Electronic Supplementary Material (ESI) for Analyst.

Supplementary Material

Acoustic detection of a mutation-specific Ligase Chain Reaction in serum coupled with Liposome amplification

Nikoletta Naoumi[1,2], Monica Araya-Farias[3,4], Maria Megariti[2], Lucile Alexandre[3,4], George Papadakis[2], Stephanie Descroix[3,4], Electra Gizeli[1,2,*]

[1] Department of Biology, University of Crete, Vassilika Vouton, Heraklion, 70013, Greece
[2] Institute of Molecular Biology and Biotechnology-FORTH, 100 N. Plastira Str., Heraklion 70013, Greece
[3]Laboratoire Physico-Chimie Curie, CNRS UMR 168, Institut Curie, PSL Research University, Paris, France
[4]Institut Pierre-Gilles de Gennes for Microfluidic (IPGG), Paris, France

***Corresponding author**

Email: gizeli@imbb.forth.gr

Section 1: Materials and Methods

S1. Acoustic experiments. Measurements were performed at 25 °C in the continuous flow rate of 50 μL/min, unless otherwise stated. Prior use, the 5MHz gold-coated crystals (AWS, S.L. Paterna, Spain) were cleaned with Hellmanex 2%, rinsed with mili-Q water, dried under N2, and treated for 30 min with UV/ozone (Ossila, Ltd., Sheffield).

S2. Acoustic detection of b-BSA and Nav. b-BSA was prepared after incubation of BSA lyophilized powder (Sigma-Aldrich) with biotin-$(AC_5)_2$-Sulfo-Osu linker (Dojindo) in a molar ratio of 1:10 for 1 h and 30 min at RT and purified using the Microcon-30kDa Centrifugal Filter Unit with Ultracel-30 membrane (Merck).

S3. dsDNA BRAF fragment production. The PCR products were mimicking a 277bp DNA region of the BRAF gene carrying or not the *BRAF* V600E mutation. For the reaction, 10 μL of the KAPA2G Fast HotStart ReadyMix (KAPABIOSYSTEMS) were mixed with 10 pmol of *BRAF*-gen-Fw and 10 pmol of *BRAF*-gen-Rv primers in a total volume of 20 uL. As template, 10 ng genomic DNA BRAF V600E 50 % or *BRAF* wt reference standard (Horizon Discovery Ltd) were added. The reaction was subjected to 30 cycles of 10 sec at 95 ºC followed by 10 sec at 57 ºC and 10 sec at 72 ºC. An initial denaturation step for 5 min at 95 ºC and final extension step at 72 ºC for 1 min were included in the amplification protocol. A no template control (NTC) was included in every run. Following amplification, PCR products were purified with the Nucleospin Gel and PCR clean-up kit (Macherey Nagel) according to manufacturer's instructions. The presence of specific amplification products was checked by agarose gel electrophoresis.

S4. LCR protocols. Regarding the initial protocol consisted of 99 cycles, as part of the optimization procedure we firstly set the annealing and ligation temperatures to 70 °C across 50 cycles of Ligase Chain Reaction (LCR). This approach resulted in non-specific ligation. In response, we adjusted the annealing and ligation temperatures to 75°C, at which point we successfully detected 10 ng of BRAF V600E 50% with high specificity. To enhance the assay's sensitivity and reduce the detection limit, we incrementally increased the number of cycles within the LCR protocol extending it to 99 cycles. This adjustment maintained the assay's high specificity significantly decreasing the detection limit.

As concerns the second and more optimized protocol consisted of 30 cycles our investigation explored a range of variables to enhance the protocol's efficacy. This included varying the enzyme amount from 1 unit to 2.5 units, adjusting the annealing/ligation temperatures among 65°C, 70°C, and 75°C, and modifying the duration of this process between 5 seconds and 10 seconds. Additionally, we experimented with the protocol's cycle number, testing 30 and 35 cycles, and the amount of probes used, comparing 2 picomoles and 5 picomoles for each probe. This comprehensive approach allowed us to meticulously assess each parameter's impact on the protocol's performance, aiming to optimize assay conditions for improved results.

