UNIVERSITY OF CRETE

DEPARTMENT OF BIOLOGY

Graduate programme in Molecular Biology-Biomedicine

Master thesis

Detection of *BRAF*^{V600E} mutation via a diagnostic system based on acoustic biosensors

Gesthimani-Ioanna Theodosi

Supervisors

Prof. Electra Gizeli

Dr. George Papadakis

Heraklion

July 2018

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Acknowledgements

The present study was carried out at the Biosensors Lab (Institute of Molecular Biology and Biotechnology-Foundation for Research and Technology, Department of Biology University of Crete).

I would like to take the opportunity to thank all the people who have helped and supported me during the last year. Firstly, I would like to thank my supervisor Prof. Electra Gizeli for giving me the opportunity to be a member of her group and my co-supervisor Dr. George Papadakis for his scientific knowledge and guidance, but also for the patience that he showed a lot of times. His name was the most heard word in the lab, as almost all the lab members used to ask him for his advice or opinion about the experiments. That says it all.

I owe my thanks also to some other people that I have met and collaborated with in the lab. First of all, I would like to express my gratitude to Maria Megariti who is one of the kindest and most helpful, patient and positive people I have ever met. I remember my first day in the lab when with her warm friendly smile made me feel very comfortable. From then we became friends and she was always available when I needed something or when I wanted to talk about non-experimental issues. Maraki thank you for everything and I wish you all the happiness for your marriage!

I would like also to say special thanks to Konstantinos Parasyris. The little gentleman, as I call him, my best colleague and my best friend in the lab. One of the most competent budding researchers I have met. Responsibility, conscientiousness, punctuality, enthusiasm and passion for experiments are some words that I can use to describe the way he works. I will not forget that you were always there for me. Thank you for your help in the lab, for all the funny moments that made me laugh when I was very stressed and for your persistent encouragement. I really appreciate everything. This year would not be the same without you! I wish you good luck on the exciting journey to the research world and “don’t let your dreams be dreams”.

Next, I would like to thank Fotini Papagavriil for our excellent cooperation in the biosensors lab but also in undergraduate labs (I will not forget our excursions with “propato stin kriti” and your apple pie), Aristea Grammoustianou who was willing to help me whenever it was necessary, Nikoletta Naoumi for our cooperation, and all the other members of the biosensors group. I would also like to thank Dr. Alexandra

Voutsina from Medical School (University of Crete) for providing the PNA for our experiments.

Finally, and most importantly, I would like to dedicate my master thesis to my parents, who always let me free to make my own decisions supporting me emotionally and financially. Thank you for your constant support and encouragement. Thank you for believing in me. Thank you for everything!!!

Abbreviations

wt: wild-type

mut: *BRAF*^{V600E} mutant

LAMP: loop-mediated isothermal amplification method

FIP: forward inner primer

BIP: backward inner primer

F3: forward outer primer

B3: backward outer primer

LF: loop forward

LB: loop backward

QCM: Quartz Crystal Microbalance

MAF: mutant allele frequency

LOD: limit of detection

ctDNA: circulating tumor DNA

cfDNA: cell free DNA

PNA: peptide nucleic acid

SD: standard deviation

ΔD: changes in dissipation

ΔF: changes in frequency

R: acoustic ratio (ΔD/ΔF)

PBS: phosphate buffer saline

Abstract

Diagnostic tests for detection of mutations in cancer-related genes are of great importance for cancer control and management. In this work, we introduce a new methodology based on isothermal amplification and acoustic biosensors for the detection of $BRAF^{V600E}$ mutation, which is very common in melanoma patients and it is the target of specific therapeutic approaches. More specifically, we tried to develop an assay that could have the potential to be applied in both solid and liquid biopsy. Thus, we employed both genomic DNA and DNA fragments as templates for loop-mediated isothermal amplification (LAMP) and quartz crystal microbalance (QCM) technology for the biosensor-based detection. The acoustic measurements were expressed as ratio of dissipation versus frequency change ($\Delta D/\Delta F$). However, the percentages of mutant ratio relative to wild-type, show clearer differences. Interestingly, a positive correlation between them and mutant allele frequency (MAF) was observed in case of genomic DNA. Peptide nucleic acid (PNA) was utilized to reduce the wild-type background. Finally, through the proposed method we managed to detect mutant allele with a frequency of 5% in genomic DNA and 0.1% in fragments, comparing every single mutant to its corresponding wild-type prepared with the same set of materials. The described assay holds promise to serve as a new simple, fast and cost-effective diagnostic solution that could contribute to an effective and safe treatment guidance for patients.

Keywords: biosensors, detection, BRAF, melanoma, diagnostics

Περίληψη

Η ανάπτυξη διαγνωστικών εργαλείων για την ανίχνευση μεταλλάξεων σε γονίδια που σχετίζονται με τον καρκίνο έχει βαρύνουσα σημασία για τον έλεγχο και τη διαχείριση της νόσου. Στην παρούσα εργασία προτείνεται μία μεθοδολογία που συνδυάζει την ισόθερμη ενίσχυση με τους ακουστικούς βιοαισθητήρες για την ανίχνευση της μετάλλαξης $BRAF^{V600E}$, που είναι συχνή στους ασθενείς με μελάνωμα και αποτελεί στόχο διαφόρων θεραπευτικών προσεγγίσεων. Πιο συγκεκριμένα, προσπαθήσαμε να αναπτύξουμε μία δοκιμή με δυνατότητα εφαρμογής και στη συμβατική και στην υγρή βιοψία. Για το σκοπό αυτό χρησιμοποιήσαμε γενωμικό DNA αλλά και τμήματα DNA, ισόθερμη αντίδραση πολυμερισμού μέσω βρόγχων (LAMP) για την ενίσχυσή τους και μικροζυγό (κρυστάλλου) χαλαζία (QCM) για την ανίχνευση. Οι μετρήσεις εκφράζονταν ως ο λόγος της αλλαγής στην απώλεια ενέργειας προς την αλλαγή στη συχνότητα (ΔD/ΔF). Τα ποσοστά, όμως, των μετρήσεων του μεταλλαγμένου ως προς το αγρίου τύπου έδιναν πιο σαφή αποτελέσματα. Μάλιστα, στην περίπτωση του γενωμικού παρατηρήθηκε μία θετική συσχέτιση ανάμεσα στα ποσοστά και στη συχνότητα του μεταλλαγμένου αλληλόμορφου. Περιορισμός της μη ειδικής ενίσχυσης του αγρίου τύπου DNA επιτεύχθηκε με πεπτιδικό νουκλεϊκό οξύ (PNA). Εν τέλει, κατορθώσαμε να ανιχνεύσουμε το μεταλλαγμένο αλληλόμορφο σε συχνότητα 5% στο γενωμικό και 0,1% στα τμήματα DNA, συγκρίνοντας κάθε μεταλλαγμένο με το αντίστοιχο αγρίου τύπου που είχε προετοιμαστεί με το ίδιο ακριβώς σύνολο υλικών. Η προτεινόμενη διεργασία είναι πολλά υποσχόμενη καθώς θα μπορούσε να εξυπηρετήσει σαν μία απλή, γρήγορη και οικονομική διαγνωστική λύση που θα συμβάλλει στην αποτελεσματική και ασφαλή καθοδήγηση των ασθενών.

Λέξεις-κλειδιά: βιοαισθητήρες, ανίχνευση, BRAF, μελάνωμα, διαγνωστικά

1. Introduction

1.1 BRAF (B rapidly accelerated fibrosarcoma) kinase

BRAF (B rapidly accelerated fibrosarcoma) is a human gene located on chromosome 7q34 that encodes a serine threonine protein kinase which is activated by Ras and subsequently activates the mitogen-activated protein kinase (MAPK)/ extracellular signal regulated kinase (ERK) signaling pathway[1]. The MAP kinase/ERK-signal transduction cascade is a normal outcome of extracellular factors binding to receptor tyrosine kinases (RTKs)[2,3] and physiologically regulates cell proliferation, differentiation and survival[2] (Figure 1.1). There is also an upstream negative feedback that serves as a mechanism protecting against constant pathway activation[3]. However, *BRAF* mutations, which are very common in human malignancies[4] and are also detected in nevi[5], cause constitutive activation of the pathway and disable the negative feedback mechanism[1]. Especially in melanoma patients, oncogenic alterations in *BRAF* are usually observed[4] with the $BRAF^{V600E}$ presenting the highest frequency[1]. More specifically, activating mutations of BRAF show an incidence of 50% and 90% of them are a single nucleotide mutation at codon 600 that results in substitution of glutamic acid (E) for valine (V) (nucleotide 1799 T > A, codon GTG > GAG)[1]. Although this specific mutation is most frequently found in melanoma it has been also associated with other types of cancers such as colorectal cancer, papillary thyroid cancer, non-small-cell lung cancer[6].

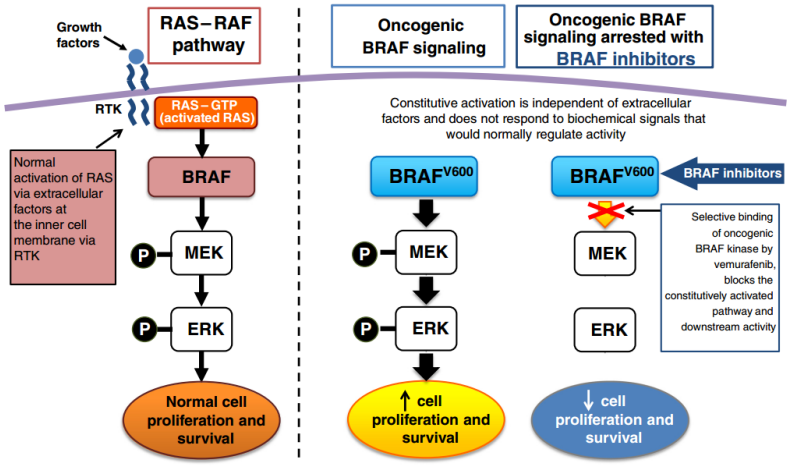


Figure 1.1: BRAF signaling pathway. Physiological activation (left), Oncogenic (middle), inhibition of oncogenic mutant (right)[1].

