensed in standard glass vials (Agilent Technologies, P/N: 5182-9697), except for the running buffer with dye which is placed in polypropylene vials (Agilent Technologies, P/N: 5182-0567) in order to reduce dye adsorption. The DNA ladder is used in glass inserts (Adelis, P/N:16-BB-DNA1K/06, volume 10  $\mu$ l) to reduce the required volume. Table 1 indicates the number of vials required depending on the number of analyzed samples.

## 5 Methods

### 5.1 Operation with microfluidic chips

#### 5.1.1 Experimental set-up

1. Place the microfluidic chips into supports that contain inlet and outlet reservoirs sealed by O-rings as well as 0.5 mm diameter holes pierced sidewalls for integrating platinum wire electrodes (Figure 1A).
2. Close the tanks with pneumatic fittings to connect the chip to a pressure controller (7 bar, Fluigent).
3. For electric field actuation, connect a high voltage power supply (Labsmith) to the electrodes. Make sure that the electric force is oriented opposite to the flow (Figure 2B).
4. Place the chip with its support on an epifluorescence microscope equipped with a light engine for cyan excitation (Lumencore) (Note 3), and an sCMOS digital camera (Andor). We use 20X or 10X air objectives for visualization.

#### 5.1.2 Experimental flow data acquisition

1. Before the experiment, fill microfluidic chips with ethanol using 0.5 bar pressure, and flush for 10 min to remove bubbles.
2. Rinse the chip with milli-Q water for 10 min at 0.8 bar.

3. Inject the working buffer with the pressure of 1.2 bar and flush for 10 min to ensure surface coating with the PVP.
4. Introduce the solution containing labeled DNA and switch the microscope to fluorescence mode. Extensive rinsing with working buffer is needed between each analysis (Note 4).
5. Apply constant pressure drop. Pressure can vary between 2 and 7 bar (see example of analysis).
6. Apply voltage in the range of 50 to 400 V (see examples of analysis, note 5).
7. The concentration and separation of DNA bands is observed immediately.
8. User can choose to take a snapshot or to record a movie of concentration and separation.
9. Post proceeding consists in extracting the fluorescence intensity along the symmetry line of the funnel ( $x$  arrow in Figure 3). Intensity profiles are then fit using the multipeak Gaussian functions to extract the position and width of each peak.

### 5.1.3 DNA separation in a microfluidic chip

A double-channel chip is used to identify DNA sizes in an unknown sample (Figure 3). The analytical sample consists of three DNA fragments of 466, 798, and 1512 bp mixed at the same concentration of 80 pg/ $\mu$ L. The reference channel contains a 100 bp DNA ladder at the concentration of 500 ng/ $\mu$ L. We used 6 bar and 82 V, corresponding to a maximum flow velocity and an electric field of 7 cm/s and 5 kV/cm, respectively.

## 5.2 Operation with the BIABooster system

### 5.2.1 Experimental set-up

The analytical protocol consists of three steps characterized by the following electrohydrodynamic actuation parameters:

1. Conditioning BIABooster device

Inlet vial	Outlet vial	Pressure (Bar)	Voltage (kV)	Duration (min)
Waste	Capillary washing solution	-10	0	3
Capillary washing solution	Waste	10	0	1.50
HCl 0.1 M	Waste	10	0	2
Capillary conditioning solution	Waste	10	0	4
Running buffer with dye	Waste	10	0	6

## 2. Sample injection

Inlet vial	Outlet vial	Pressure (Bar)	Voltage (kV)	Duration (s)
Sample	Waste	5	0	80
Water	Waste	load inlet	0	0
Water	Waste	load inlet	0	0
Running buffer with dye	Waste	5	0	10
Water	Waste	load inlet	0	0
Water	Waste	load inlet	0	0

## 3. Concentration and separation

Inlet	Outlet	Pressure (Bar)	Voltage (kV)	Duration (min)
Running buffer with dye	Running buffer with dye	10	25	0
		10	25	6
		10	10	6.4
		10	5	6.8
		10	0.6	10
		10	0.1	21
		10	0	21.1

### 5.2.2 DNA separation in capillaries

In Figure 4, a DNA1K ladder is concentrated using 10 bar and 25 kV for 6 minutes. After this concentration step, the electric field is gradually reduced from 25 to 0.1 kV in order to separate DNA. The limit of detection (signal-to-noise ratio equal to 3) of the bands of 100, 200, 300, 400 and 1000 bp are 0.3, 0.07, 0.04, 0.02 and 0.01 pg/ $\mu$ L, respectively. To determine the size profile and concentration of unknown samples, a DNA1K ladder is processed every 5 samples.



