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Faculteit Industriële ingenieurswetenschappen

master in de industriële wetenschappen: biochemie

Masterthesis

Optimization and Validation of ddPCR Assays for the Detection of *PIK3CA* Hotspot Mutations in Liquid Biopsies of Patients With Invasive Breast Carcinoma

PROMOTOR :

dr. ir. Kristel SNIEGOWSKI

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Demi Renders

Scriptie ingediend tot het behalen van de graad van master in de industriële wetenschappen: biochemie

Gezamenlijke opleiding UHasselt en KU Leuven

UHASSELT KU LEUVEN

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UHASSELT KU LEUVEN

PREFACE

During the past three months I was given the opportunity to perform my internship at the Center for Human Genetics (CHG) at UZ Leuven Gasthuisberg. Hereby, I was initiated in different aspects of the molecular diagnostics in the group of the solid tissue tumors (VATU). I was given an incredibly interesting subject on which I performed my master thesis that I proudly present in this work.

First of all I want to thank my external promotor dr. MSc Sara Vander Borght who gave me the opportunity to perform my internship at the VATU group and guided me through this whole journey. Thank you for always supporting me, for your feedback on my thesis and for all the opportunities I was offered for not only my subject but also for my personal interests in the molecular diagnostics. Thank you for leading this work to a good end. Without you, this work wouldn't be what it is now.

Secondly, I want to thank dr. MSc Lien Spans for helping me with the take-off of my project and for answering all my questions. I would also like to thank the laboratory technicians Ellen Thys, Frederik Claessens, Karen Coppesmette and Petra Sintubin for helping me with the technical side of the project and for teaching me the technical skills. I would also like to thank Apr. PhD Laurence Slembrouck who will present my obtained results on the Belgian oncoforum 2019 at UZLeuven Gasthuisberg and for answering my questions.

I also want to thank my internal promotor dr. ing. Kristel Sniegowski for her feedback and helping me write my thesis. I also want to thank my communication teacher dr. Jeroen Lievens for his feedback on the poster, framework and abstract of this thesis.

Next, I would like to thank my friends and colleagues Anneleen Coun, Isabelle Van Dyck, Julie Boonen and Wouter Van Genechten for the overall support during this journey. And last but definitely not least I want to thank my mom, stepfather, boyfriend and my entire family for the support and many lovely messages for wishing me good luck during more difficult times.

Demi Renders

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GLOSSARY

AI	Aromatase Inhibitor
BB	Binding Buffer
cfDNA	cell-free DNA
CHG	Center for Human Genetics
CNB	Core Needle Biopsy
ctDNA	cell-free tumoral DNA
DCIS	Ductal Carcinoma In Situ
ddPCR	droplet digital PCR
EB	Elution Buffer
EMA	European Medicine Agency
ER	Estrogen Receptor
EQA	External Quality Assessment
FPR	False Positive Rate
HER2	Human Epidermal growth factor Receptor-2
HPEA FT	High Pure Extension Assembly Filter Tube
HR	Hormone Receptor
IDC	Invasive Ductal Carcinoma
IDT	Integrated DNA Technologies
ILC	Invasive Lobular Carcinoma
IORT	Intraoperative Radiation Therapy
LCIS	Lobular Carcinoma In Situ
LHRH	Luteinizing Hormone-Releasing Hormone
MIBB	Minimally Invasive Breast Biopsy
NGS	Next Generation Sequencing
PBB	Paraffin Binding Buffer
PFS	Progression-Free Survival
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
PK	Proteinase K
PR	Progesteron Receptor
R	Distribution

RIZIV	Rijksinstituut voor Ziekte- en Invaliditeitsverzekering
RMD	Rare Mutation Detection
RTKs	Tyrosine Kinase Receptors
SD	Standard Deviation
SERD	Selective Estrogen Receptor Degrader
SERM	Selective Estrogen Receptor Modulator
SNV	Single Nucleotide Variation
STT	Solid Tissue Tumors
TNBC	Triple Negative Breast Cancer
UZ Leuven	University Hospital Leuven
WBI	Wash Buffer I
WBII	Wash Buffer II
WT	Wild type

ABSTRACT

The detection of *PIK3CA* mutations in invasive breast carcinoma is important to determine the treatment strategy of the patient. In combination with fulvestrant, Alpelisib, a PI3Kα-specific inhibitor, shows an average extension of 5.3 months progression-free survival in clinical trials compared to fulvestrant alone in metastatic setting. Taking a new biopsy is often not possible in this setting, whereas the use of liquid biopsies is indicated. The aim of this work is to optimize and validate singular droplet digital PCR assays to detect four *PIK3CA* hotspot mutations in plasma.

The ddPCR assays were optimized and validated based on positive/negative controls. The intra- and inter-run precision, robustness and correctness were demonstrated. Subsequently, plasma of 20 patients with known mutation status on tumor tissue (n=12 *PIK3CA* negative, n=8 *PIK3CA* positive) were tested. For 18 of 20 samples, concordant results were observed between tissue and plasma. One sample, with only a His1047Arg mutation on tissue, showed Glu542Lys, Glu545Lys and His1047Arg mutations in plasma. In the other sample, Glu545Lys was detected on tissue and Glu542Lys in plasma. The sensitivity and specificity were 87.5% and 95.8% respectively.

A good correlation was observed between the detection of *PIK3CA* mutations in tissue and plasma. The validated test will be included in the routine diagnostics and provides an opportunity for the follow-up of the mutation status and for the support of the treatment strategy.

ABSTRACT IN HET NEDERLANDS

Het opsporen van *PIK3CA* hotspot mutaties in invasief borstcarcinoom is belangrijk om de behandelingsstrategie te bepalen. Alpelisib, een PI3Kα-specifieke inhibitor, toont in combinatie met fulvestrant een gemiddelde verlenging van progressievrije overleving van 5,3 maanden t.o.v. fulvestrant monotherapie in uitgezaaide setting. Hierbij is een nieuwe biopsie vaak niet mogelijk en is een liquid biopsie aangewezen. Het opzet van dit werk is het optimaliseren en valideren van singuliere droplet digital PCR testen om vier *PIK3CA* hotspot mutaties te detecteren in plasma.

Aan de hand van positieve/negatieve controles werden de *PIK3CA* ddPCR testen geoptimaliseerd en gevalideerd. De intra- en inter-run precisie, robuustheid en juistheid werden aangetoond. Vervolgens werd het plasma van 20 patiënten met gekende weefsel mutatiestatus (n=12 *PIK3CA* negatief, n=8 *PIK3CA* positief) getest. Dit resulteerde voor 18 van 20 stalen in een concordant resultaat tussen weefsel en plasma. Eén staal, met enkel een His1047Arg mutatie op weefsel, toonde Glu542Lys, Glu545Lys en His1047Arg mutaties in plasma. In het andere staal werd de Glu545Lys gedetecteerd op weefsel en de Glu542Lys in plasma. De sensitiviteit en specificiteit bedroegen respectievelijk 87,5% en 95,8%.

Een goede concordantie werd geobserveerd tussen detectie van *PIK3CA* mutaties in weefsel en plasma. De gevalideerde test zal in de routine diagnostiek worden opgenomen en biedt een mogelijkheid voor de opvolging van de mutatiestatus en ondersteuning van het behandelingsbeleid.

1 INTRODUCTION

1.1 Context

The Center for Human Genetics (CHG) is a Belgian institution situated at Gasthuisberg Leuven and is a part of the University Hospital Leuven (UZ Leuven). It mainly focusses on genetic diseases and birth defects but also on acquired onco-hematological disorders, including leukemia, lymphomas and solid tumors [1]. It is in this latter setting that my work is situated and more specifically in the domain of invasive breast tumors.

Every year in Belgium, around 10,500 patients are diagnosed with breast cancer of which 99% are women. Therefore, breast cancer is the most common cancer in women and about 1/9 will be diagnosed before the age of 75. Despite the large number of patients, 90% of these women will survive which makes it a well-treated disease [2].