S5. Capture of ctDNA from human serum or plasma using the FB automated platform.
Collection of blood samples. Blood samples were obtained from healthy blood donors in non-fasting conditions. The samples were collected in a bag system LQT6283LE (Macopharma, France) using CPDA as an anticoagulant and centrifuged at 4800g for 10 min to separate plasma. The plasma was removed, aliquoted, and stored at −40 °C. Before analysis, the plasma was centrifuged at 10.000g for 10 min and then filtered through 0.22 μm syringe filters (Corning, NY).

Fluidized bed set-up. PDMS chips were fabricated using the standard casting method from a micromilled mold using PDMS as the material. Capture probes were complementary to the antisense and sense strand of the BRAF-mt/wt 277 bp target. To start the experiment, the FB was initially filled with PBS-BSA 1% (both from Sigma-Aldrich) and then, the 500 μg of streptavidin (SAv)-coated magnetic beads Dynabeads MyOne Streptavidin T1 (1 μm) (Invitrogen) & the Dynabeads M-270 Carboxylic Acid (2.8 μm) (Invitrogen) mixed in 1:1 ratio (250 μg each) were introduced manually and guided at the right position in the chip by moving

a magnet. After the bead loading, a magnet with a field strength of 1.47 T (NdFeB) was aligned with the microfluidic chamber and positioned at a distance of 2 mm from the chip inlet. To introduce vibration and achieve a good homogeneity of the beads during the experiment, a miniature electric motor (Model 304-101, Precision Microdrives Ltd, London, UK) coupled with a partially off-balanced mass, operated at 2.4 V and 0.04 A. The motor was strategically positioned on the inlet tubing. Following capturing of the complex "capture probe-DNA target" on the streptavidin-coated beads a last step of PBS injection was performed. Note that, Pressure and flow rates required for the experiments were controlled with All-in-One (A-i-O) software (Fluigent, France).

Sample preparation before LCR and target release from beads. Following capturing, the beads were pushed out from the chip and collected into a 1.5 mL tube. Then, the tube was placed in the magnetic rack for 3 min, the supernatant was removed and the beads were mixed with 50 µL of 4 µM biotin diluted in mili-Q water for 10-15 minutes at RT. Following incubation, three washing steps with mili-Q water took place. After the last washing step, the beads were resuspended in 16 µL mili-Q water and heated at 95 °C for 5 min. Following heating, the beads were immediately transferred in a magnetic rack placed into the ice, to prevent the rehybridization of the DNA target with the capture probes BRAF-rv-80 bp & BRAF-fw-80 bp. Finally, 15 µL of the supernatant were collected and mixed with the exponential LCR cocktail as mentioned above in the section 99 cycles and 30 cycles LCR.

Table S1: *BRAF* LCR probes, fluidized bed capture probes and 20nt-DNA-chol. The red letter represents the single nucleotide polymorphirsm or the mismatch discrimination nucleotide.

