BIABooster: On-line DNA concentration and size profiling with a limit of detection of 10 fg/µL. Application to high-sensitivity characterization of circulating cell-free DNA.

SUPPORTING INFORMATION

Comtet-Louis Andriamanampisoa[1], Aurélien Bancaud[2], Audrey Boutonnet-Rodat[1], Audrey Didelot[3], Jacques Fabre[1], Frédéric Fina[4,5,6], Fanny Garlan[3], Sonia Garrigou[3], Caroline Gaudy[7], Frédéric Ginot[1,*], Daniel Henaff[5], Pierre Laurent-Puig[3,8], Arnaud Morin[1], Vincent Picot[1], Laure Saias[1], Valérie Taly[3], Pascale Tomasini[9,] Aziz Zaanan[3,7].

[1] Picometrics Technologies, 478 rue de la Découverte, 31 670 Labège, France
[2] LAAS-CNRS, Université de Toulouse, CNRS, Toulouse, France
[3] INSERM UMR-S1147, CNRS SNC5014, Paris Descartes University, Paris, France. Equipe labellisée Ligue Nationale contre le cancer.
[4] Laboratoire de Biologie Médicale, Unité de développement technologique; Assistance Publique Hôpitaux de Marseille, France.
[5] ID-Solutions, 310 rue Louis Pasteur, 34790 Grabels, France
[6] Service d'Anatomie Pathologique et Neuropathologie ; Timone II, Assistance Publique Hôpitaux de Marseille
[7] Service de Dermatologie, Vénéréologie et Cancérologie cutanée; Assistance Publique Hôpitaux de Marseille, France.
[8] Department of Digestive Oncology, European Georges Pompidou Hospital, AP-HP, Paris, France.
[9] Aix Marseille University; Assistance Publique Hôpitaux de Marseille. Multidisciplinary Oncology & Therapeutic Innovations department, Marseille, France.

* To whom correspondence should be addressed. Tel: 33 531 61 50 90; Email: fginot@picometrics.com

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Supplementary Video 1

See separate file “SupplementaryVideo1_4X.avi”.

For this experiment, also reported in Fig. 1B, 2 µL of ΦX174 double stranded DNA at 500 ng/µL was labeled with 30 µL of YOYO-1 diluted at 10 µM with 0.5X TBE. The preparation was kept in the dark during 30 min prior to dilution in running buffer.

In the video, analysis buffer flows continuously from left to right, while counter-electrophoresis is from right to left. DNA was injected in the device by the outlet, so that DNA is present both in the small and large capillaries at the start of the video. Fluorescence builds up at the junction, because of the DNA coming by the buffer flow from the left (most of the DNA indeed), and also because of the DNA pressed against the wall and coming by counter-electrophoresis from the right.

The video is accelerated 4X versus real time.

Sizing precision

Experiment	replicate	100	200	300	400	500	517	600	700	800	900	1000	1200	1517
1A	1	98	200	300	400	501	517	600	700	800	900	996	1272	1517
	2	98	200	300	399	500	516	599	699	798	898	995	1270	1516
	3	99	200	300	399	499	514	597	694	791	887	979	1248	1494
1B	1	98	199	299	399	514	528	601	705	811	922	1025	1304	1545
	2	99	199	299	399	513	527	601	702	805	910	1011	1289	1531
	3	99	200	300	400	512	526	600	700	799	899	995	1274	1516
2A	1	101	200	301	400	503	514	600	700	801	900	1004	1269	1517
	2	100	200	301	400	502	514	599	698	800	898	1001	1264	1513
	3	101	200	301	399	503	514	600	703	803	906	1007	1271	1520
2B	1	101	199	300	400	511	524	601	700	800	900	1006	1271	1519
	2	95	200	300	400	511	525	600	700	800	901	1012	1276	1520
	3	97	200	300	400	510	524	598	697	795	894	1003	1257	1503
	mean (bp)	98.8	199.8	300.1	399.6	506.6	520.3	599.7	699.8	800.3	901.3	1 003	1 272	1 518
	SD (bp)	1.7	0.4	0.6	0.5	5.4	5.6	1.2	2.7	4.7	8.2	10.8	13.6	12.0
	CV (%)	**1.7%**	**0.2%**	**0.2%**	**0.1%**	**1.1%**	**1.1%**	**0.2%**	**0.4%**	**0.6%**	**0.9%**	**1.1%**	**1.1%**	**0.8%**

Table S-1: Triplicates of the 100 bp ladder were run four times. In each run, the size of each DNA fragment (in bp) was determined as described in Experimental Section. First line gives the size given by the manufacturer.

The average CV is calculated using all the individual data of the table, after having checked the consistency of the four experiments by one-way ANOVA analysis.

The one-way ANOVA analysis between experiments has been done first using “reduced data” [(measured value – nominal value) / nominal value] for the 13 sizes of the ladder (meaning 39 values for each of the four experiments). Using this method, no significant difference was detected between the four experiments.