The fact that in a high percentage of melanoma patients the detection of oncogenic *BRAF* mutations is a common phenomenon leaded to the assumption that these tumors are heavily linked with MEK/ERK pathway, offering the chance to design therapeutic strategies based on the inhibition of its constitutive function[4]. A selective and potent inhibitor of oncogenic mutant BRAF kinase is vemurafenib (PLX4032/RG7204/Zelboraf; Plexxikon, Roche)[1,4,6]. Vemurafenib (Figure 1.2) was the first drug approved by Food and Drug Administration (FDA) for *BRAF*-mutant cancers in 2011[1], but it was first synthesized in 2005[6]. The result of the compound's action is the interruption of BRAF/MEK step on the cascade[1] (Figure 1.1) since a decrease in MEK1/2 phosphorylation is observed[7]. Nevertheless, vemurafenib is effective only in melanoma patients that harbor the $BRAF^{V600E}$ mutation[8] having an IC_{50} (half maximal inhibitory concentration) of 44 nmol/L against mutant[7]. There are also other inhibitors though, such as Sorafenib which can inhibit BRAF but to a limited extend because it is more selective for CRAF[6] and it has a low inhibitory activity against the constitutive activation of BRAF kinase[9].

Considering that melanoma patients who do not harbor the $BRAF^{V600E}$ mutation cannot respond to Zelboraf treatment, since their malignant cells are not inhibited by vemurafenib, it is understood that there is a main restriction in relation to the unnecessary administration of such pharmaceutical products. Surprisingly, previous studies have demonstrated that RAF inhibitors paradoxically facilitate the activation of MAPK pathway and promote proliferation in melanoma cells that have a wild-type genotype concerning *BRAF*[10,11]. Thus, the cellular context is considered to be determining in the action mechanism of these particular inhibitors, since they can function either as inhibitors or as activators of the same signal transduction cascades[11]. Among the side effects that can be caused by a vemurafenib treatment is rapid development of squamous cell carcinomas (SCC) also known as keratoacanthomas[9], arthralgia, fatigue, alopecia, nausea and diarrhea[1].

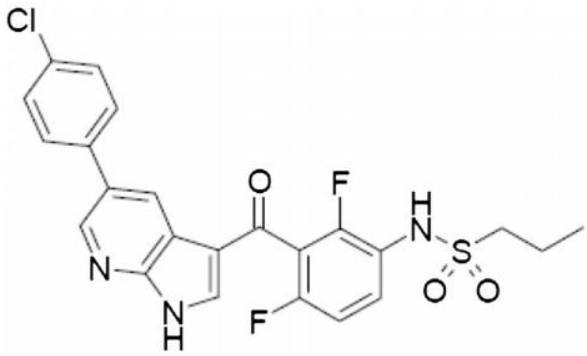


Figure 1.2: Chemical structure of vemurafenib[1].

1.2 Melanoma

Melanoma is a type of cancer that develops from melanocytes, the cells that synthesize the pigment melanin. Although melanoma typically occurs in the skin (known as cutaneous malignant melanoma), it can also be developed in other organs[12]. However, *BRAF* mutations, in which our interest is focused, usually appear in patients whose melanoma has arisen on skin without chronic sun-induced damage and rarely appear in melanoma incidents where the tumors arise from mucosal and acral sites[1]. Moreover, cutaneous malignant melanoma mostly affects people with light complexion[13,14], whereas its onset is seldom in populations with darker pigmentation[13]. Among the risk factors are multiple nevi, sun exposure, immunosuppression and family and personal history of melanoma[12]. In addition, features that indicate a malignant nevus include darker or variable discoloration, itching and enlargement (Figure 1.3) and a biopsy of the suspicious lesion can drive to the valid diagnosis[12].

Melanoma is the most aggressive type of skin cancer (there are also non-melanoma skin cancers)[15] showing an increasing prevalence over the years[13,14]. Although only 1% of diagnosed skin cancers have been attributed to melanoma, it causes the majority of skin cancer-related deaths[16]. In 2012 a number of 232,000 new cases have recorded worldwide[15] and in 2015 the active melanoma patients were 3.1 million (resulted in 59,800 deaths)[17,18]. Of note, every year approximately 132,000 people are diagnosed with melanoma worldwide according to the World Health Organization[19].

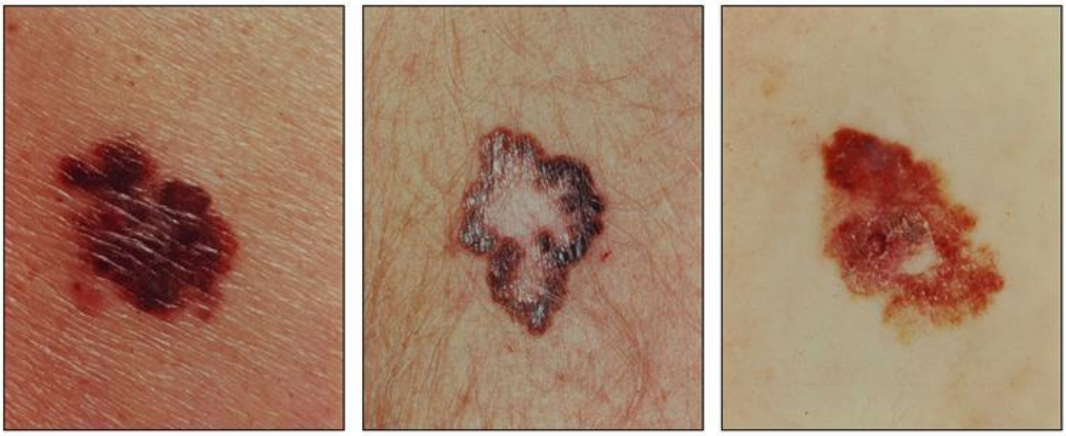


Figure 1.3: Characteristic clinical examples of melanomas (asymmetry, color variation, large diameter)[12].

1.3 Methods for detection of *BRAF* mutations

There are two types of methods for the identification of *BRAF* mutations, the DNA based and the antibody based. The antibody-based approaches are performed using immunohistochemistry protocols, but they reveal only if a protein is expressed or not. In other words, they cannot provide quantitative information and they only answer to questions of protein presence or absence in a tumor sample. The DNA-based methods employ either amplification protocols (such as typical polymerase chain reaction-PCR) and real-time PCR) or sequencing (Sanger sequencing, pyrosequencing)[16]. Nevertheless, the PCR based tests offer a faster analysis, an improved sensitivity (%mutated allele)[20] and a lower cost comparing to sequencing which requires sophisticated and expensive equipment (with a price that can reach 600,000$) and reagents that can cost thousands of dollars but offer the advantage to detect any alteration in the sequence and not only a specific one[16]. Furthermore, we have to point out that, in general, diagnostic tests have three main characteristics, sensitivity, specificity and limit of detection. Sensitivity is the ability of an assay to identify the mutation of interest correctly with a low rate of false negatives, whereas specificity is the ability to correctly identify a specific mutation with a low rate of false positives. Moreover, the threshold at which a signal (DNA harboring the *BRAF* mutation) can be distinguished from the background (*BRAF* wild-type DNA) is termed as limit of detection[16]. Consequently, there is no doubt that a high sensitivity and specificity and a very low detection limit are the three prerequisites for a successful diagnostic assay.

Some examples of such tests used in *BRAF*^{V600E} mutation identification include Cobas 4800 BRAF V600, INFINITY KRAS-BRAF and THxID-BRAF, all of which necessitate sampling of solid biopsies[16,21-23]. Cobas 4800 BRAF V600 is a real-time PCR-based test developed by Roche and can detect *BRAF*^{V600E} mutation in formalin-fixed paraffin embedded (FFPE) melanoma tissues[21,23,24]. It is characterized by a sensitivity of 95% for detecting this specific mutation. More specifically, it has a detection limit of 5% using 125 ng of DNA, obtained from a 5 μm sections, as an input[16,21] and its experimental procedure can be performed within 8 hours[21] (Figure 1.4). In addition, it is an FDA approved companion diagnostic test[16,24]. With regards to INFINITY KRAS-BRAF assay (Autogenomics), this is a laboratory-developed molecular testing specific for *KRAS* and *BRAF* mutations which uses FFPE 5μm thick sections, with a surface area of up to 250 mm^2 from melanoma tissues, requiring genomic DNA with a concentration of 15 ng/μl. The detection limit of the test is again 5% [23]. Lastly, the THxID-BRAF kit is an FDA approved companion diagnostic test[22] appropriate for both V600E and K mutations identification[16], which is real-time PCR based as cobas test, but unlike the cobas it shows high sensitivity for both V600E and K[16]. In terms of its requirements for DNA input a concentration of 10 ng/μl to 350 ng/μl is necessary[16] and a surface of 40-500 mm^2 is a prerequisite in slide samples[22].

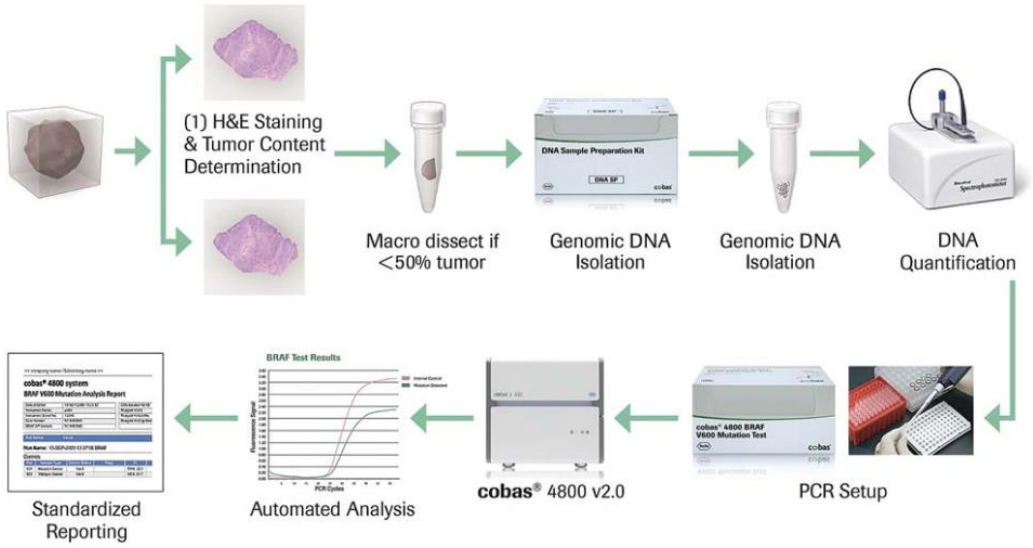


Figure 1.4: Schematic representation of Cobas 4800 BRAF V600 mutation test workflow. (H&E: Hematoxylin & Eosin, PCR: polymerase chain reaction)[21].