### 5.2.3 Analysis of cfDNA

#### 5.2.3.1 Plasma Preparation

We describe a plasma preparation method suitable for cfDNA analysis based on ref. (9) (See Note 12).

1. Centrifuge blood tubes at 1,200 g for 10 min at room temperature.
2. Transfer supernatant in a new tube, avoiding pipetting the buffy coat.
3. Centrifuge samples at 16,000 g for 10 min at 4°C (See Note 13).
4. Proceed to cfDNA extraction, or store the sample at -80°C (See Note 14).

#### 5.2.3.2 cfDNA extraction

For this step, use your preferred extraction method provided that the QIAmp Circulating Nucleic Acid Kit (QIAGEN; Note 15), the IDXTRACT kit (ID-Solutions), and the Maxwell® RSC cfDNA Plasma Kits (Promega) have been tested successfully (Note 16).

#### 5.2.3.3 RNase treatment (Notes 17 and 18)

1. Dilute the RNase1 solution at 0.2 U/μL in TE 1X.
2. Add 5 μL of this solution in 5 μL of cfDNA sample.
3. Incubate at 37°C for 30 min.

#### 5.2.3.4 BIABooster analysis

- Proceed as described above for DNA samples (see note 19). A typical chromatogram is reported in Figure 5A, and its conversion into a size distribution in 5B.
- BIABooster system is relevant to control the quality of cfDNA samples. Figure 6A shows a raw profile of a cfDNA sample containing excessive amounts of high molecular weight DNA. Figure 6B shows a degraded cfDNA sample, as inferred from the fluorescence signal at the end of the concentration step, between 3 and 6 minutes, which indicates the presence of molecules smaller than 75 bp.

### 5.2.3.5 Data treatment

Conventional data treatment designed for chromatographic or for electrophoresis systems, as described in (10), must be adapted for BIABooster analyses, because the migration of DNA fragments is not constant over time.

1. Export raw data from the Agilent CE in a csv format using the Export function:
  - a. In Data Analysis, select Export function.
  - b. Complete the parameters: export directory, unit signal.
  - c. Select the sequence file to export.
  - d. Files in CSV format are then saved in the export folder (Note 20).
2. Import data into BIABooster Analytics software and perform analysis (or perform data treatment with other means, see note 21).

## 6 Notes:

1. Different electrolytes can be selected depending on the application. The conductivity as well as to a lesser extent the viscosity depend on the electrolyte. If you change electrolyte, adjust the electric field for the concentration step.
2. There are different ways to prepare a PVP-based working buffer, including leaving it overnight at RT on a rotator, or stir it for 2 h at 40 °C on a magnetic stirrer. The viscosity of the resulting solution is largely dependent on the chosen method. We advise to prepare the running buffer with the same method for comparing experiments.
3. The excitation wavelength is chosen regarding the fluorescence dye used to label DNA. The dyes used in our studies (YOYO-1 and YO-PRO-1) are excited at 491 nm, and emit at 509 nm.
4. After conditioning the chip with a PVP-based working buffer, make sure to not introduce ethanol in between analyses. Otherwise, it leads to precipitates that

immediately clog the constriction and irreversibly damage the chip. If an air bubble arises during the experiment, try to remove it by applying the same high pressure to both reservoirs.