One-way ANOVA analysis between experiments has also been done using direct data for each DNA size (3 values in each of the four experiments); in this case, the 500/517 bands gave a significant difference between experiments: experiments 1 and 3 gave values close to the target, while experiments 2 and 4 gave increased values. In any case, the difference is smaller than 3%.

Sizing accuracy and quantification precision

	NEB 100 bp ladder	Gene DireX 50 bp ladder		Biotools 100 bp ladder	
Expected size	(duplicate)	1	2	1	2
100 bp	95	97	98	99	98
150 bp		147	146		
200 bp	198	196	196	201	199
250 bp		257	256		
300 bp	300	299	298	302	301
350 bp	-	342	340	-	-
400 bp	398	390	388	401	400
450 bp	-	446	445	-	-
500 bp	500	497	495	504	503
517 bp	512	-	-	-	-
600 bp	598	591	590	602	601
700 bp	700	691	688	695	694
800 bp	800	793	789	799	799
900 bp	919	903	896	919	920
1000 bp	1005	996	989	1028	1030
1200 bp	1207	1155/1220*	1146/1205*	-	-
1517 bp	1533	1469	1453	-	-

Expected concentration (pg/µl)	50	100		50	
Measured concentration (pg/µl)	47	129	144	84	62
Average (pg/µl)		137		73	

Table S-2: Gene DireX 50 bp ladder and Biotools 100 bp ladder were run in duplicate and sized against NEB 100 bp ladder. A duplicate of the NEB 100 bp ladder was also assessed. The table shows the exact size estimated for each DNA fragment. * manufacturer confirmed that the 1200 band is indeed a double band, without giving the exact sizes.

Signal to noise ratio and LOD for the Fragment Analyzer, DNF-474 HS kit.

Serial dilutions of the 100 bp ladder were prepared and analyzed, between a 0.5 and 50 ng/ml for the BIABooster and between 12.3 and 1000 pg/µL for the Fragment Analyzer™ system. The signal to noise ratio was measured for the 100, 200, 300, 400 and 1000 bp fragments. LOD was extrapolated at SNR=3 from the smallest concentration presenting a SNR>3. Manufacturer claims a 10% precision for the ladder concentration.

Results for BIABooster analyses are given in Table 2. Results for Fragment Analyzer™ analyses are given in table S3 below.

	SNR got from 100 bp ladder at various concentrations (13 DNA fragments, see figure 2)					LOD for a single DNA fragment (pg/µl)
	12.3 pg/µl	37 pg/µl	111.1 pg/µl	333.3pg/µl	1000 pg/µl	
100 bp	nd	nd	nd	6	26	15.9
200 bp	nd	nd	4.6	9	40.6	3.59
300 bp	nd	nd	5.8	8.5	51	3.35
400 bp	nd	nd	7.6	10	82.2	3.31
1000 bp	nd	3.9	16.9	18.4	212.5	5.42

Table S-3: SNR measured for serial dilutions of the 100 bp ladder analyzed with a Fragment Analyzer™, DNF-474 HS kit. LOD (last column) was extrapolated at SNR=3 from the smallest concentration presenting a SNR>3. nd: peak not detected by the Fragment Analyzer™ for this dilution.

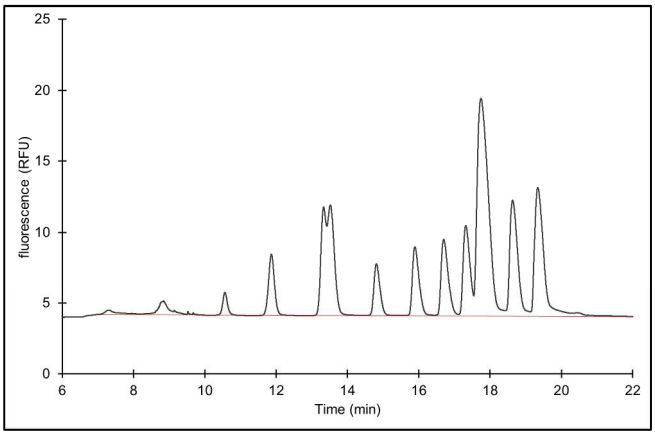
Comparison between the two digital PCR assays used in the study

Patient #	dPCR 1 (KRAS) (copies/µL)	dPCR 2 (IDQuant) (copies/µL)	ratio
83	3.3	4.8	1.46
8	10.6	9.8	0.92
102	6.6	12.1	1.82
70	11.0	6.6	0.60
79	9.8	14.6	1.50
4	17.9	13.6	0.76
13	27.4	23.8	0.87
107	27.8	26.0	0.94
27	56.2	34.2	0.61
7	47.1	32.9	0.70
31	70.4	49.6	0.70
6	64.8	33.7	0.52
57	50.9	51.2	1.01
66	52.7	52.0	0.99
30	80.7	52.4	0.65
74	53.8	73.2	1.36
36	120.4	112.0	0.93
26	183.2	182.8	1.00
87	214.9	277.2	1.29
71	402.9	572.0	1.42
		median	0.93
		mean	1.00
		25% quantile	0.70
		75% quantile	1.31

Table S-4: 20 samples of the 32 melanoma samples from sample set 2 were also assayed using the dPCR *KRAS* assay of sample set 1. Results of both dPCR assays are reported, as well as the concentration ratio between the two assays. These results demonstrate the equivalence of the two assays.