The technique that is used to screen the breast is called the mammography which uses X-rays to examine the breast tissue on abnormalities [3]. When the result of the screening comes out positive, additional examinations are performed such as core needle biopsies (CNB) or surgical biopsies [4]. These biopsies can gather specific information about the tumor e.g. the morphological type, stage and occurring mutations, and can determine whether a specific therapy can be administered to the patient. Because the surgical and core needle biopsies are fairly serious procedures, researchers started looking for less invasive methods and examined the use of liquid biopsies. Various bodily fluids can be used as liquid biopsy however in the context of breast cancer and considering the time frame, blood is used. However, a surgical or core needle biopsy still remains the golden standard for the initial diagnosis. The liquid biopsy can be used for further monitoring of the patient's disease or for the determination of next-line therapies [4].

In the liquid biopsy components such as intact tumor cells and cell-free DNA (cfDNA) from different compartments of the body, as well as cell-free tumoral DNA (ctDNA) are present and contains in this way specific information about the breast tumor. These ctDNA sequences are very fragmented whereby they can be easily isolated using specific cfDNA extraction kits. However, the fraction of ctDNA is limited in comparison with the amount of cfDNA, which indicates that a very sensitive technique has to be used to pick up the ctDNA templates [4]. A qualified method to fulfill this goal is droplet digital PCR (ddPCR).

The mutations to be detected in this work are situated in the phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*) gene, located on the long arm of chromosome 3. The four most common hotspot mutations found in the *PIK3CA* gene are responsible for over 90% of the mutations of the gene and are situated in the helical domain (exon 10, coding exon 9) and the kinase domain (exon 21, coding exon 20) [5]. The two most frequently occurring somatic hotspot mutations in the helical domain are Glu542Lys and Glu545Lys in which the amino acid glutamate is replaced by lysine. The two occurring somatic hotspot mutations in the kinase domain are His1047Leu and His1047Arg where the same position is targeted but histamine is replaced by respectively leucine and arginine [6], [7].

1.2 Problem statement

Recently the efficacy of a drug called alpelisib, a treatment for terminal metastasized breast cancer patients with a *PIK3CA* hotspot mutation, is investigated in several clinical trials.

The drug focusses on progression-free survival (PFS) and is administered to patients who already received several other treatments [8]. As mentioned in the previous section, the concentration of ctDNA isolated from a liquid biopsy is limited in comparison with the concentration of cfDNA and DNA obtained using tissue samples. Therefore, a very sensitive detection method must be used such as ddPCR [4]. This technique is already used in the routine for different purposes but not yet for the detection of *PIK3CA* mutations in blood/plasm of patients with invasive breast cancer.

Therefore, the optimization and validation of the *PIK3CA* ddPCR technique is the goal of this work. The principle of operation for the ddPCR technique is similar to the standard PCR however the embodiment is fundamentally different. Unlike the standard PCR, the reaction mixture is not put into a single well of a 96 well plate but in many small sample-oil droplets. These droplets (in oil) are transferred to one single well of a 96 well plate whereby thousands (max 20,000) of individual reactions will take place in one well. The chance of amplification with this technique is much higher compared to the standard PCR technique and therefore a higher sensitivity can be achieved [4].

1.3 Objectives

The main goal of this work is to optimize and validate four singular ddPCR assays for the four most common *PIK3CA* hotspot mutations from patients with an invasive breast carcinoma, using liquid biopsies. Therefore, Bio-Rad validated singular primer probe assays are used. In a following study, a multiplex assay can be optimized and validated (not the scope of this work).

Prior to the optimization and validation of the ddPCR assay, ctDNA has to be extracted starting from blood plasm. Therefore, the first aim of this work is to compare the obtained concentration of two ctDNA extraction methods being the manual Cobas extraction kit (Roche) starting from 2 ml and the semi-automated Maxwell extraction kit (Promega) starting from 2 and 4 ml. Depending on these results, the current ctDNA extraction kit (Cobas) can be replaced by the Maxwell ctDNA extraction kit. This kit has a few benefits being that it only requires manual preparatory actions, tolerates higher starting volume and can extract up to 48 samples at once which enhances its yield and efficiency.

The goal is to optimize and validate qualitative ddPCR assays so that *PIK3CA* mutations can be traced without generating false positive results. This goal is fulfilled by determining the correct thresholds based on positive and negative results for every assay in the ddPCR droplet reader software. In this work, the assays are only used as a qualitative method, but they can also be used for quantitative purposes. The parameters intra-run and inter-run variation, robustness and correctness are examined in the validation process.

1.4 Methodology

The first step in the process is the separation of the blood plasm, starting from whole blood of the patient, collected in special tubes to stabilize the ctDNA.

Different extraction kits/methods (cfDNA Cobas extraction vs cfDNA Maxwell extraction) are compared with respect to the obtained concentration. A higher ctDNA concentration and total input volume hopefully leads to a higher chance of obtaining reliable results. The obtained elution volume of the Maxwell kit is identical for both volumes, but lower than the Cobas extraction kit. This should certainly lead to a higher cfDNA concentration in the 4 ml eluate of the Maxwell extraction kit. If this hypothesis is confirmed by the results, the Maxwell 4 ml extraction method will be implemented in the routine.

The used Bio-Rad ddPCR assays are commercially available and therefore the validated standard protocol can be tested first. The first step in the performance of a ddPCR assay is the preparation of a reaction mix per assay in which a standard quantity of supermix buffer, probes (wild type labeled HEX and mutant labeled FAM), primers and water are mixed. The cfDNA is then added to the mix in a separate room to avoid contamination. Next, the droplets are generated in a special cartridge and are then transferred to a 96 well plate using a multichannel pipet, which is then sealed to avoid contamination and evaporation. The 96 well plate is then placed into the PCR amplifier where the actual PCR reaction takes place. The last step is the detection using the QX200™ Droplet Digital™ PCR reader which is based on a fluorometric measurement.

Based on the detected signal, the QuantaSoft™ software generates a 2D-plot with 4 clusters of droplets [9]. The boundaries of the clusters can be determined by performing the assays with positive (gBlocks or positive patient samples) and negative controls. By these experiments, the correct thresholds can be set which is a crucial step in the optimization and validation of the assays.

2 LITERATURE REVIEW

This work covers the study performed to optimize and validate new droplet digital PCR (ddPCR) assays in order to detect *PIK3CA* hotspot mutations in liquid biopsies of patients with an invasive breast carcinoma. Therefore, the first section of the literature review covers the subject breast cancer and more specifically the methods of diagnosis and the most common subtypes of breast cancer. Next, the *PIK3CA* pathway is described and illustrated. A third section shows the various types of available treatments and discusses the use of a personalized therapy called alpelisib. Then, the purpose of using liquid biopsies is illustrated, the advantages according to tissue biopsies are shown and the possibility to use it as a mutation detection tool is elucidated. At last, the principle of and analysis strategy for the ddPCR technique is illustrated.

2.1 Breast cancer

Breast cancer is the most common cancer in Belgium and every year, 10,500 new patients are diagnosed from which 99% are women. Statistics show that one in nine women will be diagnosed with breast cancer before the age of 75 of which 90% will survive [2]. This makes breast cancer a well-treated disease. Nevertheless, continuous research is important to develop or improve various therapies in order to benefit the patient's life.

2.1.1 Diagnosis

The first step in the diagnosis of breast cancer is screening the breast using a mammography device which uses X-rays to examine the breast tissue on abnormalities [3]. The technique can be used as a screening method as well as for diagnostic purposes, depending on the cause of examination [10]. The screening mammography is used for routine checks and is offered by the Belgian government for women between 50 and 69 years old, every three years [11]. If an abnormality is detected during the screening, or if a woman notices physical changes of the breast, a diagnostic mammography is pursued. During this examination, multiple X-rays are made in order to inspect the breast tissue from different viewpoints [10]. If the results are negative, there are most often no further actions required. If the results are positive, further examination is necessary.

The second step in the diagnosis of breast cancer is the initial tissue biopsy. This biopsy can be taken during a surgical procedure but most often a technique called core needle biopsy (CNB) is used, shown in Figure 1 [12]. The panel of the consensus conference III "agreed that percutaneous needle biopsy (also known as minimally-invasive breast biopsy, or MIBB) has demonstrated accuracy equivalent to open surgical biopsy and is the optimal initial tissue-acquisition procedure for image-detected breast abnormalities" [13]. This technique provides specific information "including distinction between invasive and noninvasive cancer, tumor grade, hormone receptor expression and HER2/neu oncogene overexpression" [12].