Name	**Nucleotide Sequences**	**Application**
BRAF-gen-Fw	ACC TAAACTCTTCATAATGCTTGC	PCR
BRAF-gen-Rv	TGAGACCTTCAATGACTTTCTAGT	
BRAF-p1(-b)	**biotin**-GGATCCAGACAACTGTTCAAACTGATGGGACCCACTC CATCGAGATTTC**T**	LCR probes
BRAF-p2(-chol)	CTGTAGCTAGACCAAAATCACCTATTTTTACTGTGAGGTCTTC AT GAAGA-**cholesterol**	
BRAF-cp1	**A**GAAATCTCGATGGAGTGGGTCCCATCAGTTTGAACAGTTGTC T GGATCC	
BRAF-cp2	TCTTCATGAAGACCTCACAGTAAAAATAGGTGATTTTGGTCTA GCTACAG	
BRAF-p2.2	CTGTAGCTAGACCAAAATCACCTATTTTTACTGTGAGGTCTTC ATGAAGAGGGGGTAGGAGTGTCGTT	
BRAF-cp1.2	GAAATCTCGATGGAGTGGGTCCCATCAGTTTGAACAGTTG TCTGGATCC	
BRAF-cp2.2	TCTTCATGAAGACCTCACAGTAAAAATAGGTGATTTTGGT CTAGCTACAG**A**	
BRAF-fw-80 bp	ATATATCTGAGGTGTAGTAAGTAAAGGAAAACAGTAGATCTC ATTTTCCTATCAGAGCAAGCATTATGAAGAGTTTAGGT-**biotin**	Fluidized bed Capture probes
BRAF-rv-80 bp	**biotin**-GAATTGAGGCTATTTTTCCACTGATTAAATTTT TGGCCCTGAGATGCTGCTGAGTTACTAGAAAGTCATTG AAGGTCT CA	
BRAF-mt-277bp	ACCTAAACTCTTCATAATGCTTGCTCTGATAGGAAAATGAGA TCTACTGTTTTCCTTTACTTACTACACCTCAGATATATTTCTT CATGAAGACCTCACAGTAAAAATAGGTGATTTTGGTCTAGCT ACAG**A**GAAATCTCGATGGAGTGGGTCCCATCAGTTTGAACA GTTGTCTGGATCCATTTTGTGGATGGTAAGAATTGAGGCTAT TTTTCCACTGATTAAATTTTTGGCCCTGAGATGCTGCTGAGTT ACTAGAAAGTCATTGAAGGTCTCA	BRAF target sequences
BRAF-wt-277bp	ACCTAAACTCTTCATAATGCTTGCTCTGATAGGAAAATGAGA TCTACTGTTTTCCTTTACTTACTACACCTCAGATATATTTCTT CATGAAGACCTCACAGTAAAAATAGGTGATTTTGGTCTAGCT	

	ACAG**T**GAAATCTCGATGGAGTGGGTCCCATCAGTTTGAACA GTTGTCTGGATCCATTTTGTGGATGGTAAGAATTGAGGCTAT TTTTCCACTGATTAAATTTTTGGCCCTGAGATGCTGCTGAGTT ACTAGAAAGTCATTGAAGGTCTCA	
20nt-DNA-chol	**cholesterol**-GATGAACGACACTCCTACCCCC	Acoustic detection

Section 2: Results

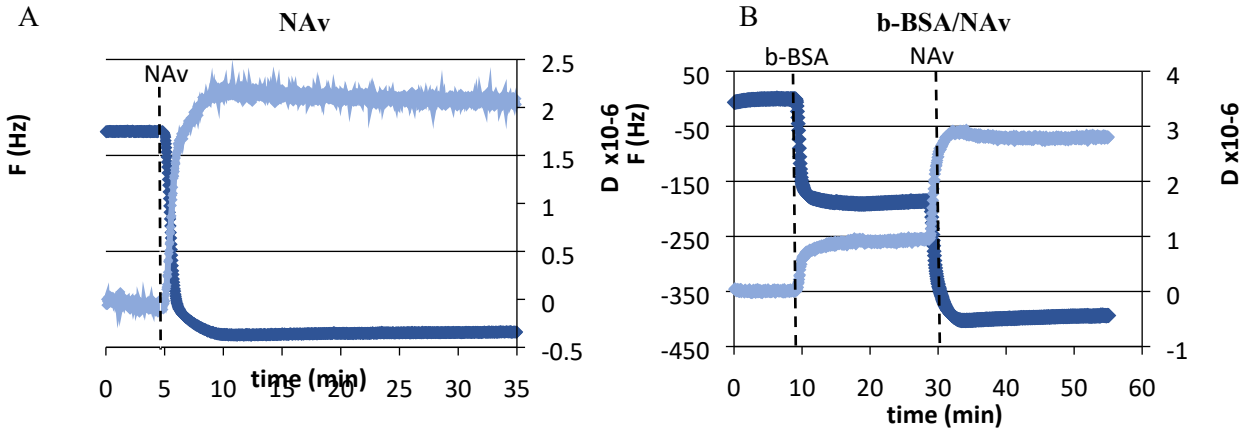
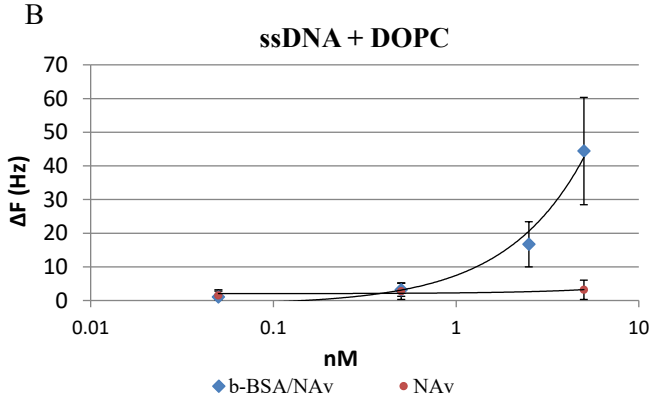