Example of calibration curves

The 100 bp NEB ladder was run according to the Experimental Section.



The two following calibration curves were then computed to determine size (left panel) and concentration (right panel) of the samples.

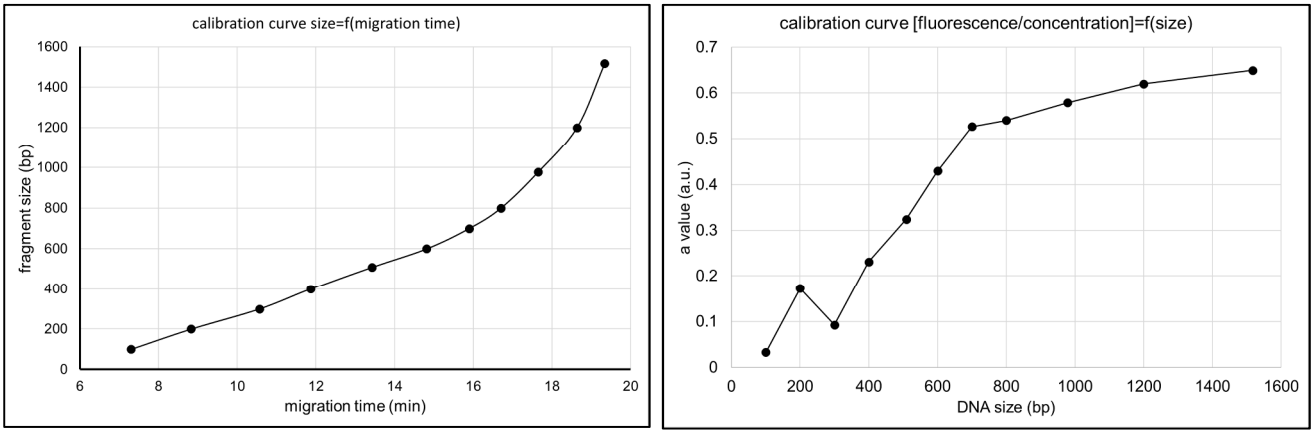


Figure S-1: left panel: calibration curve giving DNA size according to migration time. Right panel: calibration curve giving peak area for a concentration of 1 ng/mL according to DNA size.

The peak at 200 bp in the fluorescence calibration curve is reproducible; it is likely generated by the sharp changes in the temporal voltage gradient around 100 and 300 bp (see Fig. 2).

Comparison between IDXtract kit (ID-Solution) and QIAamp® Circulating Nucleic Acid Kit (Qiagen)

Blood from 24 healthy donors were collected from French Blood Establishment, (EFS-PM No. 21PLER2016-0088) in free DNA cell tubes (Roche). Plasma was prepared as described in Experimental section.

ICE DNA fragment (IDXtract, ID solutions) of 110bp was added at a concentration of 5000 copies/mL in plasma. The 4mL of plasma have then been split into two 2mL aliquots, to be extracted respectively by the IDXtract and the QiAamp CNA kits, following the protocols of each supplier.

Quantification of the ICE fragment and of nuclear cfDNA was performed using the IDQUANT kit (ID solutions; see experimental section).

The concentrations of nuclear DNA measured for each extraction technique are displayed below:

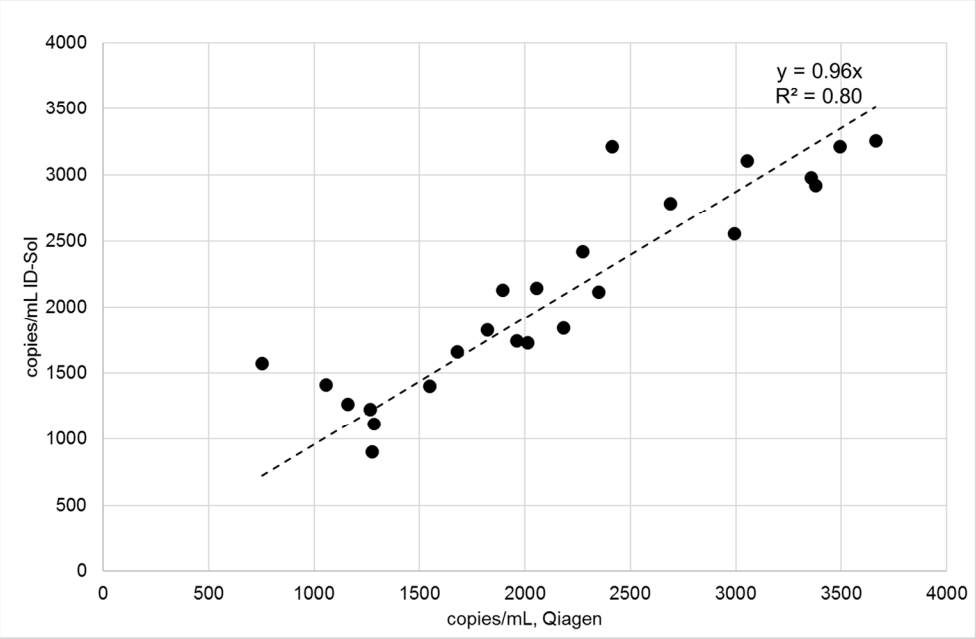


Figure S-2: Scatter plot comparing DNA concentrations recovered for 24 samples after QIAamp CNA kit (x axis) and IDXtract kit (y axis).