1.4 Companion diagnostics

Cobas and THxID tests that were described above are only two representative paradigms of companion diagnostics, since until 2016 they had been developed and approved companion diagnostic assays for 18 pharmaceutical products[25]. Taking into account that drug development for cancers usually targets oncogenic products of mutated genes in malignant cells[26], the design of companion diagnostic assays has been established as a prerequisite for a successful drug-diagnostic co-development model[25] in order to provide patients with the appropriate therapy guidance[25-27]. Additionally, diseases heterogeneity demands assays that can provide information for every single person. In other words, companion diagnostics are personalized medicine's tools directly related to patient-tailored therapy since they can distinguish candidates who are most likely to benefit from a specific treatment from those who are at high risk for side effects. They can, also, predict the outcome and monitor the response of a specific treatment, facilitating the drug development process[25,27]. As a consequence, it is considered that such a molecular testing integrated in the procedure of a certain pharmaceutical product's development have a great importance in its safe and effective use[25,27].

1.5 Solid and liquid biopsy

Conventional biopsy is the typical method for cancer diagnosis[28] and is required in a number of tests, such the aforementioned. However, it presents some important drawbacks. To begin with, it is an invasive method that causes pain to patients, it is expensive and it may take considerable time to yield an answer[29]. In addition, a single biopsy may not be indicative of tumor heterogeneity and its serial sampling may not be practical[29,30]. On the other hand, another type of biopsy the so called "liquid biopsy" has emerged as an alternative tool of cancer monitoring and management since it is non-invasive and can provide valuable insight about the entire heterogeneity of the disease and the course of treatment[28,29]. Cell free DNA (cfDNA) is one of the most studied aspects of liquid biopsies, the other one is circulating tumor cells (CTCs), but the collection and analysis of the latter demand complex equipment and it can rarely be obtained[29].

The term cfDNA describes small DNA fragments that originate from tumors or normal cells by apoptosis[28]. cfDNA is characterized by a more convenient sampling

comparing to tissue biopsy, since it can be easily obtained from serum and plasma[29]. The concentration of circulating nucleic acids in blood are at ng/ml levels corresponding to a picomolar (pM) concentration[31]. For example, the mean concentration of cfDNA in plasma, according to a study conducted by Aung et al. 2014, is ~23 ng/mL and in serum ~49 ng/mL [32]. Plasma of blood in all people contains cfDNA fragments of a length 150-200 base pairs (bp)[28] but in cancer patients, apart from the cfDNA originated from normal cells there is also circulating tumor DNA (ctDNA), cfDNA of tumor origin that can serve as a detection tool for mutations[29] (Figure 1.5).

ctDNA is not associated with cells or cell fragments and it is found in the circulation[16] constituting 0.01%-90% of cfDNA[28], having a half-life from 16 minutes to a few hours[33]. Solid tumors consisting of approximately 50 million malignant cells can release a quantity of ctDNA that is adequate to be detected in blood[28], but the mechanism of release into the blood has not been clarified yet[16,33]. Of interest, this detection limit is below of the one that radiological studies can reach, since they detect tumors of 7-10 mm containing 1 billion cells[28]. It should be paid attention in the fact that ctDNA retains the characteristics of the source tissue[34] and its abundance is a representative indication of the biological aggressiveness of the tumor[16]. Taking into account that, it can be employed as a biomarker whose quantification during the course of a medication can provide insight about a patient's response[34]. ctDNA analysis is less sensitive than solid biopsy, though[16]. The difficulty in ctDNAs detection lies in the fact that they circulate with a large number of wild-type fragments, that is in the background there is a prevailing presence of normal fragments[31]. Thus, the ability of detection is dependent to the candidate allele frequency. According to Shu et al. 2017, ctDNA mixed with cfDNA fragments that are not released from tumors have mutant allele frequencies (MAFs) below of 10%, while the MAFs of mutations in matched tumor tissues has been reported to be 23% [33]. Specifically, for $BRAF^{V600E}$ mutation the relative allelic abundance has been reported to be 1.15% in serum and 7.94% in plasma, in a previous work[29].

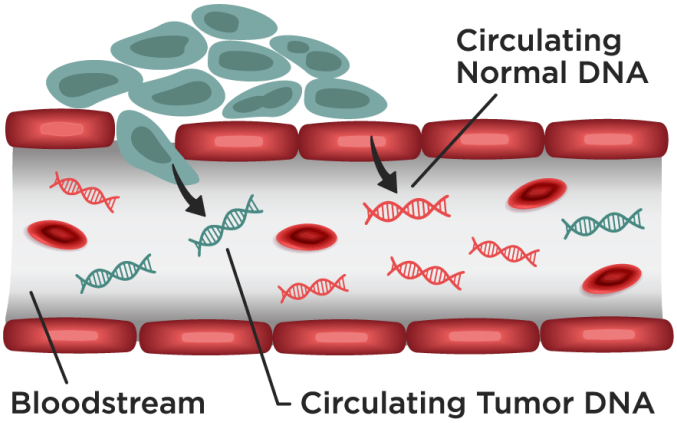


Figure 1.5: Circulating tumor DNA in bloodstream[35].

1.6 Loop-mediated isothermal amplification (LAMP)

In this work, instead of using PCR-based methods which are time and power consuming, loop-mediated isothermal amplification (LAMP) technique was employed for the amplification of target DNA. Although PCR has been used extensively for DNA amplification it has a main disadvantage, multiple thermocycling steps[36] and therefore it requires specific equipment[37]. In isothermal amplification methods, such as LAMP, there are not thermo-cycling steps, hence the time and the power consumption are reduced leading to a lower cost and a higher assay quality[36]. The fact that a thermocycler is not a prerequisite for performing the amplification step can make the development of a point-of-care (POC) system a more feasible prospect in the foreseeable future[36,37]. With LAMP more than 10^9 copies can be synthesized from less than 100 copies of template DNA within an hour[38] and there are several procedures of LAMP amplicons detection that have been used over the years, including the typical electrophoresis, lateral flow assays, optical and pH-sensing mechanisms[36]. During the last decade, LAMP has been widely used, with many implementations in detection of pathogens[39,40] and mutations[37,41]. For example, Toumazou et al. 2013 have used successfully LAMP for $BRAF^{V600E}$ mutation detection via a pH-sensing semiconductor system[41]. Herein, we followed the same amplification approach, but the amplicons detection was accomplished combining acoustic biosesnors and biotin-neutravidin capture system.

1.7 Acoustic wave biosensors

The onset of biosensors as alternative tools in pathogens[42,43] but also in mutations detection[44,45] demonstrate their importance in diagnostics. Biosensors are analytical devices, utilized for the detection of an analyte, that achieve a combination of biological

components with physicochemical detectors[46,47]. Particularly, in terms of the operation principles of acoustic sensors, employed in this study, in general the propagation characteristics of an acoustic wave, i.e., its velocity and energy are affected by the presence of an analyte at the sensor's surface and the effects are subsequently monitored as changes in frequency (ΔF) and energy dissipation (ΔD) which are indicative of the amount of the adsorbed mass and viscoelastic properties of the bound molecules, respectively[48]. Acoustic wave devices are divided in two categories; the *bulk acoustic wave* (BAW) device, which is used to generate bulk waves and the *surface acoustic wave* (SAW) device, which is used to generate surface waves. Quartz Crystal Microbalance (QCM), which is used for the purposes of this study, is the most common BAW device[49] and herein it is combined with biotin-neutravidin capture system for the detection of LAMP amplicons. With regard to biotin-neutravidin capture system, this is a widespread immobilization method because of its high affinity interaction, which is non-reversible under normal assay conditions[49].

1.8 Study objectives

The detection of mutations related to cancers is of great importance for the disease monitoring and management, since it could be determining in prevention of problems related to health and safety providing information for candidates of targeted therapies. However, the traditional methods are cost-prohibitive and time-consuming and are coupled with complicated and laborious steps. Therefore, the aim of the current project was to introduce a new rapid, sensitive and simple methodology based on isothermal amplification and acoustic biosensors, that would be applicable in the foreseeable future for clinical diagnosis using either solid (conventional) or liquid biopsy. More specifically, we focused our interest in the detection of $BRAF^{V600E}$ mutation which is very common in melanoma cases and it is the target of specific therapeutic approaches, that require appropriate guidance. Our purpose, hence, was to develop a protocol that could achieve a 5% limit of detection for genomic DNA and 0.1% for fragments that resemble cfDNA and liquid biopsy.

2. Materials and Methods

2.1 LAMP reactions

2.1.1 LAMP method

Loop-mediated isothermal amplification (LAMP) employs a DNA polymerase with strand displacement activity and a set of six primers that recognize distinct sequences on the target DNA, thereby ensuring that the target sequence will be amplified with high specificity and rapidity[50]. In the initial steps, the combined action of the first two sets of primers, forward inner primer (FIP), forward outer primer (F3), backward inner primer (BIP) and backward outer primer (B3) leads to the formation of a structure with stem-loops at each end, which is known as a "dumbbell-like structure" (Figure 2.1)[50]. In the next stage, after the elongation and cycling amplification steps formations of multiple sizes that contain alternately inverted repeats of the target on the same strand are created[50,51]. Furthermore, the addition of loop primers has as a result cauliflower-like formations (Figure 2.2) with multiple loops, accelerating the whole process since exponential proliferation is achieved[50,52]. Although LAMP can be effective using only FIP, BIP, F3, B3,[53] the use of loop primers can result in higher sensitivity[50].