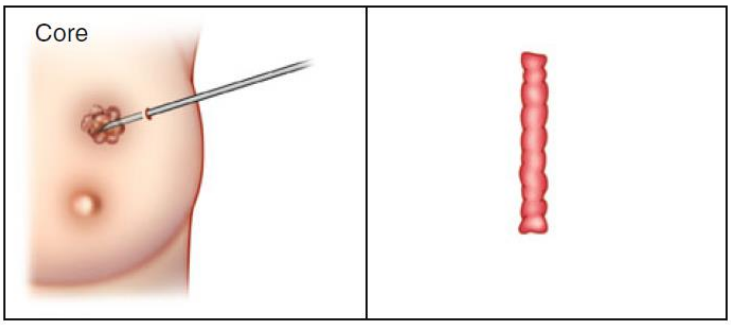

Figure 1 Presentation of a tissue core needle biopsy [14, p. 21]

2.1.2 Subtypes of breast cancer

Various subtypes of breast cancer can be defined, however two main types can be appointed being ductal and lobular carcinoma. When looking at the anatomy of the breast shown in Figure 2, it is constructed out of lobes which produce milk and ducts which lead the milk to the nipple and fatty tissue [2].

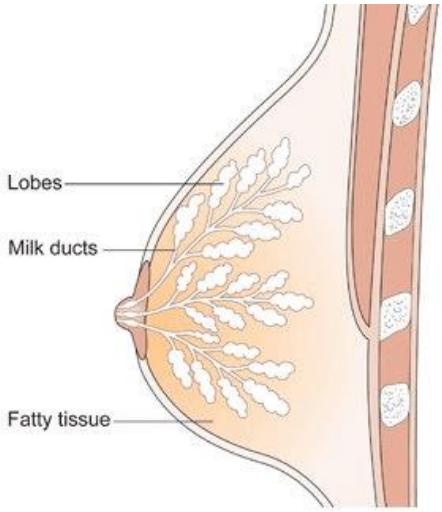

Figure 2 Anatomy of the breast [2]

When the tumor originates from the milk ducts, ductal carcinoma is diagnosed which can be invasive as well as non-invasive [15]. The non-invasive type is also known as Ductal Carcinoma In Situ (DCIS) and is an early stage of breast cancer which has not yet spread to surrounding tissue and is well treatable. If no treatment is administered, the cancer can migrate to the surrounding tissues which is then called Invasive Ductal Carcinoma (IDC). Approximately 70-80% of all women diagnosed with breast cancer have IDC which makes it the most common subtype of breast cancer [2].

Tumors originating from the lobes are called lobular carcinomas and can also appear in two forms. The first, called Lobular Carcinoma In Situ (LCIS), is an accumulation of abnormal cells in the lobes and indicates that there is an increased risk to develop an invasive type of breast cancer. Invasive Lobular Carcinoma (ILC) originates in the lobes of the breast followed by a metastasis to the surrounding tissues and possibly the lymph nodes and other parts of the body [2].

2.2 *PIK3CA*

2.2.1 PI3K Pathway

The *PIK3CA* gene is part of the PI3K pathway which regulates crucial processes such as cell proliferation, cell division, protein synthesis, apoptosis, … [16]. The PI3K-AKT pathway, shown in Figure 3, is initiated by the activation of the tyrosine kinase receptors (RTKs) by molecules such as growth factors, cytokines and hormones. Binding of these molecules induces dimerization of two RTK monomers and phosphorylation of the intracellular tyrosine kinase domain. The PI3K enzyme, indicated in the black circle in Figure 3, phosphorylates PIP_2 to PIP_3 from whence a series of reactions follow, which eventually leads to the regulation of the crucial processes as described above [17]. Phosphorylation by the PI3K enzyme is initiated by its catalytic P110α-subunit which is encoded by the *PIK3CA* gene [6], [18], [19]. The mutations studied in this work are situated in this gene and will be elucidated in the section below.

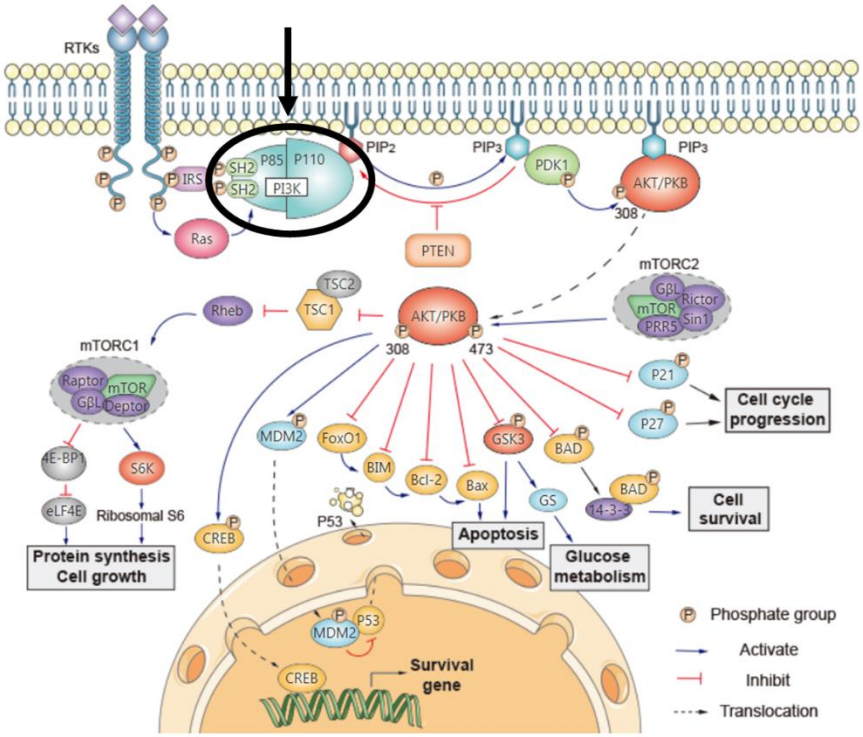

Figure 3 PI3K pathway with the indication of the position of the PI3K enzyme in the signaling pathway [17]

2.2.2 *PIK3CA* hotspot mutations

PIK3CA mutations can be induced like mutations in other genes by hormonal regulation, toxic agents, environmental conditions, …. This leads to alterations of one or more amino acids whereby the primary structure of the protein is altered and thereby also its function. In case of the *PIK3CA* gene, the somatic hotspot mutations result in a more constitutional character of the protein which leads to an uncontrolled PI3K pathway and eventually the development and maintenance of (invasive) tumors [6].

The four most common hotspot mutations found in the *PIK3CA* gene are all single nucleotide variations (SNV) and are responsible for over 90% of the mutations of the gene and are situated in the helical

domain (exon 10, coding exon 9) and the kinase domain (exon 21, coding exon 20) as shown in Figure 4 and Figure 5 [7].

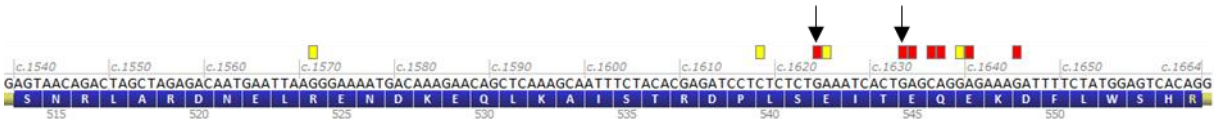

Figure 4 Alamut wild-type (normal) DNA sequence coding exon 9 [5], the red marks indicated the occurred hotspot mutations and their occurrence at UZ Leuven. The yellow marks indicate the less frequent probably passenger mutations.