The two methods give clearly correlated results, with a linear regression following the equation: $[cfDNA]_{IDXtrac} = 0.96\ [cfDNA]_{QIAamp}$. So, the two methods are equivalent in terms of yield, as estimated by dPCR of a single copy gene, with an amplicon size of 65 bp.

To reinforce the comparison for small DNA fragments, representative of fragmented cfDNA, the 110 bp fragment spiked in the plasma was also quantified by dPCR. For each sample, the ratio of copy number for ID-Xtract and QIAamp was calculated. The mean value is 0.92 (median: 0.91) with a coefficient of variation (CV) of 16%. This value is not significantly different than the 0.96 value found above for the nuclear cfDNA.

The effectiveness of the two extraction techniques are very close and allow to compare the % of cfDNA in the first peak between the different tumor populations and healthy donors, regardless of the DNA extraction process.

Serial dilutions of the 100 bp ladder for LOD assessment

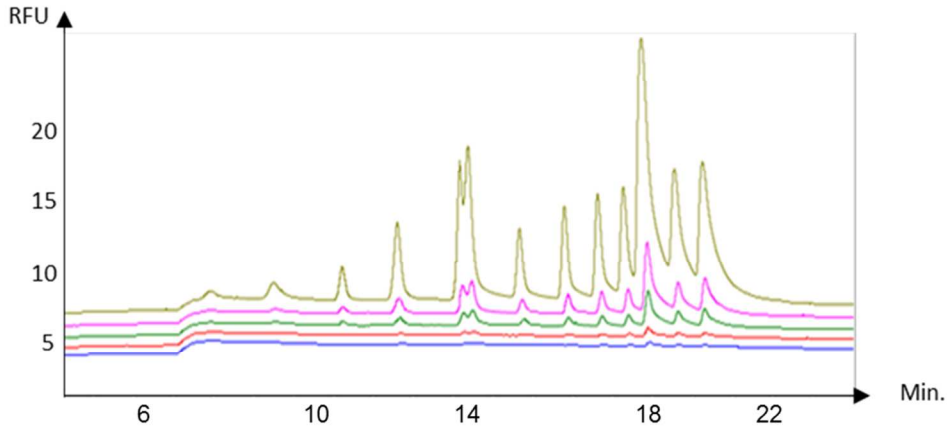


Figure S-3: Analysis of 100bp ladder serially diluted at 50ng/mL (yellow), 10ng/mL (pink), 5ng/mL (green), 1ng/mL (red), 0.5ng/mL (blue). Curves were shifted along the y axis for visibility. These analyses were used to determine LOD reported in Table 2.

Profiling circulating cell-free DNA

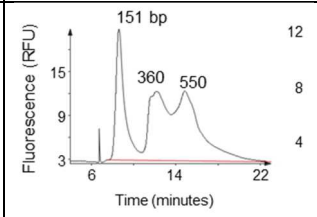
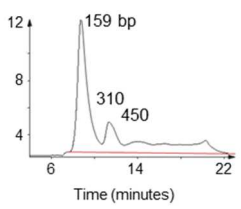
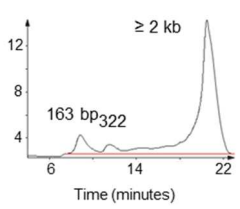
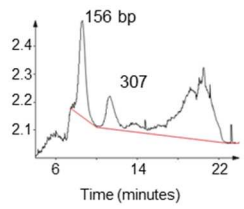
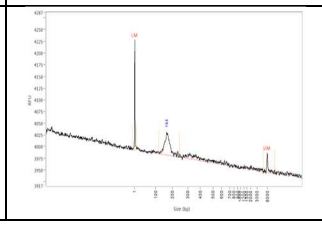
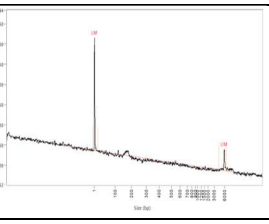
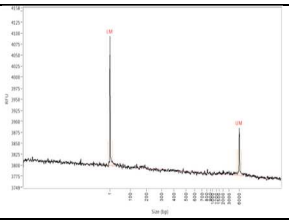
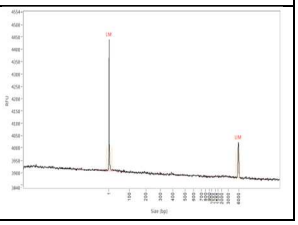
Sample conc.	500 pg/µL	184 pg/µL	49 pg/µL	1pg/µL
BIABooster				
Fragment Analyzer				

Figure S-4: Fluorescence traces representing typical profiles and typical concentrations of ccfDNA samples from melanoma patients. Upper traces: BIABooster analyses. Lower traces: Fragment Analyzer™ traces, DNF474 high sensitivity kit. The two thin peaks in each of the Fragment Analyzer™ traces are internal markers added by the system for calibration.