5. A 300 nm layer of silicon oxide silicon is grown on silicon surfaces to avoid breakdown at high electric fields. We suggest to operate at tensions lower than 300-400 V.
6. The CE-LIF system simultaneously delivers high pressure (up to 10 bar) and high voltage (25 kV). If you have a CE-LIF equipment in your lab, Adelis can study a specific adaptation of the BIABooster device to your CE-LIF system.
7. For this application, the fluorescence detectors Zetalif LASER 488 nm and Zetalif LED 480 nm are equivalent. The limit of detection is due to the background signal in the running buffer, which contains the dye.
8. A short electrode is used on the inlet side in order to avoid any contamination of the sample. The short electrode (ref: G7100-60033, Agilent Technologies) is 5 mm shorter than the standard.
9. For a home-made buffer, you may use TBE 1X with PVP, as for microfluidic chips. It is preferable to use TBE 0.5X to decrease electric current, hence Joule heating, when applying 25 kV or more.
10. You may also use YOPRO-1, YOYO-1 or Sybr Green (Molecular Probes, Invitrogen). The dye/DNA base pair ratio is 1:8 for YOYO-1 and YOPRO-1.
11. The filtration step removes fluorescent microaggregates which generate small spikes in the fluorescence traces. The running buffer + dye solution is stable for one day at room temperature in the carousel of the CE.
12. Any plasma preparation suitable for cfDNA analysis by PCR or sequencing will work. The pre-requisites are (i) the preparation of plasma within the first 6 hours after blood drawing using standard K2-EDTA collecting tubes, and (ii) the use of a double centrifugation to completely eliminate cells and cell debris. Tubes specifically designed

for cfDNA analysis (Cell-free Collection Tube (Roche Diagnostics), PAXgene Blood ccfDNA tube (Qiagen), and Streck BCT tubes (Streck)), which allow keeping the blood at room temperature for several days before preparing plasma, have been used successfully for cfDNA sizing.

13. Centrifuging 10 mL tubes at 16,000 g at 4°C requires an appropriate centrifuge. We also ran the second centrifugation at 4,500 g at room temperature. The resulting plasma is less clear when a turbid plasma is obtained after the first centrifugation. However, cfDNA size profiles are reliable. In any case, use exactly the same preanalytical method for all the samples of a given study.
14. In order to avoid multiple freezing/thawing cycles, it is recommended to aliquot plasma samples before freezing. In our hands, freezing/thawing plasma samples one time has no impact on cfDNA size distribution, though it may impact the total concentration.
15. This analytical method is suitable for unsalted samples. The concentration step can be adapted to perform cfDNA sizing without prior extraction, starting from 100 µL of plasma (results under publication).
16. We noticed differences in the size distribution of cfDNA according to the DNA extraction method. cfDNA kits have generally been set up for small fragments extraction, and the yield for high MW molecules varies significantly. For instance, the concentration of long molecules (>500 bp) is lower with the IDExtract kit than the QIAamp CNA kit. Also, the size of the main mononucleosomal peak slightly could change from one kit to another, e.g., the peak is 3 bp smaller with the QIAamp CNA kit than the IDExtract kit. The main features of the size profile are nevertheless conserved between the different procedures.
17. When using a DNA extraction procedure with a carrier to improve extraction yield, note that the carrier is likely to interfere with the size analysis, especially if it contains

nucleic acids. For instance, the carrier in the Qiagen CNA kit contains RNA. We recommend not to use the carrier given that we repeatedly compared the yield of cfDNA extraction with and without carrier, and never detected any difference, in agreement with ref. (11). If you cannot avoid the carrier, treat the samples with RNAses.

18. It is preferable to treat cfDNA samples with RNases because DNA and RNA are sometimes extracted, and endogenous RNA may distort the size distribution profile. It was the case in one project with the Cobas® cfDNA sample preparation kit (Roche).
19. Above 150 pg/μL, the first peak of the cfDNA sample may saturate the fluorescence detector (fluorescence greater than 50 RFU; this is almost the case in Figure 6B). It is then necessary to dilute the sample in TE 1X.
20. Thanks to the support of Agilent, we propose a macro to export all the curves of a run in a single step upon request.
21. To use other data treatment tools than BIABooster Analytics software, follow the three main steps: i) curve smoothing (using a size window of 1 to 5 sec), ii) time/size and fluorescence/concentration calibration based on the fluorescence trace of the reference ladder and linear interpolation between the peaks of the ladder, iii) apply fluorescence/concentration calibration to the fluorescence trace of the samples, and integrate the signal between the desired time intervals. Use the time/size calibration curve to know the time interval corresponding to the desired size interval. The Agilent sequence given herein analyzes one reference ladder sample every 5 samples to ensure excellent analytical results. If you do not need maximum precision, for instance if you just want to check the degradation of your sample, you can fewer reference analysis.