Figure S1. Real time frequency and dissipation monitoring of (A) NAv & (B) b-BSA/NAv absorption on gold sensor surface. Light and dark blue curves correspond to dissipation and frequency changes, respectively.

A

	NAv		b-BSA/NAv	
[DNA] nM	ΔD x10^{-6}	SD	ΔD x10^{-6}	SD
0	0.12	0.011	0.15	0.083
0.05	0.19	0.071	0.21	0.053
0.5	0.48	0.125	0.27	0.063
2.5	2.32	0.580	Not tested	
5	4.35	1.310	0.38	0.159

B



	NAv		b-BSA/NAv	
[DNA] nM	ΔF (Hz)	SD	ΔF (Hz)	SD
0	0.00	0.00	0.00	0.00
0.05	1.05	2.10	1.47	1.32
0.5	3.18	1.90	2.76	2.48
2.5	16.70	6.76	Not tested	
5	44.41	16.00	3.18	2.88

Figure S2. A) Comparison of dissipation changes observed upon addition of 200nm DOPC liposomes on various concentrations of the 50nt ssDNA pre-absorbed on NAv and b-BSA/NAv coated surfaces. B) Comparison of frequency changes observed upon addition of 200nm DOPC liposomes on various concentrations of the 50nt ssDNA pre-absorbed on NAv and b-BSA/NAv coated surfaces. The Figure is presented in logarithmic scale.

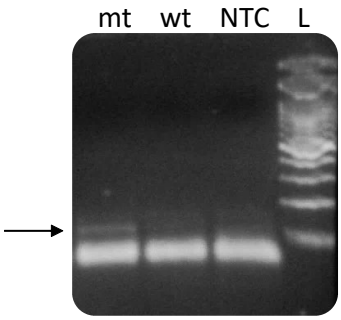


Figure S3. Exponential LCRs of 99 cycles were performed for various amounts of BRAF dsDNA template. Finally, we detected as few as 1.67x10^5 copies of mt DNA (mt) which resulted in clearly visible products. The wt (3.34x10^5 copies) and NTC controls produced no detectable products, demonstrating the high specificity of our assay. The black arrow shows the 100bp LCR product. A 100 bp DNA ladder (L) was used. Note that, the mt target consisted of 50 % V600E (mt) sequence and 50 % wt but here only the mt number of molecules are mentioned.