This method was adopted because enables the amplification of the target DNA 10^9-10^{10} times within 15-60 min[51] under isothermal conditions[50,51,53] providing with the opportunity to avoid the use of a thermocycler if it is necessary. In addition, it does not demand complicated equipment or special reagents, diminishing thus the total expenses[51].

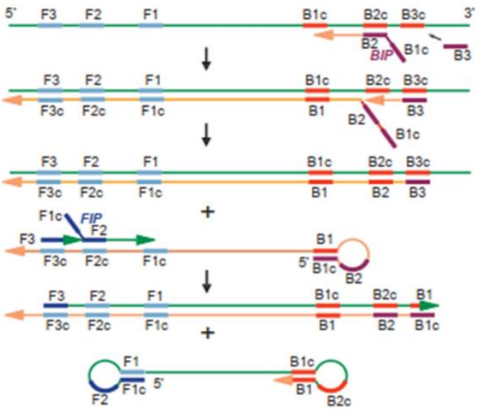


Figure 2.1: The steps of "dumbbell-like structure" formation[50].

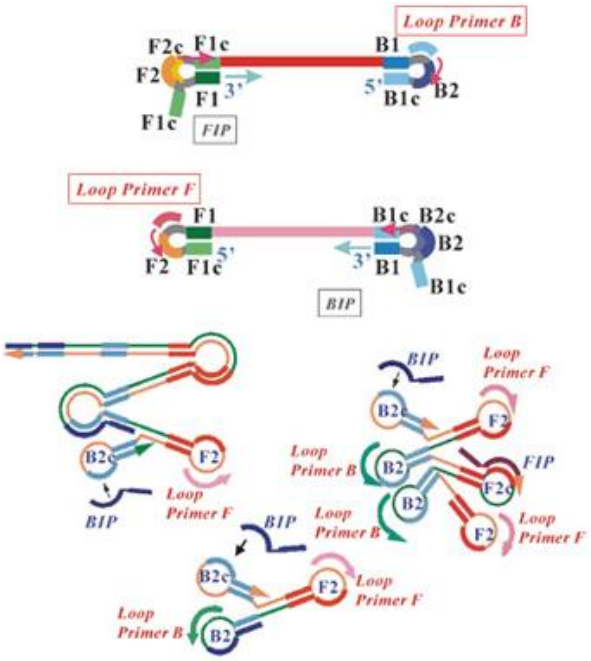


Figure 2.2: The formation of cauliflower-like structures[52].

2.1.2 Template DNA

2.1.2.1 Genomic DNA

The two genomic DNAs that were employed for our experiments are BRAF Wild Type Reference Standard 50 ng/μL (Horizon) and BRAF V600E Reference Standard (heterozygous-50% frequency of mutant allele) 50 ng/μL (Horizon).

2.1.2.2 Fragments

Polymerase chain reaction (PCR) was used to synthesize fragments of 277 bp that can resemble successfully the cfDNA fragments of a real sample. Preparation of the fragments (gel extraction or PCR cleanup) was performed using the Macherey-Nagel kit according to the manufacturer's protocol.

2.1.3 LavaLAMP^{TM} DNA Master Mix

LavaLAMP^{TM} DNA Master Mix (Lucigen) is an optimized form of the conventional LAMP mix since it limits optimization to target specific conditions (concentration of primers and template and reaction temperature). Furthermore, due to the fact that the mix is heat stable at 90°C for ~5 min it allows us to add a preheat step in order to denature the DNA template if it is necessary. The manufacturer recommends a preheat

step at 90°C for 5 min or less and for the amplification suggests 68°C - 74°C for 30-60 min[54].

2.1.4 DNA oligonucleotides

The primers used for the LAMP reactions were purchased from metabion international AG. Their sequences have been retrieved from supplementary material of Toumazou et al. 2013 research work[41] and are summarized below:

wt FIP(B) wt BIP	5'-Biotin-ACTGTAGCTAGCAGATATATTTCTTCATGAAGACCT-3' 5'-TGAAATCTCGATTCCACAAAATGGATCCAGA-3'
mut FIP(B) mut BIP	5'Biotin-TCTGTAGCTAGCAGATATATTTCTTCATGAAGACCT-3' 5'-AGAAATCTCGATTCCACAAAATGGATCCAGA-3'
F3 B3	5'-GGAAAATGAGATCTACTG-3' 5'-TCTCAGGGCCAA-3'
Loop F Loop B	5'-ACCAAAATCACCTATTT-3' 5'-GGAGTGGGTCCC-3'

2.1.5 Betaine

For the first experiments betaine (5M, Sigma-Aldrich) was used in a final concentration of 0.3M. Betaine facilitates DNA amplification by limiting the formation of secondary structure in GC-rich DNA regions[55], decreasing the melting temperature (T_m) and assisting the hybridization between the primer and the target sequence.

2.1.6 Peptide nucleic acid (PNA)

Peptide nucleic acids (PNAs) are synthetic homologs of DNA with a length no greater than 30 bases. In PNAs there is a pseudo-peptide polymer with linked nucleotides instead of a phosphodiester backbone. A main difference between PNA and nucleic acids is the lack of a charge, which makes the PNA-DNA duplexes more stable because of the absence of electrostastic repulsion. Another advantage of PNAs is that they cannot serve as primers or template to be amplified, since polymerases cannot recognize them and thus they cannot interact directly with them. PNAs can prevent the amplification since they can bind to the template or compete with the primers blocking their elongation[56,57]. PNA (50 μM) employed for our experiments was provided by Dr.

Alexandra Voutsina (Medical School, University of Crete) and it was specific for wild-type template, in order to block non-specific hybridization of mutant specific primers in wild-type DNA. In reactions its final concentration was 0.25 μM.

2.1.7 Preparation of LAMP reactions

For our reactions we followed the protocol of LAMP. LAMP reactions were performed in a total 25 μL mixture containing 1.6 μM each of FIP and BIP, 0.8 μM each of LF, LB and 0.2 μM each of F3 and B3. The addition of LavaLAMP mix and the specified amounts of betaine, PNA (betaine and PNA were used only for a number of experiments), ddH_2O and double stranded DNA was the next step. The mixture was incubated firstly at 90°C for 5 min in order to achieve denaturation and next at 68°C for 20-35 min depending on the experiment, using a thermocycler (Peqstar 2x VWR Peqlab). The last step included incubation on ice to stop the enzyme activity. Clean View UV Cambinet (Clearer Scientific Ltd) was employed during the preparation of the mixture in order to avoid aerosol cross-contamination.

More specifically, the first step of the procedure was the preparation of LavaLAMP mix and primers which were added in the same eppendorf in volumes that are mentioned below:

- 12.5 μL Lava LAMP mix (2x)
- 1 μL mut FIP primer (Biotinylated) 40 μM
- 1 μL mut BIP primer 40 μM
- 0.5 μL F3 primer 10 μM
- 0.5 μL B3 primer 10 μM
- 1 μL LoopF primer 20 μM
- 1 μL LoopB primer 20 μM

For specific experiments the quantities of the extra components were:

- 1.5 μL Betaine 5 M
- 1.25 μL PNA 5 μM

2.1.8 Gel electrophoresis

After the end of the incubation in the thermocycler, 5μL of the reaction mixture with 1μL loading dye (loading buffer 6x, Takara Clontech) were loaded in 2% agarose gels (in 0.5x TBE buffer for electrophoresis), in order to confirm if the reaction was successful. DNA ladder of 100 bp (NIPON Genetics, Europe) was used as marker for the identification of the products size and negative controls (reactions without template) were used for sample quality and contamination assessment. For the synthesis of 100 mL of agarose gel we mixed 100mL of 0.5x TBE buffer, 2g of agarose (NIPON Genetics, Europe) and 10 μL GelRed (Biotium). TBE 10x (250mL) was prepared from 27g Tris-base (PanReac AppliChem, ITW Reagents), 13.75g Boric acid (MERCK), 10ml EDTA (SIGMA-ALDRICH) and dH_2O until the desired volume. Electrophoresis took place in an electrophoresis chamber combined with Power Pack Basic (BIO-RAD) at 130 or 140 volts. The gel photos were taken by IXUS Canon camera, on an ultraviolet (UV) transilluminator (G. Kisker).

2.1.9 PCR (polymerase chain reaction)

As mentioned above polymerase chain reaction (PCR) was used to synthesize fragments of 277 bp that can resemble successfully the cfDNA fragments of a real sample. The sequences of the primers employed for PCR are described below and they were the same for wild-type and mutant template, since we supposed that the difference in the point of mutation could remain after the amplification.

The primers were purchased from metabion international AG and they were designed using the primerquest tool of INTEGRATED DNA TECHNOLOGIES (IDT)[58].

Forward	5'-ACCTAAACTCTTCATAATGCTTGC-3'
Reverse	5'-TGAGACCTTCAATGACTTTCTAGT-3'

Components for 20μl final volume:

- 10 μL KAPA 2G Fast HotStart Ready mix (KAPABIOSYSTEMS)
- 1 μL Forward primer 10 μM
- 1 μL Reverse primer 10 μM

- 6 μL ddH_2O
- 2 μL genomic DNA (wild-type/mutant) (5ng/μL)

PCR protocol:

- 95° C for 5 min
- 95° C for 10 s
- 57° C for 10 s
- 72° C for 10 s

These steps were repeated for 35 cycles for the first experiments and 30 cycles when problems of contamination began. The last step of the procedure was gel extraction, using the Macherey Nagel kit as mentioned above. However, in the last experiments we decided to avoid the exposure of the PCR product in UV and thus we followed only the PCR cleanup protocol of the kit.