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## 8 Tables

Required number of vials depending on number of samples								
Number of samples	HCl 0.1 M	water	Capillary washing solution	Capillary conditioning solution	Running buffer DNA 1K	Running buffer DNA 1K with fluorescent dye	DNA ladder 1K	Waste vials
from 1 to 5	2	3	1	1	1	2	1	3
from 1 to 10	2	3	2	1	2	4	2	5
from 1 to 15	3	3	2	2	3	6	3	6

*Table 1: number of vials used in the CE carousel for different numbers of analysis*

## 9 Figure captions

Figure 1: (A) Design and dimensions of  $\mu$ LAS chip. (B) Bright-field image of the funnel region. (C) Design of the single or double channels chips.

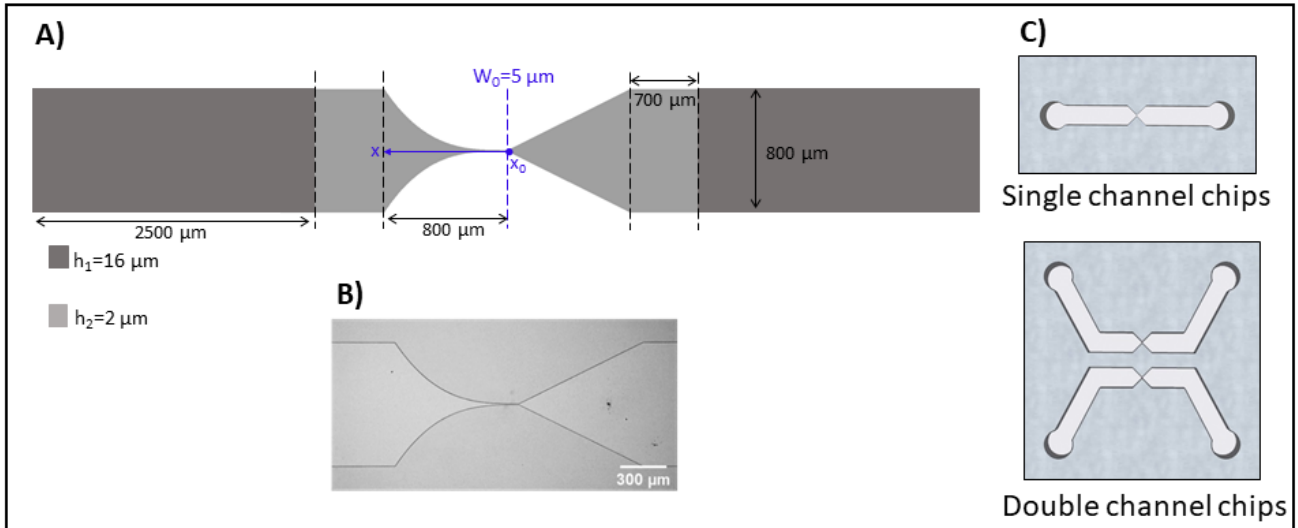


Figure 2: (A) Microfluidic chip integration for its operation. (B) Strategy for electrohydrodynamic actuation and observation by fluorescence microscopy (image extracted from (8)).