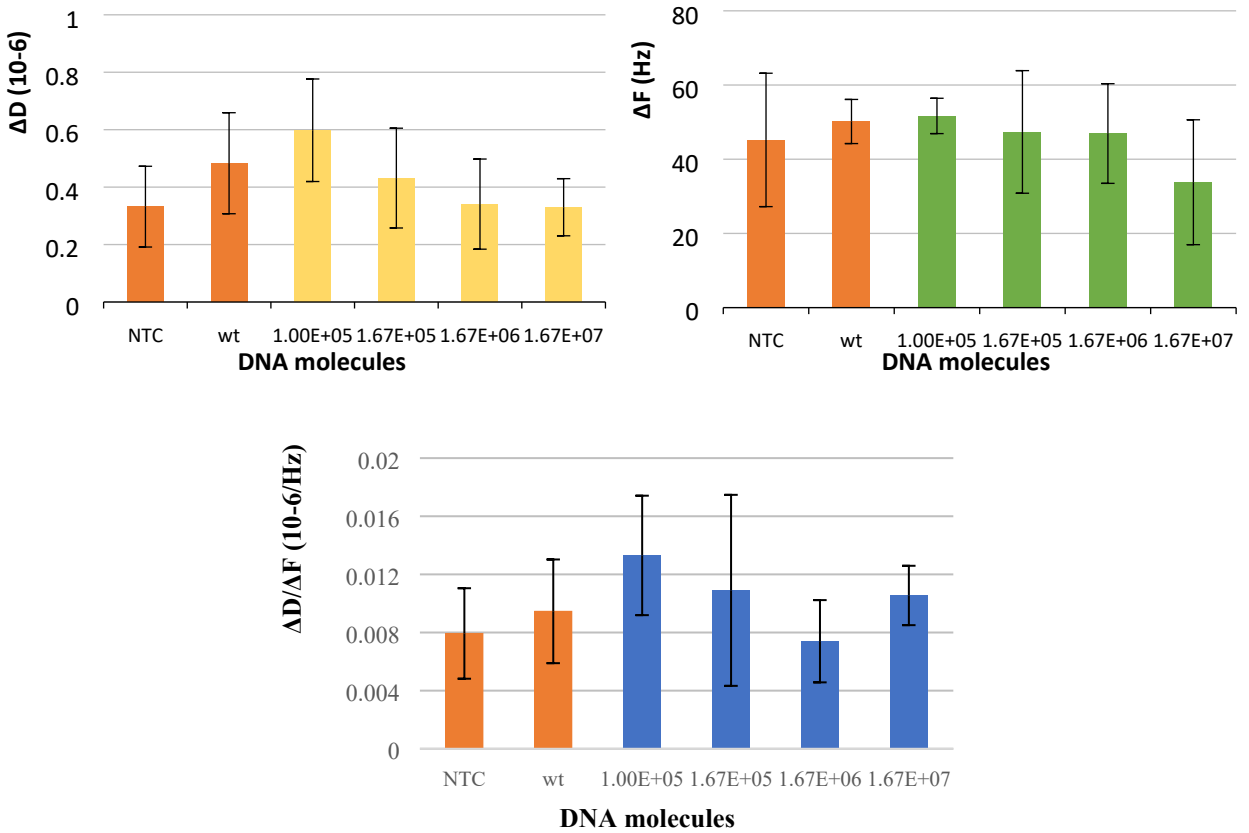


Figure S4. ΔD, ΔF and ΔD/ΔF values obtained from the addition and direct detection (no liposomes) of various LCRs containing *BRAF* V600E DNA on b-BSA/NAv coated surface. Orange columns corresponds to reactions containing no template (NTC) and 1.67E+06 wt molecules, respectively.