With Fragment Analyzer ™, only the most concentrated sample, at 500 pg/µl, gives a profile of circulating DNA, with the 2nd and 3rd peaks so weak that they cannot be quantified reliably by the system. Only the main first peak is detected for the sample at 184 pg/µl. For the two samples at 49 and 1 pg/µl respectively, they are not detected at all. In total, 22 samples from melanoma patients were analyzed both with the BIABooster and the Fragment Analyzer™: 13 were not detectable with the Fragment Analyzer™, 8 were detected only by their main first peak, and 3 gave a profile giving some insight beyond the first main peak.

Individual values of the indicator "percentage of DNA in the first peak"

Sample set 1		Sample set 2			
CRC	**Healthy 1**	**Healthy 2**	**Melanoma**	**NSCLC**	**prostate**
77.00	42.0	34.2	74.5	50.1	65.2
39.00	48.0	60.0	67.7	69.0	75.1
75.00	30.0	30.2	74.4	74.2	79.0
33.00	54.0	49.3	66.6	61.0	
75.00	52.0	59.8	69.7	61.6	
73.00	59.0	60.3	65.2	57.1	
86.00	37.0	52.1	64.0	46.7	
65.00	61.0	46.5	59.6	80.6	
68.00	42.0	60.5	66.2	91.9	
81.00			59.5	81.9	
79.00			53.6	63.9	
61.00			59.8	82.4	
83.00			64.4	65.6	
81.00			57.1	66.2	
			38.1	59.0	
			76.9	62.8	
			63.4	70.4	
			51.2	60.6	
			36.5	62.7	
			52.4	66.4	
			71.0	57.5	
			40.5	77.2	
			63.1	62.2	
			65.0	74.9	
			67.3	86.6	
			49.0	56.8	
			85.5	46.7	
			85.3	36.5	
			82.8	48.0	
			53.3	66.1	
			60.7	68.7	
			68.0	56.8	

Table S-5: Individual values of the indicator "percentage of DNA in the first peak", meaning [75-239bp]/[75-1549bp]x100.

The distributions of healthy 1 and healthy 2 are completely equivalent, with a Mann-Whitney test giving a p-value of 0.48.

Results for the indicator "percentage of DNA in the first peak" without pooling healthy donors

Even without pooling healthy samples from the two data sets, the indicator "percentage of DNA in the first peak" is significant, as illustrated below:

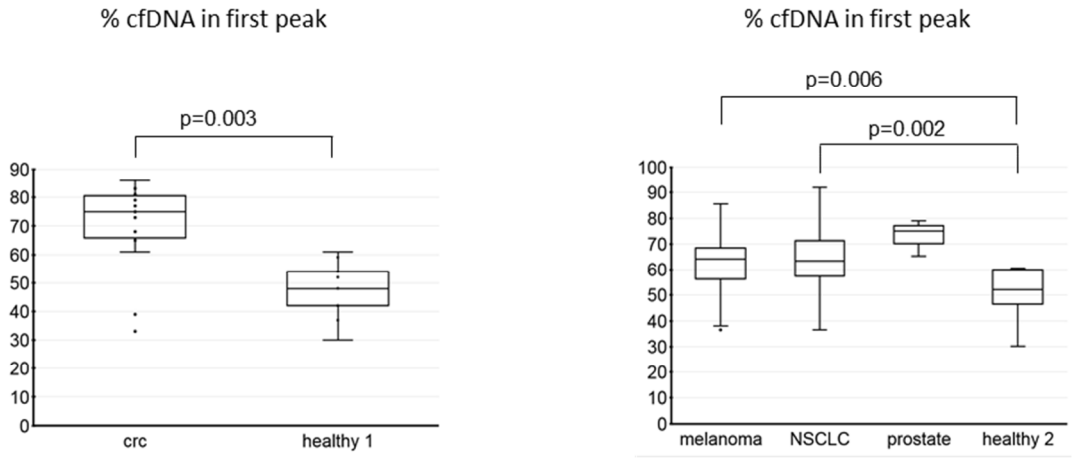


Figure S-5: box and whiskers plots corresponding to that of Figure 5 right panel, with no pooling of healthy donors from each sample set.