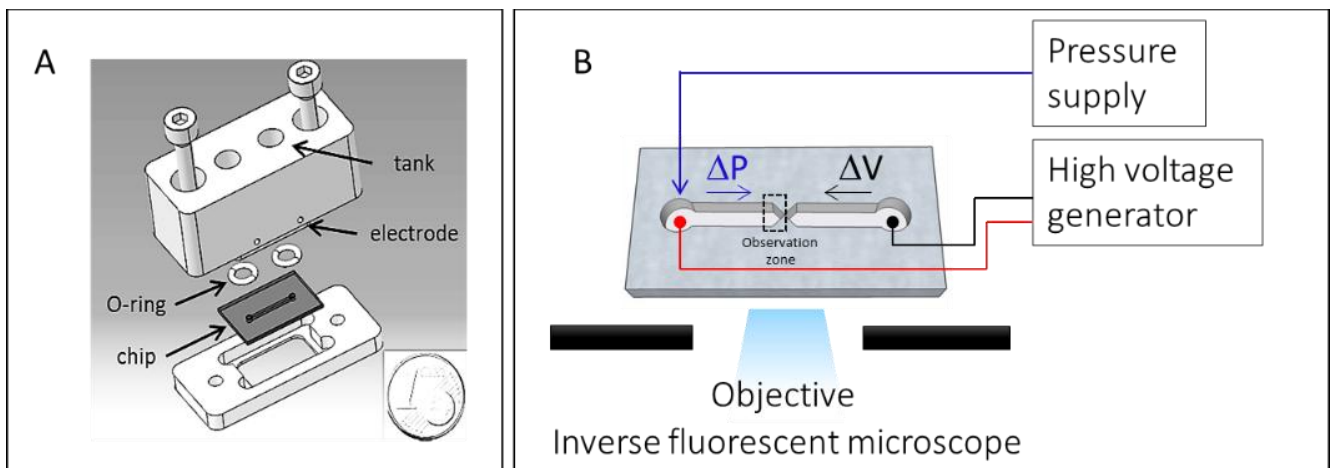


Figure 3: (A) A fluorescence micrograph with the two channel chips after a concentration time of 30 sec. (B) The fluorescence intensity profiles corresponding to each micrograph are shown in black and



the corresponding fits using Gaussian functions in blue and red. The scale bars correspond to 300  $\mu\text{m}$ .  
 (adapted from (7)).

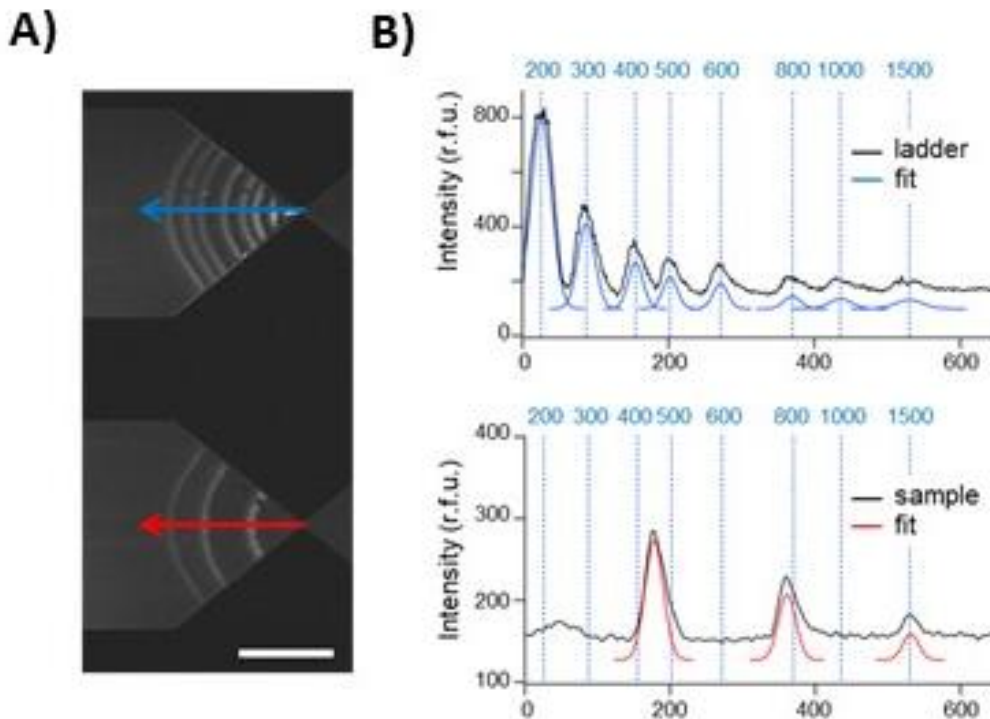


Figure 4: BIABooster analysis of DNA1K ladder (0.1-1.5 kb) at a concentration of 32  $\mu\text{g}/\mu\text{L}$ . The black and blue curves correspond to the fluorescence signal and the applied voltage during analysis, respectively. Above each peak, the fragment size is reported in bp.