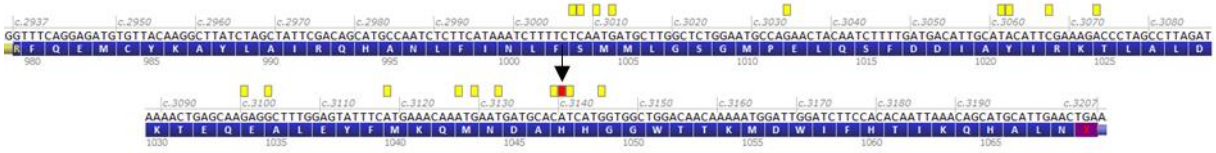

Figure 5 Alamut wild-type (normal) DNA sequence coding exon 20 [5], the red marks indicated the occurred hotspot mutations and their occurrence at UZ Leuven. The yellow marks indicate the less frequent probably passenger mutations.

Two frequently occurring somatic hotspot mutations in the helical domain are Glu542Lys and Glu545Lys in which the amino acid glutamate is replaced by lysine. Two frequently occurring somatic hotspot mutations in the kinase domain are His1047Leu and His1047Arg where the same position is targeted but histamine is replaced by respectively leucine and arginine [6], [7]. Based on these data, it is evident that the selectivity of the ddPCR assays is very important in order to distinguish the difference between the correct reference DNA sequence and the mutated DNA sequence with just one substituted base.

2.3 Therapy

After defining the exact tumor type and its characteristics, based on the results of the core needle biopsy/surgical biopsy, a personalized therapy program is defined by the clinicians. This program can include surgery, radiation therapy, chemotherapy, hormone therapy, targeted therapies or combinations [20].

2.3.1 Conventional therapy

The term conventional therapy includes all treatments that are non-specific, which means that it does not target a specific type of molecule e.g. proteins that regulate a pathway, but can potentially strike all body cells. The treatments covered in this category are surgery, radiation therapy, chemotherapy and hormonal therapy [20].

2.3.1.1 Surgery

Surgery is quite often a basic step included in a patient's therapy program and can be subdivided in the breast-conserving surgery, also called lumpectomy, and the mastectomy.

The lumpectomy is a surgical procedure where the tumor (lump) and a part of the healthy surrounding tissue is removed and can be performed on patients with a small breast carcinoma. A part of the healthy surrounding tissue is removed to assure that all the tumor cells are removed to avoid a relapse [21], [22]. Some of the lymph nodes can also be removed to examine if tumor cells have spread and to

determine whether the breast carcinoma is invasive or not [22]. In addition, radiation therapy can be administered shortly after the lumpectomy to eliminate remaining tumor cells in the surrounding tissue [22].

The mastectomy is a surgical procedure where the entire breast and axillary lymph nodes are removed and can be performed with or without reconstruction. The mastectomy is performed on patients with large and/or metastatic tumors or on a healthy person with a high risk of developing breast cancer due to hereditary factors [23], [24].

2.3.1.2 Radiation therapy

As mentioned in the previous section, radiation therapy can be administered to assure all the remaining tumor cells, in the surrounding healthy tissue, are eliminated [25]. There are three main types of radiation therapy being external whole-breast radiation, internal radiation and the intraoperative radiation therapy (IORT) [26].

The external whole-breast radiation therapy is the most frequently used radiation therapy in which a linear accelerator machine sends a beam of high-energy radiation to the breast. Hereby the breast is targeted from different angles and the energy dose, expressed in Gray, can be variated depending on the amount of sessions [27]. The internal radiation therapy uses small pieces of radioactive material, also called seeds, and places them in the space where the tumor was located. In this way, the remaining tumor cells are targeted from the inside of the body [28]. During the intraoperative radiation therapy, a high dose of radiation is administered during the operation, right after a lumpectomy is performed [29].

2.3.1.3 Chemotherapy

Chemotherapy is the administration of a drug, more specifically a cytostatic, to slow down the growth of tumor cells or to eliminate them. It can be used after surgery to remove any remaining tumor cells or it can be used in advance of a surgery to reduce the tumor size. It is a systemic therapy that can be administered orally, intravenous or intramuscular and can affect all cells. This is one of the big disadvantages of the therapy because it causes a lot of side-effects such as hair loss, nausea, fatigue, etc. Many different types of chemotherapy are available and can be administered as such or a cocktail of cytostatics can be administered [30].

2.3.1.4 Hormonal therapy

Hormonal therapy is a qualified therapy for women with hormonal induced breast cancer. Approximately 2/3 tumors are hormone receptor-positive which means their growth and spread is stimulated when high levels of estrogen (ER-positive) or progesterone (PR-positive) are present. This because the tumor cells carry estrogen and/or progesterone receptors which are activated when estrogen and/or progesterone binds. The therapy can be used as an adjuvant treatment, after surgery to prevent relapse, or as a neoadjuvant treatment, before surgery to facilitate the surgical procedure. There are different types of hormonal therapy available but the two most common types are based on blocking the hormonal receptor and decreasing the amount of hormone [31], [32].

The hormone receptor blocking drugs can be subdivided into the selective estrogen receptor modulators (SERMs) and the selective estrogen receptor degraders (SERDs). The SERMs work as an anti-estrogen in the breast cells but as an estrogen in other parts of the body e.g. the uterus and the bones. Examples of this drug are tamoxifen which is administered to patients with non-invasive breast cancer and toremifene which is used in metastatic context. The SERDs work as an anti-estrogen in the

entire body and block and damage the estrogen-receptors. An example of a SERD is fulvestrant which is used in metastatic context [31].

The hormone decreasing drugs can also be subdivided into two groups being the aromatase inhibitors (AIs) and the ovarian suppression drugs. The AIs are used in post-menopausal women and stop the estrogen production. Examples of these drugs are letrozole, anastrozole and exemestane and are mostly used as an adjuvant therapy. The ovarian suppression drugs are used in pre-menopausal women and remove or shut down the ovaries to decrease the amount of hormones. This makes them post-menopausal whereby other therapies can be administered. The removal or shutdown of the ovaries can be achieved by surgically removing the ovaries, called an oophorectomy, by using Luteinizing hormone-releasing hormone (LHRH) analogs which stops the estrogen production by the ovaries and last by using chemotherapy in which the ovaries are damaged [31].

2.3.2 Personalized therapy

The term personalized therapy includes the administration of a chemical drug that targets a specific molecule e.g. a specific receptor or key molecule in a pathway, in order to obtain an increased therapy response with less side effects [33]. It is not a type of immunotherapy since the administered drugs are of chemical origin. The therapy is based on the characteristics and microenvironment of the tumor and on the characteristics of the tumor and therefore a specific personalized therapy program is composed [33].

The personalized therapy that qualifies for the treatment of patients with a *PIK3CA* gene mutation induced breast cancer described in this work is called alpelisib (Novartis). It is a specific PI3K alfa-inhibitor (BYL719) and shows positive results in combination with fulvestrant in the global Phase III SOLAR-1 trial [8]. “The trial evaluated the efficacy and safety of alpelisib in postmenopausal women with *PIK3CA* mutated hormone-receptor positive (HR+), Human Epidermal growth factor Receptor-2 negative (HER2-) advanced or metastatic breast cancer that progressed on or after an aromatase inhibitor with or without a CDK4/6 inhibitor” [8].

Alpelisib, known as a very toxic drug, is administered to terminal patients with metastatic breast cancer who already received several other therapies and focusses mainly on progression-free survival (PFS). The study showed a median PFS of 11 months for the combination alpelisib/fulvestrant compared to a PFS of 5.7 months for fulvestrant alone [8]. The most frequent side-effects of alpelisib are rash, gastro-intestinal problems and the development of hyperglycemia during the treatment [8], [34]. The hyperglycemia can be controlled by administering anti-diabetic medication [8]. Alpelisib is still examined in several studies and is not yet commercially released and approved by the European Medicine Agency (EMA).

2.4 Liquid biopsies

A liquid biopsy is a simple and minimal invasive alternative to a surgical biopsy in order to achieve specific information about a tumor. Various body fluids can be used as a liquid biopsy e.g. blood, saliva, urine, lumbar fluid, … however in the context of this work, blood is used [4].

When diagnosing a patient, currently an initial surgical tissue biopsy must be taken from which the DNA has to be extracted and analyzed in order to gather information about the patient’s tumor. This is a serious and unpleasant procedure and in order to execute it, “the tumor must be accessible and already be of detectable size, and in many cases a mass is not detected at an early enough stage for effective treatment” [35]. Therefore, a liquid biopsy can offer benefits such as being a less invasive detection method and it can potentially be used in the future as an early detection method [35]. The

liquid biopsy is also an interesting material source that can be used for the further monitoring of the patient. Hereby surgical procedures can be avoided and the patient can be monitored in time to check on treatment response.