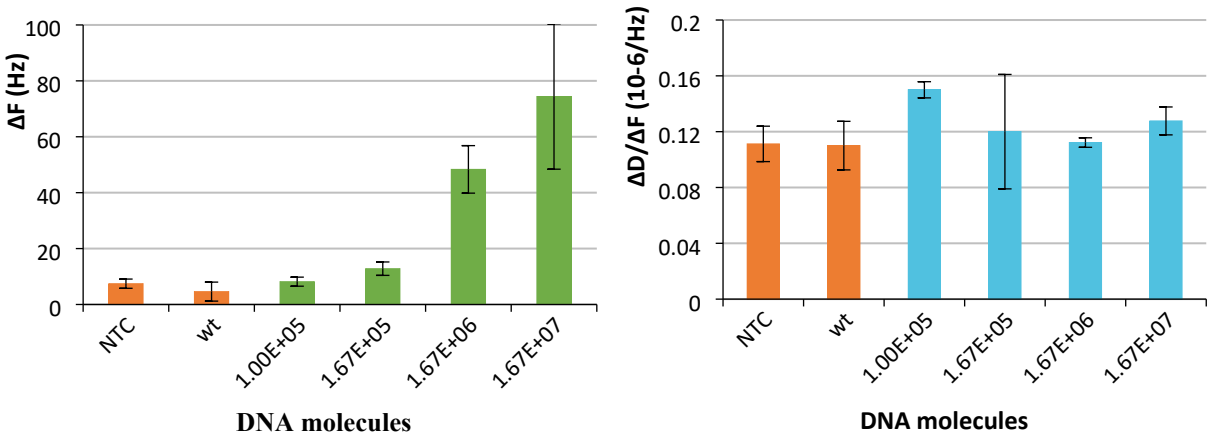


Figure S5: ΔF and ΔD/ΔF values obtained from the detection of LCR of various amounts of the BRAF V600E DNA (1.00E+05 – 1.67E +07) through 200 nm POPC liposomes. Orange columns correspond to reactions containing no template (NTC) and 1.67E+06 wt molecules, respectively. Note that the large error bars are mainly attributed to variability within different LCRs.

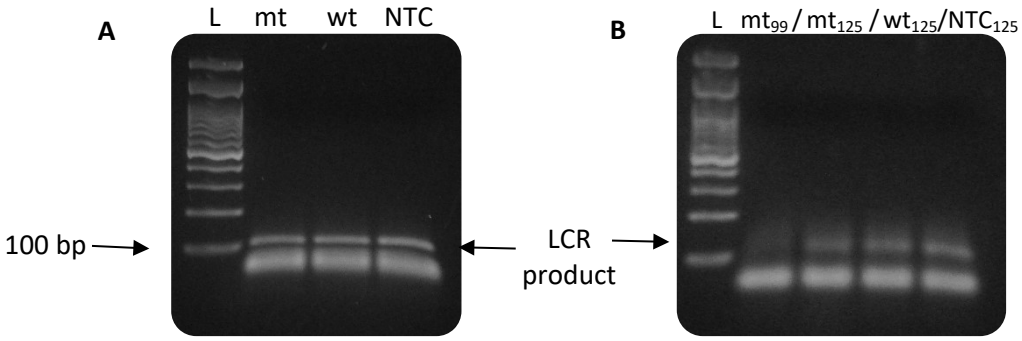


Figure S6. LCR analysis through gel electrophoresis. A) 3 x10^4 mt (50 %), 3 x10^4 wt molecules of BRAF sequence and no template (NTC) were subjected to LCR of 149 cycles. B) 3 x10^4 mt (50 %) (mt_{99} and mt_{125}), 3 x10^4 wt molecules of BRAF sequence or no template (NTC) were subjected to LCR of 99 (lane 1) or 125 cycles (lanes 2-4). A 100 bp DNA ladder (L) was used.

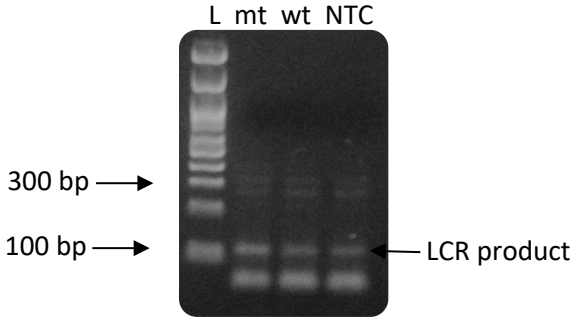


Figure S7. Target-independent ligation following a reaction of 35 amplification cycles. 1.67 x10^5 *BRAF* V600E (50%), 3.34 x10^5 *BRAF* wt molecules & no template control. Wt and negative reactions generated by-products visualized in 2% agarose gel through target-independent ligation.