Separation after concentration is insensitive to salt

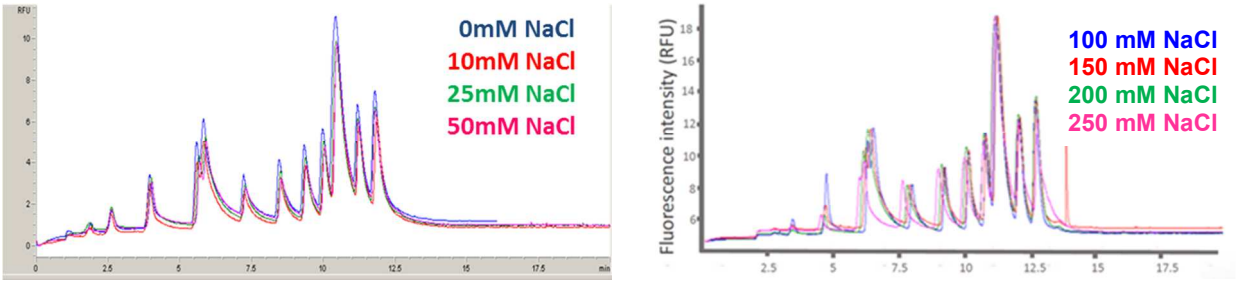


Figure S-6: Increasing amount of NaCl were added to the NEB 100 bp ladder. These samples were then analyzed with the BIABooster, with an increased time for the concentration phase (not shown). The migration profile of the ladder is not modified by the presence of salts.

Experimental Section – supplementary information

Peak-to-peak noise definition and LOD calculation

Noise is the average of the background noise over a lag of one minute before and after the peak. The background noise is calculated between the maximum peak and the minimum peak. This background noise does not consider the drift of the baseline (see graphical illustration).

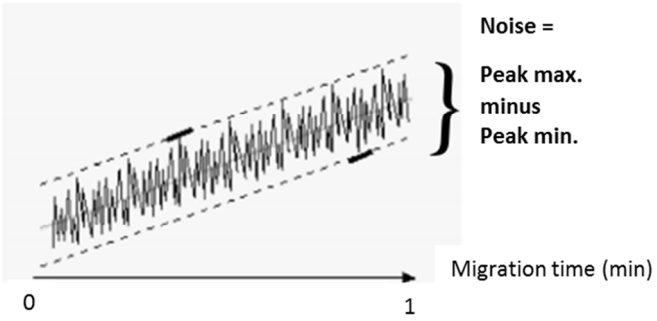


Figure S-7: Graphical illustration of the peak-to-peak noise definition: The x axis spans 1 minute. Bold segments of the dotted lines indicate how the maximum and minimum signals are taken for noise calculation.

The signal to noise ratio (SNR) is the ratio between peak height (baseline deduced) and noise.

The LOD was calculated by extrapolating the DNA concentration for a signal to noise ratio (SNR) of 3 from the less concentrated sample giving a SNR greater than 3, according to equation $LOD_{Xbp} = (3 \times C_{A\,x}\,\%Xbp) / SNR_A$, where LOD_{xbp} is LOD for fragment of size Xbp, C_A is the lowest concentration presenting a SNR >3 for fragment Xbp, SNR_A is the signal to noise ratio at concentration C_A for fragment Xbp, and %Xbp is the proportion in mass of fragment Xbp within the ladder, according to manufacturer specification. Fig. S3 gives the BIABooster fluorescence traces used to measure SNR and LOD.

dPCR for sample set 1: detailed method and results of positive and negative controls

Samples used in this study were selected among those of the clinical PLACOL study which have either the G12V or the G13D mutation in the *KRAS* gene.

For both *KRAS* assays, the sequences of the forward and reverse primers were 5'-AATATAAACTTGTGGTAGTTGGAGC-3' and 5'-GCTGTATCGTCAAGGCACTCT-3' respectively. For the *KRAS* G12V assay, the sequence of the wild type probe was 5'/VIC/-CTACGCCACCAGCTC-/MGBNFQ*/3' and the sequence of mutant probe 5'/6FAM/-ACGCCAACAGCTC-/MGBNFQ*/3'. For the *KRAS* G13D assay, the sequence of the wild type probe was 5'/VIC/-TGGTGGCGTAGGCA-/MGBNFQ*/3' and the sequence of mutant probe 5'/6FAM/-CTGGTGACGTAGGCA-/MGBNFQ*/3'.

Reactions were performed in a 25 µL reaction volume which consisted of 5-9 µL of extracted DNA, 0.8 µM of the primers and 0.4 µM of TaqMan® probes. Emulsion was generated using the RainDrop Digital PCR Source (RainDance Technologies, Billerica, US). During this step, samples are compartmentalized into millions of 5 picolitre droplets. The emulsions were thermal-cycled using a BioRad® thermal cycler (MJ-Mini, S1000, or C1000 touch) as follows: 2 min at 50°C, 10 min at 95°C (using a 0.6°C/second ramp rate), followed by 45 cycles of: 95°C, 15 s and 64°C, 1 min (using a 0.6°C/min ramp rate), with an ultimate step of 10 min at 98°C. After completion, the emulsions were either stored at 4°C or processed immediately to measure the end-point