The blood used for the liquid biopsy contains intact tumor cells, cfDNA originating from various tissues of the body and ctDNA origination from and mimicking the (metastatic) tumor tissue as shown in Figure 6 .The ctDNA and cfDNA are already strongly fragmented and this characteristic can be used to extract and isolate the DNA using extraction kits. However, the isolated ctDNA fraction is much lower than the cfDNA fraction which indicates that a very sensitive detection technique has to be used, being ddPCR [4].

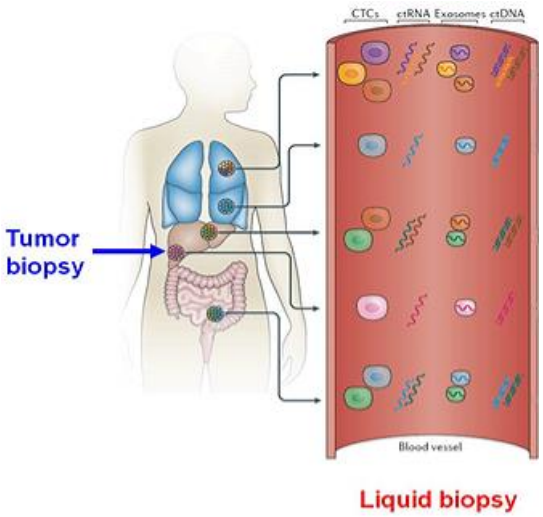

Figure 6 Components of a liquid biopsy [4]

2.5 droplet digital PCR (ddPCR)

2.5.1 Principle

Droplet digital PCR is a technique similar to the well-known standard PCR but is much more sensitive because of its unique embodiment. Unlike the standard PCR, ddPCR is performed using thousands of nanoliter (nl) sized sample droplets which are generated by a droplet generator that disperses the reaction mix containing the DNA fragments in droplet generation oil by using vacuum (see Figure 7) [36]. The obtained droplets are transferred to one single well of a 96 well plate whereby in amplification, thousands (max 20,000) of individual reactions can take place in one well [4].

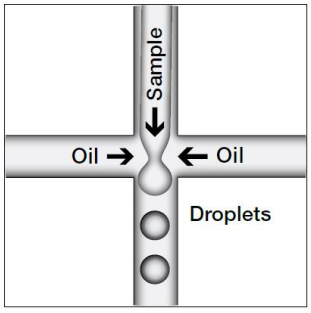

Figure 7 Droplet generation by the Bio-Rad QX200 Droplet Generator [36, p. 3]

After amplification, the plate is analyzed with the droplet reader which is based on fluorescence detection. Therefore, a specific Bio-Rad PrimePCR™ ddPCR™ Rare Mutation Detection (RMD) assay is used. "Rare mutation detection (RMD) refers to detection of a sequence variant that is present at a very low frequency in a pool of wild-type (WT) background. The challenge for RMD is the discrimination between two highly similar sequences, one of which is significantly more abundant than the other" [9]. The assay uses two competitive probes that are each labeled with a different fluorophore, shown in Figure 8. The fluorophores used in the assays are FAM (blue) and HEX (green) and detect respectively the mutated DNA and the wild type DNA [36].

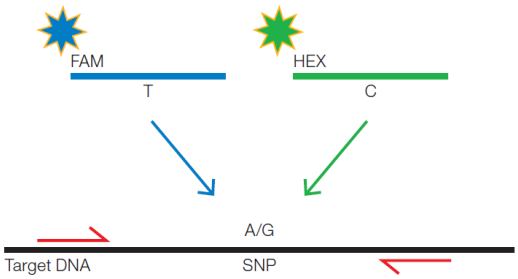

Figure 8 Presentation of the two competitive probes, FAM (blue) binding the mutant DNA and HEX (green) binding the wild type DNA [9, p. 5]

As mentioned above the analysis is performed by the ddPCR droplet reader which reads the labeled amplicons by individually screening them using a two channel fluorescence detector, shown in Figure 9. Hereby "positive droplets, which contain at least one copy of the target DNA molecule, exhibit increased FAM fluorescence compared to negative (no DNA) droplets" [36].

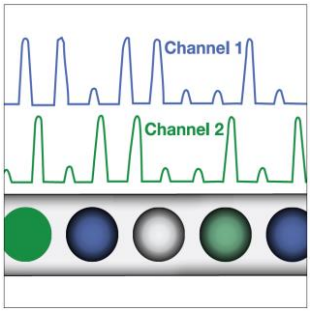

Figure 9 Fluorescence reading using two channels [36, p. 5]

2.5.2 Analysis of the results using the QuantaSoft™ 1.7.4 software

Once the droplets are screened, a 2-D plot is generated in which the "channel 1 fluorescence (FAM) is plotted against channel 2 (HEX) for each droplet" as shown in Figure 10 [36]. The droplets are clustered in to four groups and colors:

- the black cluster in the left bottom corner are double negative (FAM and HEX) droplets that do not contain any DNA;
- the green cluster in the right bottom corner are HEX positive droplets that contain only wild-type DNA;
- the blue cluster in the left upper corner are FAM positive droplets that contain only mutated DNA;
- the orange cluster in the right upper corner are double positive (FAM and HEX) droplets that contain both mutated and wild type DNA [36].

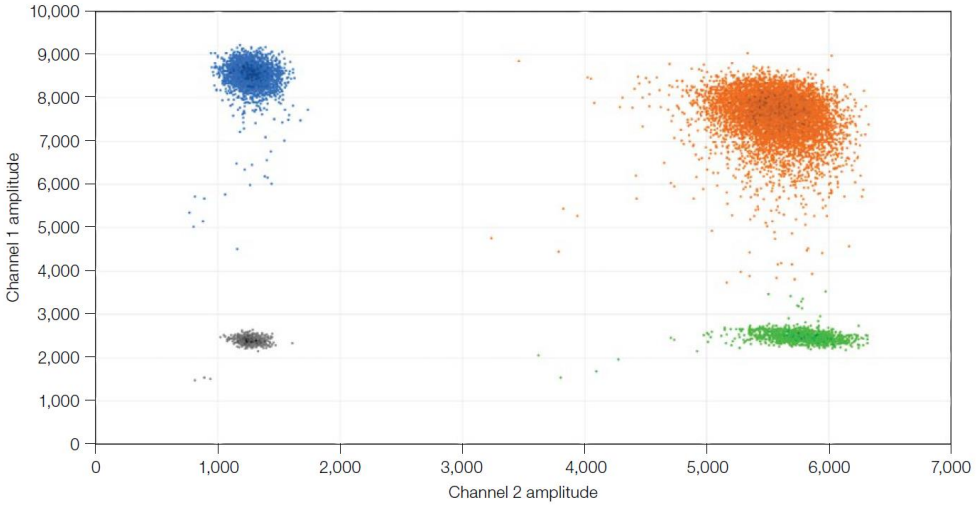

Figure 10 2-D plot generated in the QuantaSoft™ software [36, p. 6]

After counting the amount of positive and negative droplets, "the software then fits the fraction of positive droplets to a Poisson algorithm *(1)* to determine the starting concentration of the target DNA molecule in units of copies/µl input" [36].

$$Copies\ target\ DNA\ per\ droplet = -ln\left(1 - \frac{positive\ droplets}{total\ amount\ of\ droplets}\right) \quad (1)$$

The 2-D plot in Figure 10 shows well-separated clusters which makes it relatively easy to manually determine the correct thresholds. When working with patient samples, the clusters are not always well separated and therefore positive controls (gBlocks) are additionally used to manually set these thresholds. The use of the gBlocks will be further elucidated in the material and methods.

3 MATERIAL AND METHODS

3.1 Patient population

All 21 patients selected for this work are female and diagnosed with a metastasized invasive breast carcinoma. The patient population is further specified in Table 1 below. The sample of patient 16 is not included in the ddPCR analysis because the used blood sample was highly hemolyzed which can possibly generate non-representative results.