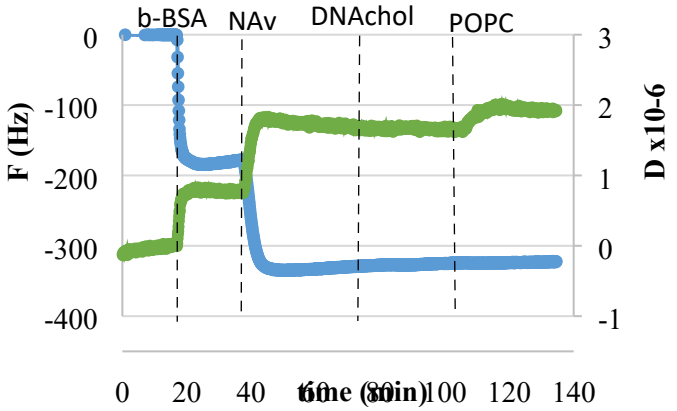


Figure S8. Specificity assay; the addition of the 20nt chol-probe produced negligible changes in the acoustic signal. Green and blue curves correspond to dissipation and frequency changes, respectively.

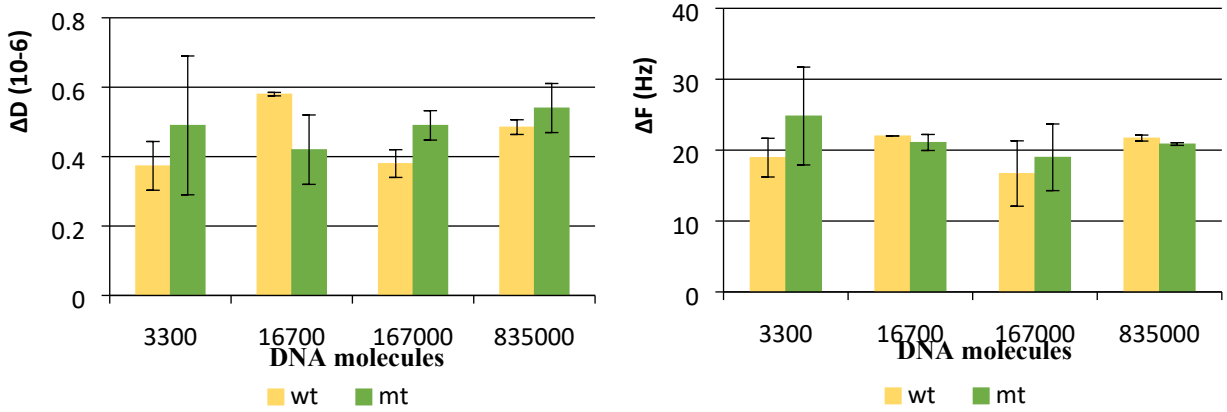


Figure S9. ΔD & ΔF values obtained from the direct detection (no liposomes) of various BRAF V600E LCR reactions on b-BSA/NAv.