2.1.10 Digests

TspRI restriction enzyme (New England BioLabs) was utilized for digest of DNA at the end of the LAMP reaction. TspRI recognizes NNCASTGNN (S: C or G) and hence it digests only the wild-type but not the V600E mutated DNA. Furthermore, none of the other V600 mutations are recognized by this enzyme[59]. However, there are two restriction sites in the target sequence (wild-type) the first one involves the nucleotide that differs between wild-type and mutant and the second one is located 81 bases along this nucleotide. To destroy the second restriction site we designed a new B3 primer using the PrimerExplorer V5. In addition, we designed new FIP and BIP primers that lack the nucleotide that differs between wild-type and mutant. The sequences of new primers are summarized below and they were synthesized by metabion international AG. The sequences of the rest primers for LAMP remained the same as previously.

Primers (for TspRI)

FIP	5'-Biotin-CTGTAGCTAGCAGATATATTTCTTCATGAAGACCT-3'
BIP	5'-GAAATCTCGATTCCACAAAATGGATCCAGA-3'
B3	5'-AGGGCCAAAAATTTAATTAGT-3'

For the digests two experimental procedures were tested.

- Digest in 40μL final volume after the end of LAMP reaction:
  - 20 μL LAMP reaction (with 15 ng genomic DNA, mutant 50%)
  - 4 μL enzyme buffer
  - 2 μL TspRI enzyme (10000 units/mL)
  - 24 μL ddH_2O

Incubation at 65°C for 30 min using either the thermocycler Peqstar 2x (VWR Peqlab) or Fast gene ultra cycler (NIPON Genetics).

- Digest in 25 μl final volume, with only addition of enzyme at the end of LAMP reaction:
  - 25 μL LAMP reaction (with 15 ng genomic DNA, mutant 50%)
  - 0.5 μL TspRI enzyme (10000 units/mL)

Incubation at 65°C for 15 min in the thermocycler.

2.2 Acoustic measurements

2.2.1 Chemicals

Phosphate Buffered Saline (PBS, P4417) was purchased from Sigma. Neutravidin biotin-binding protein was obtained from Invitrogen (A2666). The reason why we used neutravidin, instead of avidin, is that it has a pI of 6.3 and hence it is negatively charged at pH 7.5. Thus, non-specific adsorption of the negatively charged DNA is prevented.

2.2.2 Quartz Crystal Microbalance (QCM) experimental setup and procedure

The acoustic experiments presented in this work were performed at 35 MHz using the commercially available QCM-D gold sensors (AWSensors SNS 000042 A) (Figure 2.3) and the Q-Sense E4 instrument (QSense, Sweden) (Figure 2.4). This equipment enables the acoustic signal monitoring of four sensors simultaneously. Prior to any experimental measurements the QCM devices were cleaned with ethanol and etched for 2.30 min at high power with a Harrick Plasma Cleaner using air. Afterwards, each one of the four sensors was transferred in its chamber and filled with PBS buffer using a peristaltic pump. A volume of 200 μL neutravidin (200 μg mL^{-1}) were loaded on each one sensor under a constant flow of approximately 50 μL min^{-1} followed by PBS rinsing. The next step was the addition of LAMP reactions (15 μL) or digests (30 or 15 μL depending on its total volume) diluted in a final volume of 200 μL. The final step was buffer rinsing again.

Figure 2.3: Quartz crystal microbalance (QCM) gold device[60].

Figure 2.4: Experimental equipment for acoustic experiments. Q-Sense E4 instrument and peristaltic pump.

3. Results and Discussion

In the current work, our aim was to develop a new rapid and sensitive diagnostic assay based on isothermal amplification and acoustic biosensors for the detection of *BRAF*^{V600E} mutation in a 5% allele frequency for genomic DNA and 0.1% for fragments of a length that resembles cell free DNA (cfDNA). More specifically, our assay employs the loop-mediated isothermal amplification (LAMP) technique for the amplification of the DNA target, performed in a thermocycler which is a typical research lab equipment, while the acoustic measurements were acquired via Qsense instrument and quartz crystal microbalance (QCM) devices which are commercially available by various manufacturers. LAMP has been used previously in the study of Toumazou et al.[41] for the identification of the same mutation, with the difference that herein the LAMP products are biotinylated (since one of the LAMP primers is

biotinylated) and the amplicons detection is based on acoustic biosensors and biotin-neutravidin interaction.

Several protocols were tested to achieve the most efficient and specific amplification in the shortest time possible. After a number of unsuccessful efforts, we managed to amplify our target applying an additional preheat step. The denaturation step was the reason why we decided to use Lava LAMP mix instead of the typical LAMP mix, because it contains an enzyme that is heat stable at 90°C for ~5 min. Thus, the protocol that we finally followed included two steps; preheat at 90°C for 5 min in order to accomplish denaturation and next amplification at 68°C for 20-35 min depending on the experiment. All the reactions were performed in a final volume of 25 μL using mutant allele specific primers. Wild-type specific primers were used only in the first tests (data not shown) to verify that the wild-type template could be amplified successfully.

3.1 Investigating the limit of detection in genomic DNA (gDNA)

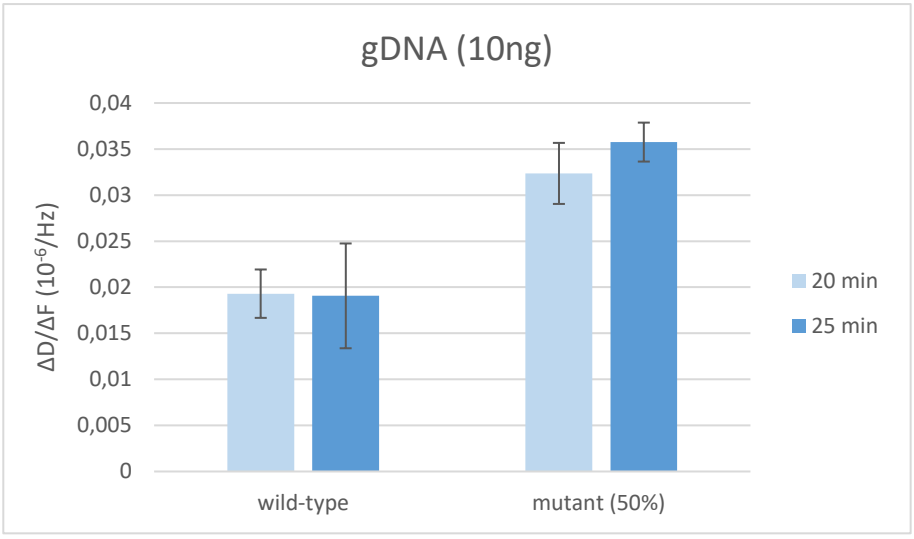
3.1.1 Detection of mutant allele in a frequency of 50%, using 10 ng genomic DNA and betaine

At a first stage, we conducted experiments using 10ng of both wild-type and mutant genomic DNA (gDNA), as suggested in Toumazou et al. work[41], to investigate if it was feasible for the sensor to discriminate between them, which in turn would imply that the approach had the potential to serve as a successful diagnostic assay. However, it should be paid attention in the fact that mutant genomic DNA used for the purpose of our study is heterozygous, that is the mutant allele frequency (MAF) is 50%. To facilitate the denaturation and promote the specificity of the amplification betaine was also used (reported also in Toumazou et al. protocol[41]). Figure 3.1 depicts the cumulative results of the reactions performed for either 20 or 25 min with 10 ng template DNA in the presence of betaine. In terms of acoustic experiments, the measured values correspond to changes in dissipation (ΔD) and frequency (ΔF), but herein only the calculated acoustic ratio (R) ΔD/ΔF is presented, since after a number of experiments we concluded that the differences in R were markedly clearer comparing with ΔD or ΔF separately. As it is shown from the chart the mutant (50%) is successfully detected from the sensor since there is obvious difference between the wild-type and mutant reactions. The significant change that the wild-type causes in

acoustic signal can be attributed to the formation of primer dimers or the single stranded biotinylated primer present in the reaction.

Although according to the bar graph we cannot claim that the additional 5 min of the 25 min reactions cause a significant difference, the agarose gel verification indicates that the mutant template reaction performed within 25 min is more efficient than the corresponding one of 20 min (Figure 3.1B). Nevertheless, it is remarkable that the LAMP presents a variation because the same protocol did not have the same efficiency in every individual experiment. This suggests that technique's efficiency is dependent on the particular set of materials used. That is the fresher is the set of materials, the more efficient is the reaction. Furthermore, the quantity of 10 ng DNA in these particular experiments seems to be ideal for achieving the specific amplification of mutant but not the wild-type template, but we assumed that betaine has also played a role in this. We should also note that the amplicons of LAMP have various sizes as it is obvious from the gel, which means that we do not expect a single product characterized from a certain number of base pairs.

(A)



(B)

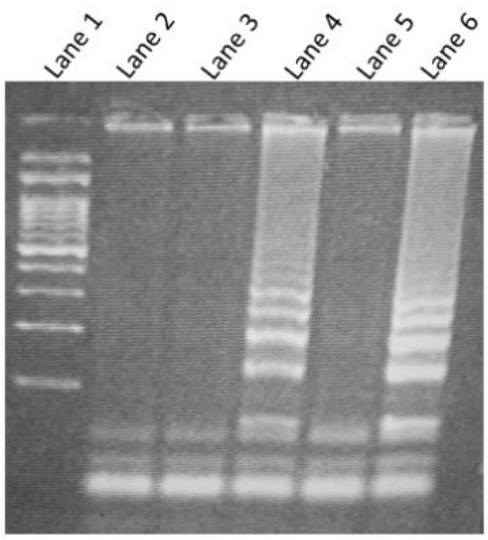


Figure 3.1: Detection of mutant allele with 50% frequency, using 10 ng genomic DNA and 0.3M betaine. **(A)** Acoustic measurements of LAMP reactions performed using 10 ng genomic DNA and 0.3M betaine for 20 and 25 min. In the graph representing the ratio ΔD/ΔF, the light blue bars correspond to 20 min reactions, whereas the dark blue bars correspond to 25 min reactions. Data are shown as mean +/- SD [n=4 for 20 min reactions, and n=3 for 25 min reactions (n=number of independent experiments)]. **(B)** A 2% agarose gel where the DNA LAMP products of 20 and 25 min reactions are displayed together. Lane 1 corresponds to ladder, Lane 2 is no template reaction, Lane 3 corresponds to wild-type of 20 min, Lane 4 represents mutant of 20 min, Lane 5 corresponds to wild type of 25 min and Lane 6 to mutant of 25 min. Based on gel verification it seems that the mutant template reaction performed within 25 min is more efficient than the corresponding one of 20 min, but acoustic measurements reflect only a slight difference between 20 and 25 min.