fluorescence signal from each droplet using the RainDrop Digital PCR Sense. Data were analysed using the Raindrop Analyst II software as described by the manufacturer. The probe bearing VIC-fluorophore (λ_{ex} 538 nm / λ_{em} 554 nm) was designed to be specific to the WT *KRAS* allele, while the probe bearing FAM fluorophore (λ_{ex} 494 nm / λ_{em} 518 nm) was able to specifically hybridize to the mutated *KRAS* sequence (p.G13D or p.G12V). The Limit Of Blank was calculated equal to 1 for those assays, from the analysis of wild-type human genomic DNA (n= 9) and commercially available plasma DNA samples of healthy donors (n= 17 from which 7 were purchased from Biological Specialty Corporation and 10 from Biopredict), as described earlier (see ref. 19 of the paper). The mean number of droplets red per experiments was 4,442,158 droplets (Standard deviation of 187,621 droplets). For further analysis, total number of droplets was normalized to 5 million as previously described[19].

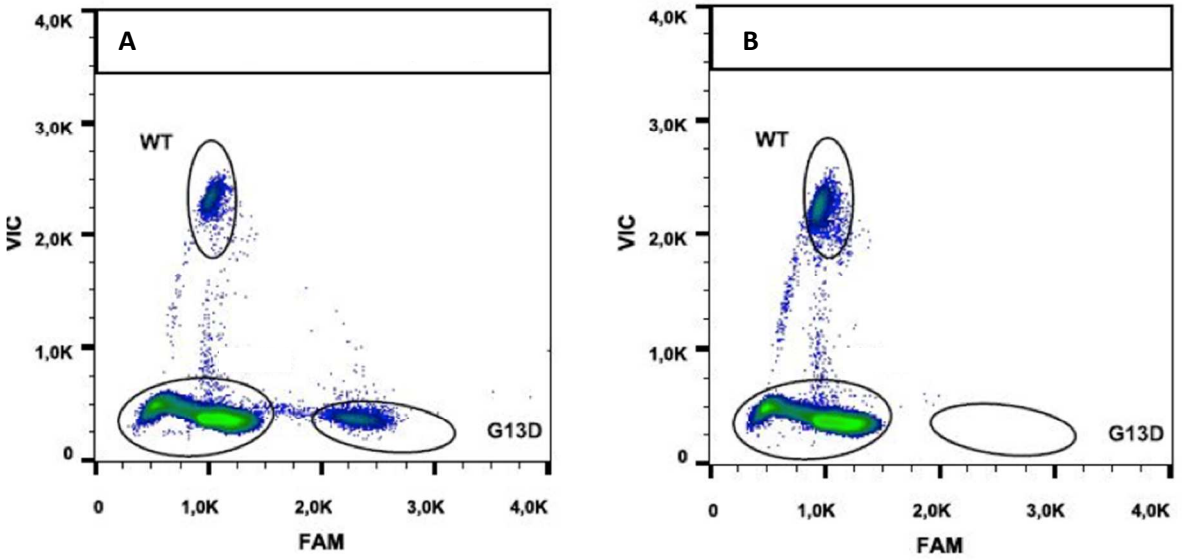


Figure S-8: Example of KRAS G13D detection digital PCR assay (60bp amplicon): Plots were obtained from dPCR analysis using home-made Taqman assay targeting KRAS G13D mutation (FAM probe) and corresponding KRAS wild-type sequence. As positive and negative controls, LoVo ATCC® CCL229™ human cell line DNA positive control (A) and human wild-type genomic DNA (B) have been used, respectively. 20ng of input DNA were used per experiment.

Samples (see above)	Wild-type (WT) droplets	Mutant (Mut) droplets	Number of analysed droplets	dPCR measured concentrations ng/µL*	Qubit measured concentrations ng/µL
LoVo ATCC® CCL229™ (human cell line) (Positive control)	1,975	4,137	4,523,876	10.1	10
Wild type genomic DNA Promega (Negative control)	6,017	0	3,856,546	11.7	10

Table S-6: This table shows results of positive and negative controls of the duplex dPCR for sample set 1. The ratio between positive and accepted droplets is converted in concentration (ng/µL; 3.3 pg/copy). Controls were also assayed by fluorimetry (Qubit™; last column). * droplets were normalized to 5 million for this calculation.

dPCR for sample set 2: detailed method and results of positive and negative controls

cfDNA was quantified by digital PCR using IDQUANT kit (id-Solutions, Grabels, France). This kit comprises a duplex ddPCR™ which allows quantification of a single copy human gene (67 bp amplicon) as well as an exogenous DNA fragment of 110 bp commonly used for inter-assay normalization of cfDNA extraction. The kit uses TaqMan® probes.