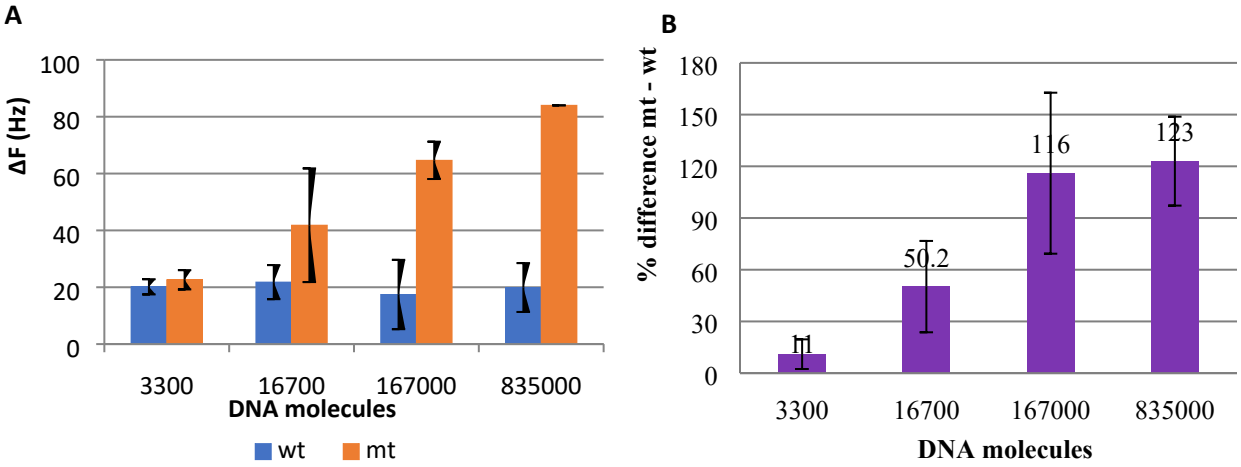


Figure S10. ΔF changes obtained during the addition of POPC liposomes on various BRAF V600E LCR reactions and their corresponding wt controls. A) Comparison of ΔF changes observed between the mt/wt reactions. B) % difference between mt and wt reactions of the same LCR preparation and acoustic experiment. Note that, in all cases, the wt control contains wt molecules in a concentration of 2 x C_{mt}.

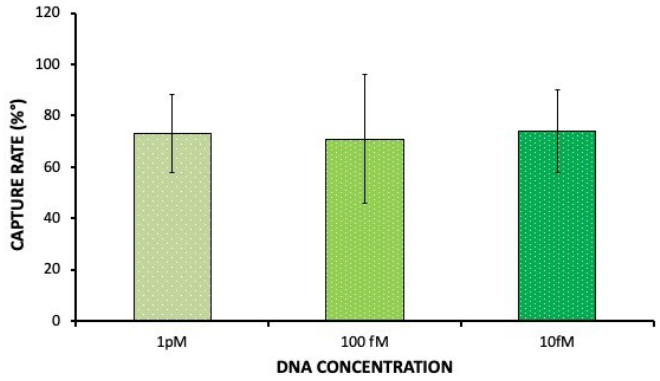


Figure S11. Capture Rate (%) of mt dsDNA 277 bp *BRAF* in human plasma by using FB. Error bars represent mean ± SD of triplicate.

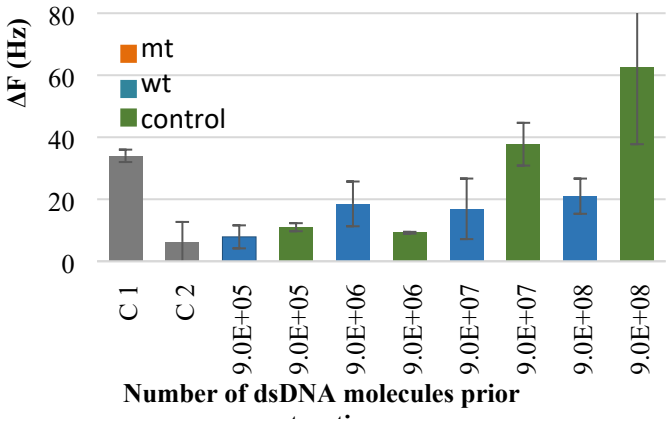


Figure S12. ΔF changes obtained changes recorded at the 200 nm POPC liposome step during the acoustic analysis of LCR derived from dsDNA 277 bp BRAF mt/wt targets spiked in human plasma samples following capturing on FB. C1 and C2 correspond to negative control plasma samples with and without capture probes. Note that, capture efficiency was about 70% ± 10%.