Table 1 Specifications of the patient population

Patient	Age	Tumor status	*PIK3CA* status*	Metastasized tumor localization
1	73	ER+/PR+/HER2-	Glu542Lys	liver
2	70	ER+/PR+/HER2-	Glu545Lys	abdomen
3	69†	ER+/PR+/HER2-	His1047Arg	axillary lymph node
4	71†	ER+/PR+/HER2-	Glu545Lys	breast
5	69	ER+/PR+/HER2-	Glu545Lys	supraclavicular lymph node
6	63	ER+/PR+/HER2-	Glu545Lys	liver
7	62†	ER+/PR+/HER2-	Glu545Lys	liver
8	48	TNBC**	Glu545Lys	skin chest wall
9	78	ER+/PR+/HER2-	WT	breast
10	61	TNBC	WT	Unknown
11	52	ER+/PR+/HER2-	WT	right mammary gland
12	65	TNBC	WT	supraclavicular lymph node
13	57	TNBC	WT	lung, top right
14	61	TNBC	WT	skin chest wall
15	49	ER+/PR-/HER2-	WT	ovary
16	31	Unknown	Unknown	lung, bottom left
17	48	ER-/PR+/HER2+	WT	liver
18	46	ER+/PR-/HER2-	WT	liver
19	74	ER+/PR+/HER2-	WT	liver
20	74	ER+/PR-/HER2-	WT	liver
21	59	ER+/PR+/HER2-	WT	liver

*The *PIK3CA* (coding exon 9 and 20*)* status is determined on tissue sample using the SeqCap assay (Roche, IDT)

**TNBC = Triple Negative Breast Cancer (ER-/PR-/HER2-)

The personalized therapy alpelisib is studied in clinical trials on patients with a HR+/HER2- (=ER+/PR+/HER2-) tumor status as mentioned in section 2.3.2. However, not all patient material used in this work matches this specific profile.

3.2 cfDNA plasma extraction kits

Two cfDNA extraction methods being the Cobas® cfDNA Sample Preparation Kit (Roche) and the Maxwell® RSC LV ccfDNA Kit, Custom (Promega) are used in this work.

3.2.1 Cobas® cfDNA Sample Preparation Kit (Roche)

The Cobas® cfDNA Sample Preparation Kit is a manual extraction kit and requires 2 ml blood plasm. The blood plasm was first vortexed and transferred to a labeled 15 ml falcon tube where 250 µl of proteinase K (PK) was added to digest the present proteins. Then 2 ml of DNA paraffin binding buffer

(PBB) was added and the falcon tube was mixed by shaking it five times. After an incubation of 30 minutes at room temperature, 500 µl isopropanol was added to create an organic phase to which all the hydrophobic components could migrate. The tube was then mixed by shaking it five times and the content was transferred to a High Pure Extension Assembly Filter Tube (HPEA FT), shown in Figure 11. This tube contains a small chromatography column (indicated as Filter Tube in Figure 11) with a glass fiber filter insert that binds the cfDNA.

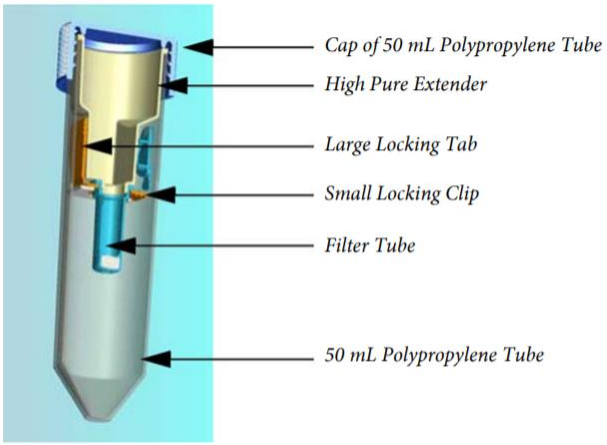

Figure 11 High Pure Extension Assembly Filter Tube (HPEA FT) [37, p. 13]

The tube was then centrifuged for 5 minutes on 4000xg where after the chromatography column was placed on a collection tube. The chromatography column was then washed using Wash Buffer I (WBI), centrifuged for 1 minute on 8000xg and transferred to a new collection tube. This step was repeated using Wash Buffer II (WBII) and then the glass fiber filter insert was dried by centrifuging the column for 1 minute on 16,000-20,000xg. The column was then placed on an elution tube and 100 µl of DNA Elution Buffer (EB) was added in the center of the filter's surface. After an incubation of 5 minutes, the elution tube with column was centrifuged for 1 minute on 8000xg. As last, 80 µl of eluate was transferred to a labeled Eppendorf safe-lock tube without touching the pellet that was formed on the bottom of the elution tube. This Eppendorf safe-lock tube now contains the extracted cfDNA. For the exact protocol see ANNEX 1.

For the determination of the extracted cfDNA concentration, the Qubit® dsDNA HS Assay Kit Q32851 (Invitrogen) was used. The use of this kit will be described in section 3.2.3.

3.2.2 Maxwell® RSC LV ccfDNA Kit, Custom (Promega)

The Maxwell® RSC LV ccfDNA Custom Kit on a Maxwell RSC device can process up to 48 samples at once and was performed with 2 ml and 4 ml of blood plasm. The blood plasm was first vortexed and transferred to a labeled 15 ml falcon tube where an equal amount of Binding Buffer (BB) was added (2 or 4 ml). Then 140 µl of magnetic resin was added where after an incubation of 45 minutes followed. The tube had to be kept in suspension during the entire incubation so therefore a rotator shaker was used. After the 45 minute incubation, the tube was centrifuged for 2 minutes on 1000xg to pellet the magnetic resin and the supernatant was removed. The pellet was then resuspended by transferring the content of well 1 of the Maxwell® RSC LV ccfDNA Custom Kit cartridge, shown in Figure 12, to the 15 ml falcon tube.

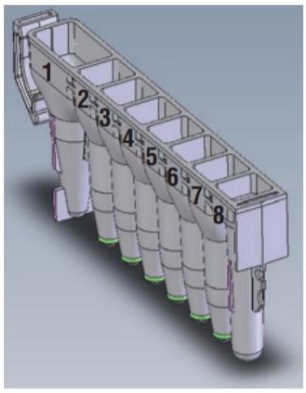

Figure 12 Maxwell® RSC LV ccfDNA Custom Kit cartridge [38, p. 4]

Once the pellet was resuspended, the content was transferred back to the first well of the cartridge. A plunger was placed in the eighth well of the cartridge and an elution tube filled with 75 µl EB was placed in the Maxwell Deck Tray, shown in Figure 13. The 'RSC ccfDNA Plasma method' program was then selected and after approximately one hour, the run was completed. As last, the content from the elution tube was transferred into a labeled Eppendorf safe-lock tube while placing a magnet alongside the elution tube to prevent the transfer of any remaining magnetic resin. For the exact protocol see ANNEX 2.

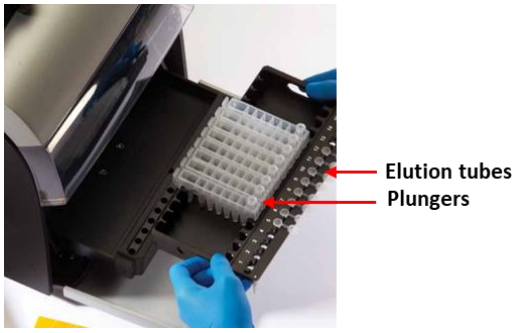

Figure 13 Maxwell Deck Tray with cartridges, plungers and elution tubes [39, p. 10]

For the determination of the extracted cfDNA concentration, the Qubit® dsDNA HS Assay Kit Q32851 (Invitrogen) was used. The use of this kit will be described below.