The human gene used for quantification of cfDNA in the IDQUANT kit is a proprietary information of ID-Solutions. Amplicon is 65 bp long. Forward primer is located in an exon, and reverse primer is located in an intron, to avoid mRNA amplification. The associated TaqMan™ probe is labeled with FAM. The internal standard spiked in plasma, before DNA extraction, is a DNA fragment 110 bp long, with no homology with a human sequence. The associated TaqMan™ probe is labeled with HEX. The validation file of the PCR design is given in the supplementary file "ValidationFile IDQUANT.pdf".

ddPCR™ was performed using a QX200 system (Bio-Rad; software version 1.7). Reactions were performed in duplicate in a 21 µL reaction volume containing 5 µL of extracted DNA, 3 µl water, 13 µL of ARM-Mix. Droplets were generated using the droplet generator of the system, after having added 70 µl of oil. 40 µl of the generated emulsions were amplified in a PCR plate with a C1000 Touch™ Thermal Cycler (BIO-Rad) as follows: 10 min at 95°C (polymerase activation), 40 cycles of denaturation at 95°C for 15 s followed by elongation at 60°C for 60s, 10 min at 98°C for droplet maturation, then cooling down at 12°C. Quantification was performed by the system, according to manufacturer instructions. The mean number of droplets red per replicate was 15 000. The duplicates were summed up to give the results.

Examples of positive and negative controls are given below:

Sample	Conc(copies/µL)	TotalConfMax	TotalConfMin	Positives	Accepted Droplets
TPC IDQUANT (positive control)	41,5	45	38,1	565	16292
NAC	0,0785	0,375	0,0033	1	14980

Table S-7: This table shows results of positive and negative controls of the duplex ddPCR™ for sample set 2 (BioRad system). The ratio between positive and accepted droplets is converted in concentration (copies/µL). Counting statistics are displayed as 95% confidence maximum and minimum concentrations respectively.

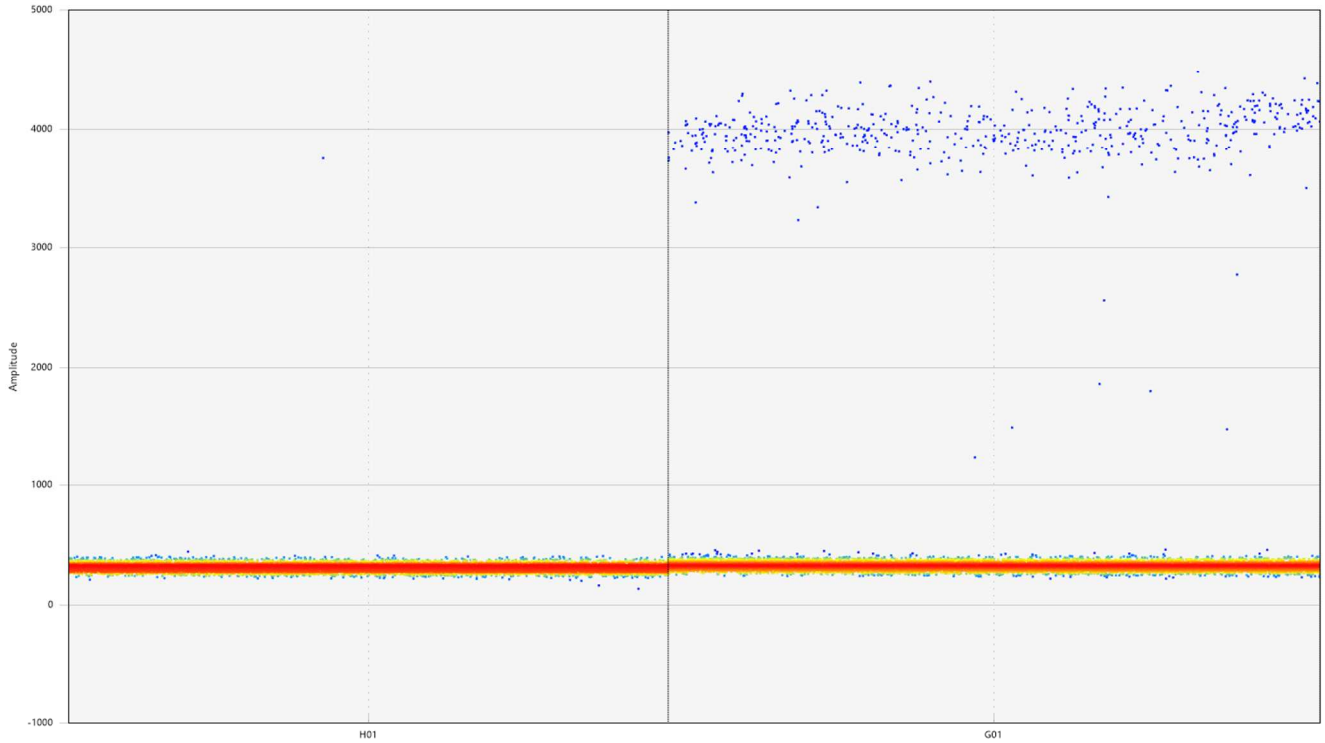


Figure S-9: This graph displays raw fluorescence of droplets from the positive and negative controls. These raw data correspond to the concentration results given in the table above.