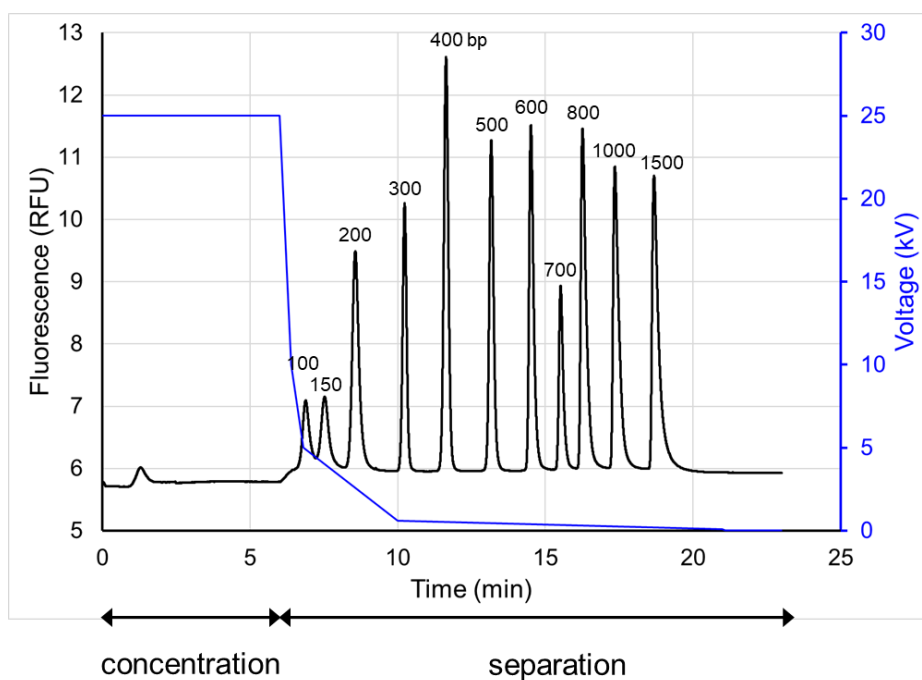


Figure 5: Typical cfDNA size profile. (A) The black curve corresponds is the fluorescent trace with two main peaks the size of which matches that of mono and di-nucleosomes. The red line is the base line. (B) The fluorescence intensity plot of A is converted into a concentration per bp over size.

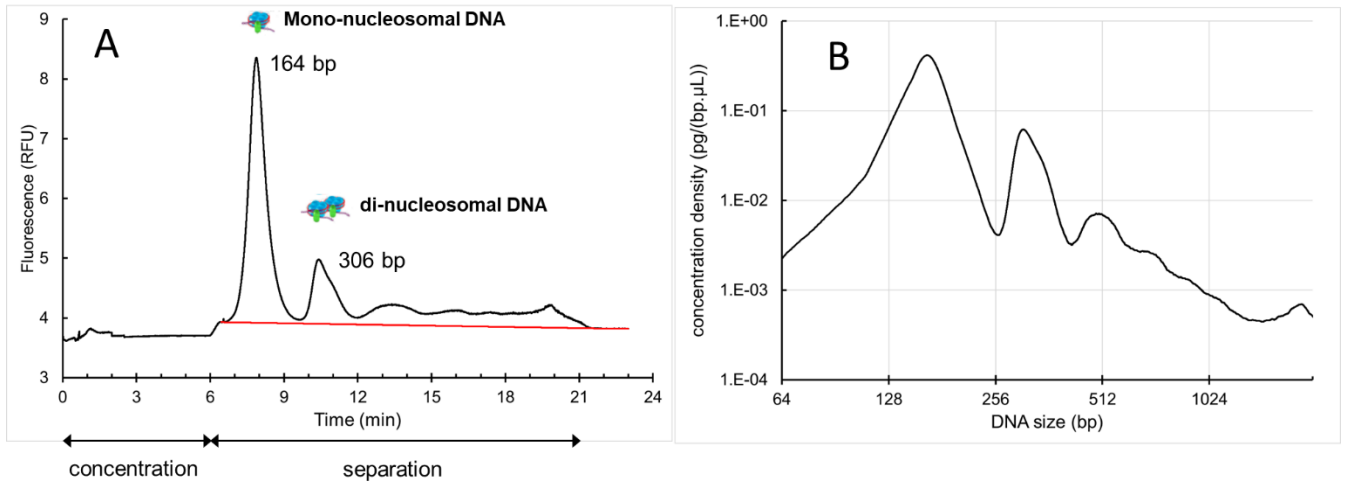


Figure 6: Anomalous cfDNA profiles. (A) The profile contains a high amount of high molecular weight DNA. It is typically obtained with blood samples collected in EDTA tubes, after a few days of aging at RT before plasma preparation. (B) cfDNA profile containing a lot of small DNA (<75bp).