3.1.2 Detection of mutant allele in frequencies 10-50%, using 15 ng genomic DNA without betaine

To investigate what is the limit of detection (LOD) of the assay using the genomic DNA we tried to detect mutant allele in different frequencies. For this reason, we mixed mutant with wild-type template in various ratios in order to achieve different relative abundancies of 10, 20 and 33%. However, we began with 50% mutant, which was detectable in the previous experiments and did not require to prepare a mix of wild-type and mutant since it already contained 50% of both of them. With regards to the quantity of genomic DNA that was used, we should mention that although 10 ng was sufficient, as it is obvious from the experiments described above, we supposed that 15 ng could result in more significant differences. Apart from that, 15 ng was a more convenient

amount for the mixes of wild-type and mutant that we had to prepare to attain the specific ratios. Furthermore, we increased the time of amplification to 30 min, because reactions with low MAFs were not successful in less than 30 min. With a combination of 15 ng and 30 min non-specific amplification was emerged as a point of concern though, since LAMP products were synthesized from wild-type template (Figure 3.2). This would be also a problem due to the lack of betaine, because for these experiments and all those that will be presented next betaine was excluded, since we realized that it was not necessary for the successful amplification and although it could reduce the wild-type background in reactions, it probably affected the acoustic experiments as the differences between wild-type and mutant in acoustic values were clearer in experiments without the betaine.

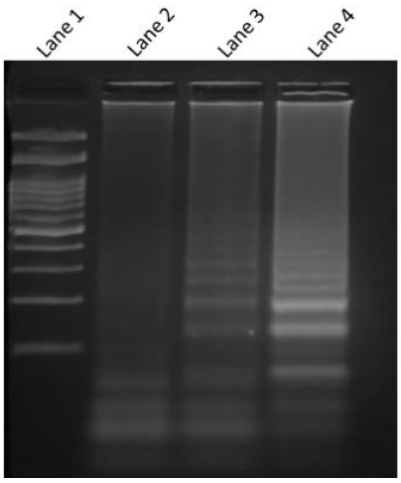
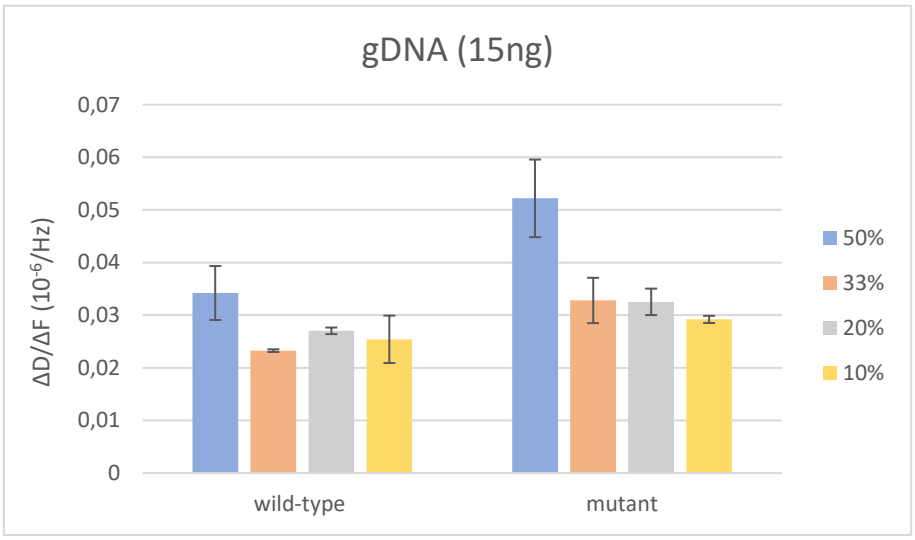


Figure 3.2: A 2% agarose gel where the LAMP products of 30 min reactions with 15 ng genomic DNA and without betaine are displayed. Lane 1 corresponds to ladder, Lane 2 is no template reaction, Lane 3 corresponds to wild-type and Lane 4 to mutant.

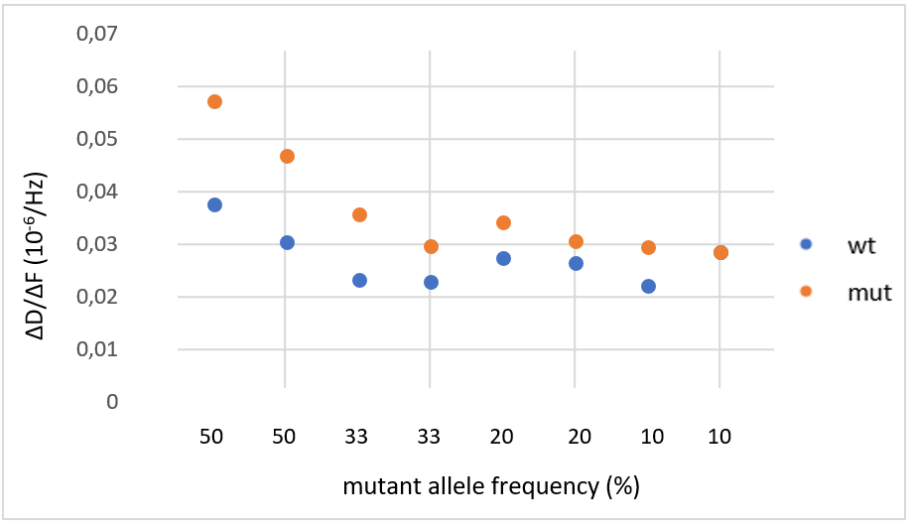
The acoustic experiments of this part are depicted in Figure 3.3. Firstly, we tried to represent acoustic ratio ΔD/ΔF of the reactions as means categorized by the corresponding MAFs (Figure 3.3A). However, in this way of representation the conclusion was that probably the wild-type and mutant cannot be distinguished very effectively. Thus, we tried to illustrate the results in a plot, which depicts wild-type and mutant as individual values (dots of single measurements) and not as an average (Figure 3.3B). In this way, we succeeded to understand that when every single mutant is compared with its corresponding wild-type, which was prepared in the same LAMP process with the same set of materials, considerable differences between them could be observed. The reason why the differences are not apparent when the averages are used

could be the variation which characterizes the efficiency of LAMP, since we noticed that between all the wild-types, which had been prepared following exactly the same protocol, there were significant differences. We can assume that probably this happens due to the fact that LAMP's ingredients and dilutions of DNA are not new each time. In addition, in Figure 3.3B we observed that when the MAF is decreased the acoustic ratio is decreased too. Therefore, we decided to express the mutant acoustic ratios as percentages of wild-type ratios [((mutant-wild type)/wild type)*100%]. Figure 3.3C reveals that there is a positive correlation between the MAFs and the percentages of acoustic experiments. Indeed, every individual MAF seems to resemble to its corresponding average of percentages, since a mean percentage of ~53% corresponds to a MAF of 50%, ~41% corresponds to 33% MAF, ~20% to a MAF of 20% and ~17% to 10% MAF. In this way we managed to reach a LOD of 10%. In this set of experiments, we tested also a MAF of 5% but no amplification was carried out.

(A)



(B)



(C)

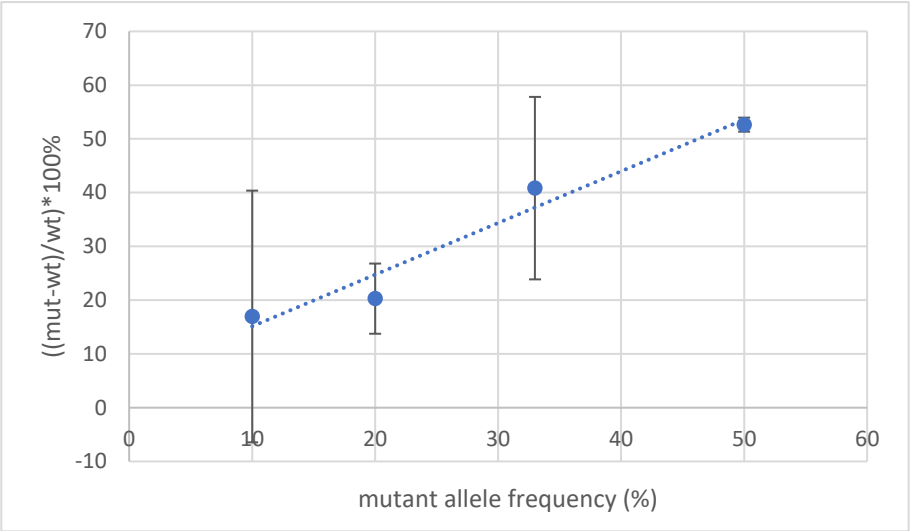


Figure 3.3: Detection of mutant allele with frequencies 50, 33, 20 and 10%, using 15 ng genomic DNA in 30 min LAMP reactions. **(A)** Acoustic ratio ΔD/ΔF of LAMP reactions performed using 15 ng genomic DNA (without betaine) for 30 min. The blue bars of the chart correspond to a MAF of 50%, the orange to 33%, the gray to 20% and the yellow corresponds to 10%. Data are shown as mean +/- SD [n=2 (n=number of independent experiments)]. **(B)** A different representation of the same measurements in a plot which depicts wild-type (wt) with blue dots while mutant (mut) with orange dots, as individual values and not as an average as in (A). **(C)** A graph presenting mutant acoustic ratios as percentages of wild-type ratios. The acoustic measurements that were used for the calculation of percentages are the same that were used for (A) and (B). Data are shown as mean +/- SD [n=2 (n=number of independent experiments)]. Overall, the results of the three types of representation suggest that the differences are clearer when every single mutant is compared with its corresponding wild-type, which is prepared in the same LAMP process with the same set of materials. In addition, the representation of mutant acoustic ratios as percentages of wild-type ratios reflects a positive correlation between the MAFs and the percentages of acoustic experiments. Indeed, the individual MAFs seem to resemble to the average of their corresponding percentages.