3.2.3 Qubit® dsDNA HS Assay Kit

The Qubit® dsDNA HS Assay Kit was used to determine the concentrations from the obtained cfDNA extracts and is based on a fluorescence measurement. The first step was to prepare the Qubit Working Solution by mixing 1 µl of Qubit® dsDNA HS Assay/sample with 199 µl of Qubit buffer solution/sample. The Qubit fluorometer had to be recalibrated before each use and therefore two standards where used. The working solution was vortexed and distributed into assay tubes being 190 µl/standard and 199 µl/sample. Then 10 µl of standard or 1 µl of cfDNA extract was added and the assay tubes where vortexed using VWR® Light-Duty Tissue Wipers to avoid interference from impurities sticking to the outside of the assay tube. After an incubation of 2 minutes, the standards where measured and a calibration curve was drawn where after the sample concentrations could be measured. For the exact protocol see ANNEX 3.

3.3 *PIK3CA* ddPCR analysis

3.3.1 Preparation *PIK3CA* ddPCR controls

A series of controls were included in each ddPCR experiment to guarantee that the obtained results were correct. This series included (in duplo) two positive controls with different % mutant fraction, one negative control and Nuclease-Free water (ThermoFisher Scientific) as no template control.

A negative control is a wild-type DNA sequence that does not contain the studied mutations and is used to determine the false positive rate (FPR). It must have a high ploidy correspondence to a normal human DNA sequence and must be available in large amounts. Therefore, the use of a cell line was chosen as negative control. The archive of the CME cell culture lab contains various cell lines and based on literature research the DLD-1 cell line, derived from a colorectal adenocarcinoma [40], and the PA-1 cell line, derived from an ovary teratocarcinoma [41], were ddPCR screened. After screening, the concentration of the selected cell line was measured and diluted in TE (1x) pH 8.0 low EDTA + poly A buffer to a concentration of 7.5 copies/µl.

The Nuclease-Free water was used as no template control to exclude any contamination.

The commercially available gBlocks (Integrated DNA Technologies, IDT) were used as positive controls and are artificial DNA sequences that contain the examined hotspot mutation. A positive control should be almost identical to a positive patient sample and therefore the gBlocks were diluted and mixed with the negative control wild-type cell line and TE (1x) pH 8.0 low EDTA + poly A buffer. The mutated gBlock gene sequences for all four hotspot mutations discussed in this work are shown in ANNEX 4, along with their characteristics.

The first step in the positive control preparation was to resolve the gBlock powders since they were delivered in lyophilized form. Therefore, the tube was centrifuged for 3-5 seconds and 50 µl of TE (1x) pH 8.0 low EDTA + poly A buffer was added to 500 ng of the lyophilized powder whereby a concentration of 10 ng/µl was obtained. The tubes where then vortexed and incubated for 20 minutes at 50°C. Since the unit of the ddPCR software (QuantaSoft™) is copies/µl, the concentration of the resolved gBlock were converted using equation 2 which resulted in a concentration of 1.95×10^{10} copies/µl.

$$number\ of\ copies/\mu l = \frac{X\ ng * 6{,}022 * 10^{23}\ molecules/mole}{M\ g/mole * 1 * 10^{9}\ ng/g} \quad (2)$$

With X = amount of amplicon in ng
M = molecular weight in g/mol

This stock solution was then diluted as shown in Figure 14. The first 3 dilutions, 1:1000 dilutions, were made by mixing 1.5 µl from the previous tube with 1498.5 µl of TE (1x) pH 8.0 low EDTA + poly A buffer. For the fourth dilution, a 1:10 dilution, was made by mixing 150 µl of dilution three with 1350 µl TE (1x) pH 8.0 low EDTA + poly A buffer.

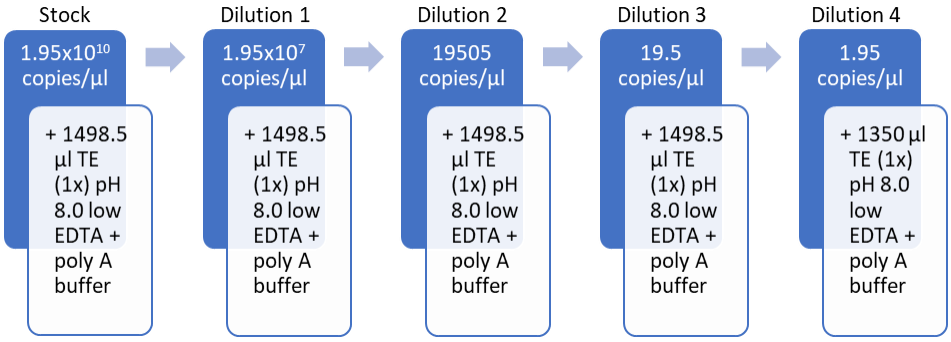

Figure 14 Serial dilution scheme gBlocks

Finally, the gBlocks were mixed with the negative control wild-type (WT) DNA cell line. In this work, two positive controls were used, one with a high and one with a low number of copies/µl of gBlock. The concentration of wild-type DNA was constant in every positive control. The high positive control was used to check if the assay was working, the low control was used to determine the detection limit for this assay. Therefore, several low controls were made.

The target concentrations for the high positive control was 10 copies/µl gBlock. For the low positive control following concentrations were screened: 0.6 copies/µl and 0.8 copies/µl. The exact calculations for the final used controls can be found in ANNEX 5.

3.3.2 Performance ddPCR mutation analysis

A typical ddPCR mutation analysis experiment was performed using both the positive controls, the negative control, the Nuclease-Free water and the patient material, all in duplo.

In the first step of the mutation analysis, the ddPCR reaction mix was prepared as shown in Table 2. The required volume for one ddPCR reaction (1 well) is 22 µl which consists out of 14 µl ddPCR reaction mix and 8 µl patient DNA (standard setting). The ddPCR reaction mix was made in a pre-PCR room where no DNA was allowed. Once the reaction mix was made and aliquoted in eps (14 µl/reaction) they were transferred to a separate room where the DNA was added (8 µl/reaction). The mixture was then vortexed and is from now on called 'sample'.

Table 2 Required reagents with their amounts for the ddPCR mix

Reagent	Amount/reaction
ddPCR mix (14 µl/reaction)	11 µl ddPCR Supermix for Probes (No dUTP) 1 µl 20x mutant/WT primer/probe* 2 µl Nuclease-Free water

*The specifically used ddPCR assays (Bio-Rad) are:

- ddPCR™ Mutation Assay: *PIK3CA* p.Glu542Lys, Human, Unique Assay ID: dHsaMDV2010073;
- ddPCR™ Mutation Assay: *PIK3CA* p.Glu545Lys, Human, Unique Assay ID: dHsaMDV2010075;
- ddPCR™ Mutation Assay: *PIK3CA* p.His1047Leu, Human, Unique Assay ID: dHsaMDV2010123;
- ddPCR™ Mutation Assay: *PIK3CA* p.His1047Arg, Human, Unique Assay ID: dHsaMDV2010077.

The second step in the process was the droplet generation. Therefore, a DG8™ Cartridges for QX200 Droplet generator was placed in the DG8™ Cartridge holder as shown in Figure 15a, and 20 µl of every sample was pipetted in the middle row of the cartridge. Once all samples where pipetted, 70 µl of Droplet generation oil for Probes was added in the bottom row.

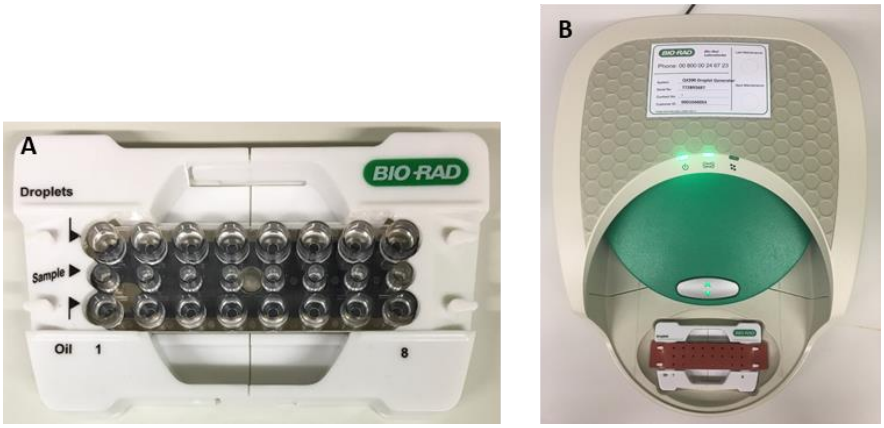

Figure 15 (A) DG8™ Cartridge holder with cartridge (B) Cartridge holder with gasket placed in QX200™ Droplet Generator

After placing a DG8™ Gaskets on top, the cartridge holder was placed into the QX200™ Droplet Generator, shown in Figure 15b. The obtained droplets were then transferred to a ddPCR™ 96-Well Plate using a multichannel pipet. After transferring, the plate was sealed on 180°C in the PX1™ PCR Plate Sealer to prevent evaporation and contamination.