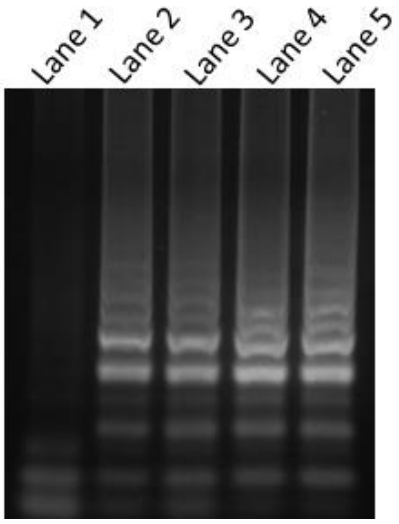
3.1.3 Experiments to reduce the signal from wild-type background

Considering that the non-specific amplification of wild-type affected previous experiments to an extent, we examined two different approaches in order to reduce or even avoid the signal from wild-type background; digests with TspRI restriction enzyme and peptide nucleic acid (PNA) specific for wild-type sequence.

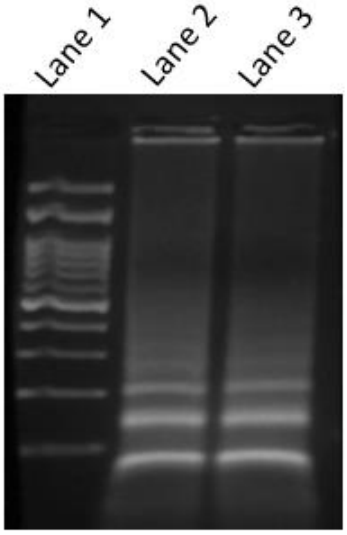
3.1.3.1 TspRI digest is not an appropriate approach for reducing the wild-type background

TspRI restriction enzyme was used for digest of DNA at the end of the LAMP reaction. TspRI recognizes and digests only the wild-type but not the V600E mutated DNA. The same enzyme has been previously employed in another assay for *BRAF*^{V600E} mutation proposed by Panka et al.[59] We examined two different experimental procedures for the digests, using LAMP reaction of 15 ng template and MAF 50%. In the first one, we performed a 40 μL digest of the LAMP products for 30 min at 65°C preparing a typical digest mix and the second one included the digest of the LAMP reaction with the addition of only 0.5 μL of the enzyme and the incubation was carried out at 65°C for 15 min (the procedures are described in detail in materials and methods section). After the digest we anticipated the biotinylated products of wild-type reaction to have a shorter length, which in turn would imply a weaker acoustic response. Nonetheless, our findings contradicted this assumption. Surprisingly, only a slight difference was observed in gel verification (Figure 3.4 A&B) and the variety of the LAMP products size did not allow us to know what the exact length of the expected products was. According to Figure 3.4C the digest does not provide an appropriate approach for the reduction of the wild-type background, since the wild-type and mutant were discriminated in a lower degree comparing to previous experiments of 50% MAF and the calculated percentages were much lower (data not shown). This could occur possibly due to the fact that the biotinylated amplicons of wild-type do not have a smaller size as we expected. In contrast, they may have a large length, more than 600 bp, that causes saturation on the sensor's surface as suggested by a previous study[61]. Thus, even if the biotinylated products of wild-type and mutant have a difference in length the sensor cannot detect it.

(A)



(B)



(C)

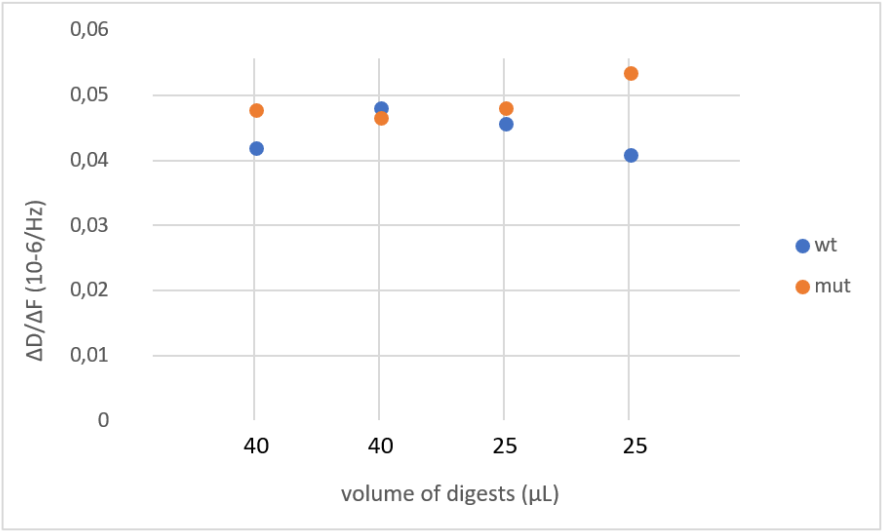
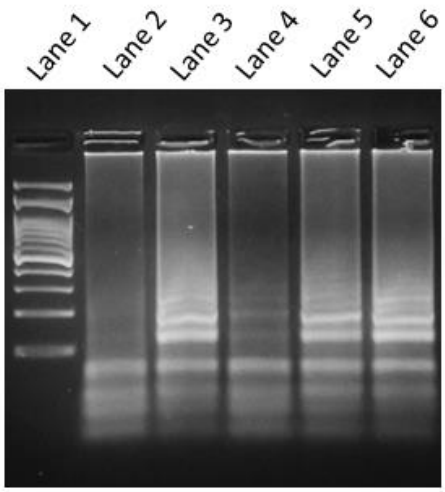


Figure 3.4: TspRI is not an appropriate approach for reducing the wild-type background. **(A)** A 2% agarose gel depicts the products of the 25 µL digest (15 min). Lane 1 corresponds to no template reaction, Lanes 2 and 3 correspond to wild-type and Lanes 3, 4 to mutant (50% MAF). **(B)** A 2% agarose gel depicts the products of the 40 µL digest (30 min). Lane 1 corresponds to ladder, Lane 2 to wild-type and 3 corresponds to mutant (50% MAF). **(C)** A plot which represents wild-type (wt) with blue dots while mutant (mut) with orange dots, as individual values. As it is apparent, the digest does not provide an appropriate approach for the decrease of the wild-type background, since the wild-type and mutant were discriminated in a lower degree comparing to previous experiments of 50% MAF.

3.1.3.2 A 5% LOD is feasible using PNA as an approach for the reduction of wild-type background

To evaluate peptide nucleic acid (PNA) specific for wild-type sequence as a means that can decrease or even prevent the non-specific amplification of wild-type template, we firstly tested it in LAMP reactions with 50% MAF and the blocking was successful without affecting mutant amplification significantly (data not shown). Consequently, we decided to examine its efficiency in reactions of 5% MAF in which the mutant template was not amplified in our previous efforts as it was mentioned above. To enhance even more the amplification of mutant with this low incidence we increased the time of LAMP to 35 min. As seen from Figure 3.5A, comparing Lanes 3 and 4, PNA can effectively reduce the non-specific amplification of wild-type. Furthermore, as it shown in the table of Figure 3.5B we achieved the desired LOD of 5%, in reactions performed using PNA, since in three independent experiments mutant was detectable with the calculated percentages of mutant related to wild-type being ~10-39%. On the other hand, in reactions without PNA the acoustic ratios obtained from wild-types were greater comparing to the corresponding mutants, therefore the resulted percentages had a negative value in all replications. As a consequence, we concluded that PNA is a very promising strategy to eliminate the wild-type background, which seemed to affect highly the ability of the sensor to discriminate mutant from it. Taking everything into account, the increase of time in combination with PNA made the detection of mutant in a frequency of 5% feasible.

(A)



(B)

Number of experiment	((mutant-wild type)/wild type)*100%	
	without PNA	PNA
1	-15,4064027	10,11617862
2	-5,936990096	38,80651213
3	-4,583711926	31,17360479

Figure 3.5: PNA is an appropriate approach for reduction of the wild-type background and a LOD of 5% is achieved via PNA use. **(A)** A 2% agarose gel depicts the products of LAMP reactions performed employing PNA in 35 min. Lane 1 corresponds to ladder, Lane 2 corresponds to no template reaction, Lanes 3 and 4 correspond to wild-type without and with PNA respectively and Lanes 5, 6 to mutant (5% MAF) without and with PNA respectively. It seems that PNA can effectively prevent the amplification of wild-type. **(B)** A table represents mutant acoustic ratios as percentages of wild-type ratios without PNA and with PNA, in three independent experiments. Every single percentage has resulted from one mutant value and its corresponding wild-type. The MAF in all these reactions was 5%. Overall, these findings suggest that PNA facilitates the reduction of the wild-type background allowing the detection of $BRAF^{V600E}$ mutation in a frequency of 5%, in combination with increased time of amplification.

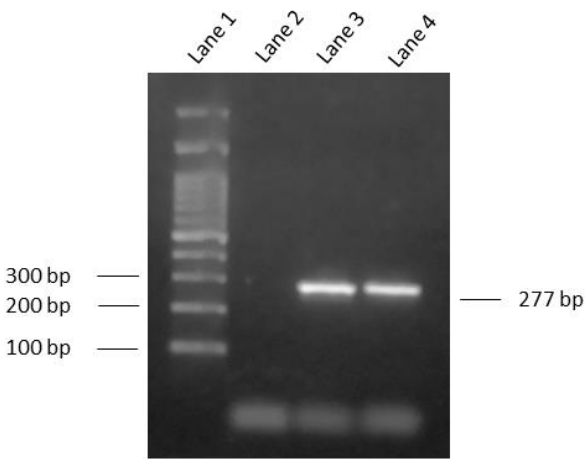
3.2 Investigating the LOD in fragments that resemble cfDNA

3.2.1 A LOD of 1% is feasible in a wild-type background of 10,000 copies

To define the detection limit concerning liquid biopsy we followed an experimental procedure employing polymerase chain reaction (PCR) to synthesize fragments of a short length (277 bp) that resemble cfDNA fragments of a real sample (Figure 3.6A). The LAMP reactions were performed again at 68°C for 30 min (with a preheat step of 5 min at 90°C), as the most of the LAMP reactions in the case of genomic DNA. Firstly, we prepared reactions with 1000 copies of mutant in a wild-type background of 10,000 copies in order to examine if a 10% MAF was detectable and according to our findings it was (Figure 3.6B). Consequently, we next examined a reaction mixture of 100 mutant copies in a wild-type background of 10,000 copies, corresponding to 1% MAF and the detection was successful again since the sensor was able to discriminate between them. As it is indicated from the graph and based on the calculated percentages which were ~32% and 26% for MAF 10% and ~12% and 56% for MAF 1% there is no positive

correlation between the MAF and the calculated percentages of acoustic values, as it was observed in the previous experiments with genomic DNA. In fact, in one case of MAF 10% it has been observed less significant difference than the corresponding one of 1%, but further investigation is necessary to reinforce these findings. In addition, we tried to perform reactions of 0.1% MAF, that is 10 copies of mutant in 10,000 copies wild-type background, but the amplification was not possible.

(A)



(B)

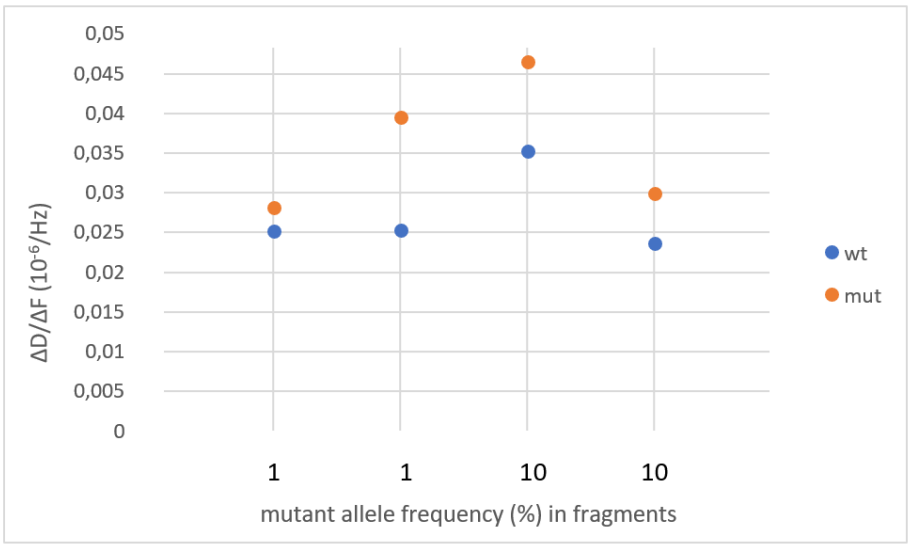


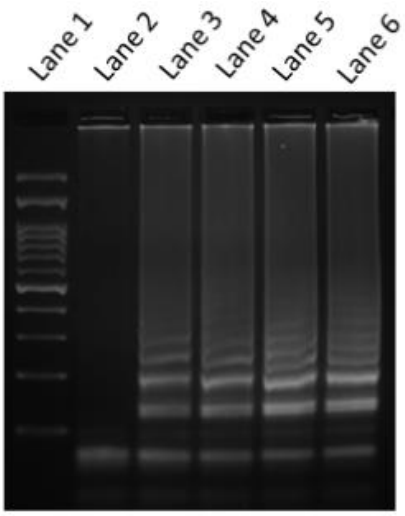
Figure 3.6: A LOD of 1% is feasible in the case of fragments in a wild-type background of 10,000 copies. **(A)** An 1% agarose gel illustrates the products of PCR reactions. Lane 1 corresponds to ladder, Lane 2 corresponds to no template reaction, Lanes 3 and 4 correspond to wild-type and mutant PCR products of 277 bp (base pairs) respectively. **(B)** A plot which illustrates wild-type (wt) with blue dots and mutant (mut) with orange dots, as individual values. As the plot indicates, wild-type and mutant are discriminated effectively but there is no positive correlation between the MAF and the percentages of acoustic ratios as it was noticed in the

previous experiments with genomic DNA, since in the second case of 10% a less significant difference is indicated comparing to the corresponding one in the second case of 1%.

3.2.2 A LOD of 0.1% is possible in a wild-type background of 100,000 copies

Given that 10 copies of mutant were not sufficient for achieving amplification, we considered to use a different combination in terms of the number of mutant and wild-type copies. In other words, we mixed 100 copies of mutant with 100,000 of wild-type, which is again a ratio that corresponds to 0.1% MAF. This effort was successful since the amplification took place and the sensor could discriminate between wild-type and mutant (Figure 3.7A&B). The time of amplification remained 30 min. However, the problem of non-specific amplification of wild-type caused some restrictions concerning the ability of differentiating between the two. We should mention that although PNA was not tested in these experiments, and hence the amplification of wild-type was not prevented, its use may improve the results. Despite the fact that more experiments are necessary, these preliminary results could suggest that a LOD of 0.1% is probably feasible using this assay.

(A)



(B)

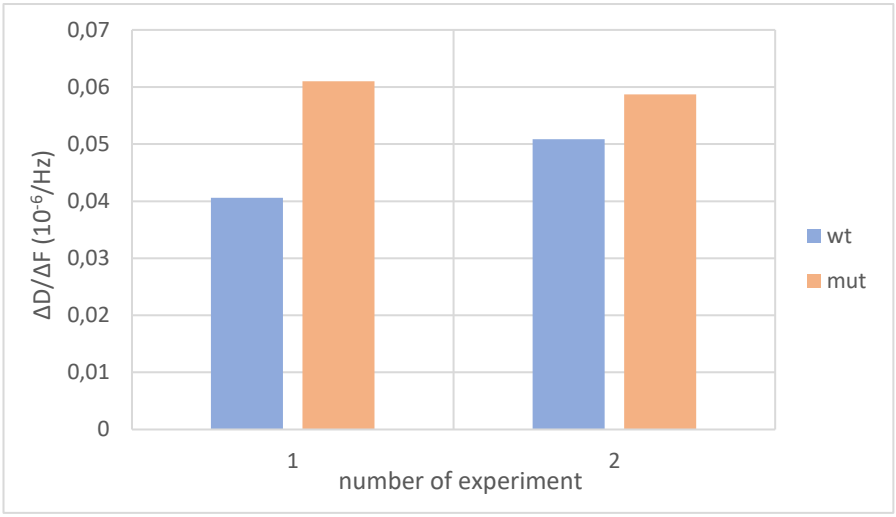


Figure 3.7: A LOD of 0.1% is feasible in the case of fragments in a wild-type background of 100,000 copies. **(A)** A 2% agarose gel depicts the products of LAMP reactions. Lane 1 corresponds to ladder, Lane 2 corresponds to no template reaction, Lanes 3 and 4 correspond to wild-type and Lanes 5 and 6 correspond to mutant (0.1%). **(B)** A bar graph represents the acoustic response of 100,000 wild-type copies (blue bars) and 100 mutant copies in a wild-type background of 100,000 copies (orange bars). The graph has resulted from two experiments and the bar of each mutant (0.1%) is depicted next to its corresponding wild-type for direct comparison. It seems that the detection of 0.1% mutant may be feasible through the proposed assay.

4. Conclusion

In the above study, we describe a new methodology for the detection of *BRAF*^{V600E} mutation which is very common in melanoma patients and it is the target of specific therapeutic approaches, that require appropriate guidance in order to ensure candidate's health and safety. Our aim was to develop a new rapid, sensitive, simple and cost-effective diagnostic assay based on isothermal amplification and acoustic biosensors for the detection of the mutant allele in a 5% frequency for genomic DNA and 0.1% for fragments of a size that resembles cfDNA. More specifically, our assay employed the loop-mediated isothermal amplification (LAMP) technique for the amplification of the DNA target, while the acoustic measurements were acquired via the commercially available Qsense instrument and QCM devices. Our findings led us to some important conclusions which are reported below:

- Only the calculated acoustic ratio (ΔD/ΔF) is able to reveal the differences between mutant and wild-type reactions clearly; ΔD or ΔF separately, cannot provide valuable information.
- Every single mutant sample must be compared with its corresponding wild-type one, which is prepared in the same LAMP process with the same set of materials. This is due to the fact that LAMP is characterized by a variation because the same protocol did not have the same efficiency in every individual experiment, suggesting that its efficiency depends on the particular set of materials that were used for the preparation of the same reaction mix.
- Expressing the mutant acoustic ratios as percentages of wild-type ratios [((mutant-wild type)/wild type)*100%] provides an appropriate means of interpreting the data, as their calculation indicate that there is a positive correlation between them and the MAFs. Indeed, the individual MAFs seem to resemble to the average of the corresponding percentages (for example a mean percentage of ~53% corresponds to a MAF of 50%).
- TspRI digest is not an appropriate approach for reducing the wild-type background, which was the main problem that we had to address. In more detail, the biotinylated amplicons of wild-type perhaps have a large size and not a smaller one as we expected and sensor's surface may be saturated. Thus, even if the biotinylated products of wild-type and mutant has a difference in length the sensor cannot detect it.
- PNA is an effective approach for the reduction of wild-type background since the 5% LOD was feasible only through the procedure including it.

Future approaches could include more experiments with fragments using PNA for the reduction of background and next, tests to evaluate the applicability of the proposed assay in real samples to demonstrate its potential clinical utility. To conclude, although further optimization and more experiments are necessary, our findings could suggest that we managed to achieve our aim which was the detection of the mutant allele in a 5% frequency for genomic DNA and 0.1% for fragments. Our methodology is very promising since it provides with the opportunity to yield an answer in a short time through a simple and cost-effective assay. Therefore, we consider that it could assist the evolving efforts of new diagnostic solutions and improve melanoma control and management to advance public health perspectives.

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