Next, the actual ddPCR amplification was performed in the C1000 Touch™ Thermal Cycler T-87316 using the ddPCR mutation detection program shown in Table 3.

Table 3 PIK3CA ddPCR mutation detection program

	Temperature	Time	#cycli	Ramp rate
Enzyme activation	95°C	10 min	1	2°C/sec
Denaturation	94°C	30 sec	40	
Annealing/extension	55°C	1 min		
Enzyme deactivation	98°C	10 min	1	
Hold	12°C	Infinite	1	1°C/sec

After amplification the plate was transferred to the QX200™ Droplet Digital™ PCR reader where a fluorescence measurement was performed as described in section 2.5.1.

3.3.3 Result interpretation

The obtained results were interpreted and checked on four specifications.

For the first specification, the total amount of detected droplets had to be higher than 10,000 and lower than 20,000 per well as shown in Figure 16. If lower than 10,000; the well was not accounted as reliable and the result was not analyzed.

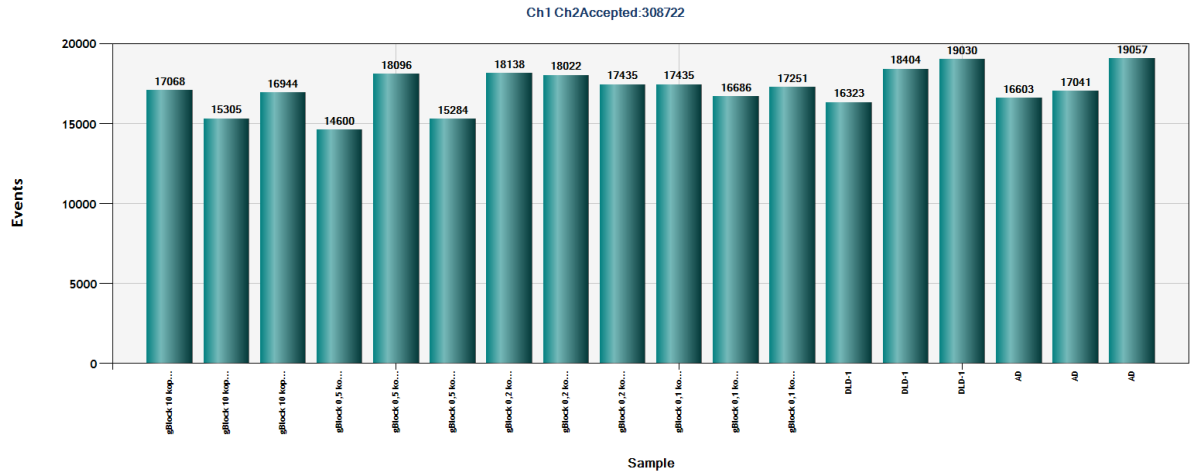

Figure 16 Illustrative example total amount of droplets per sample (>10,000 and <20,000)

For the second specification, the no template control had to be completely negative so only empty, black droplets were allowed as shown in Figure 17. If the no template control contained any kind of DNA (WT or mutant), the experiment was considered as contaminated and had to be repeated.

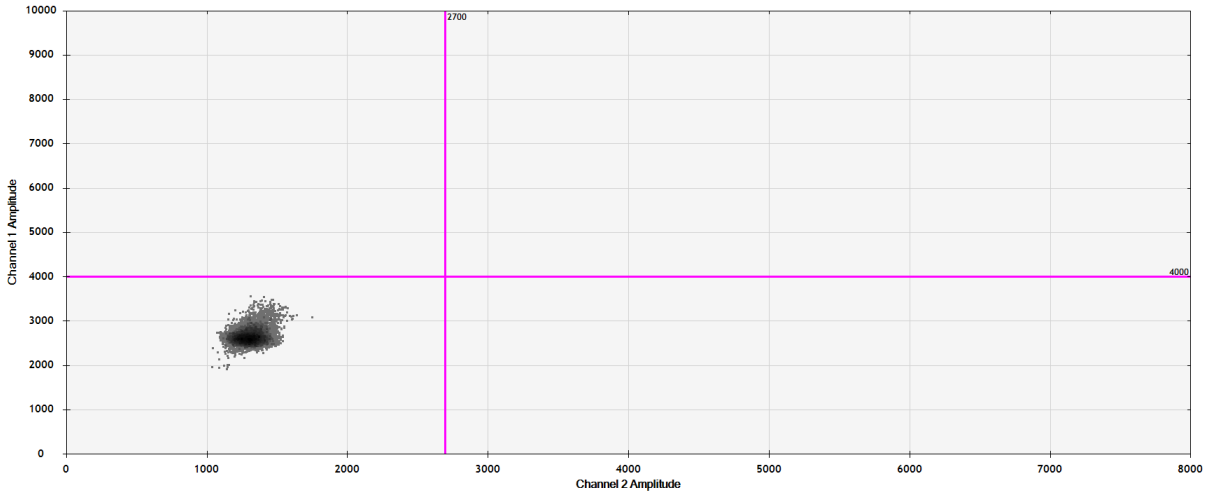

Figure 17 Example no template control containing only empty droplets

To meet the third condition, the negative control had to contain black empty droplets and green WT DNA (HEX) droplets as shown in Figure 18. If the negative control contained a blue (FAM) or orange positive (FAM+HEX) droplet, false positive results were generated which are unacceptable.

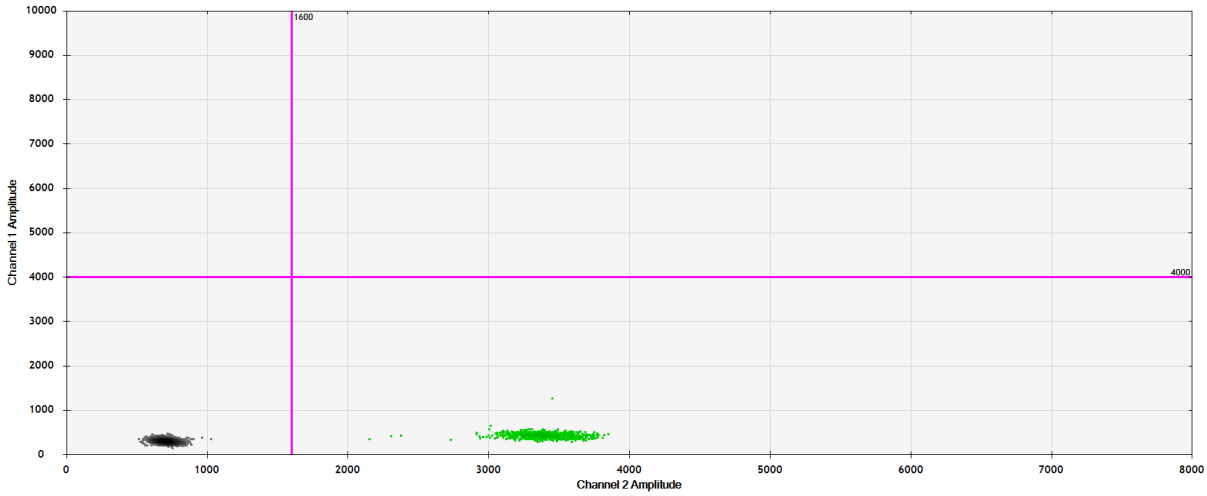

Figure 18 Example negative WT DNA control containing empty (black) an WT (green) droplets

As last, the positive control had to contain black empty droplets, green WT DNA (HEX) droplets and blue PIK3A mutated DNA (FAM) droplets as shown in Figure 19. If the positive control did not contain any blue *PIK3CA* mutated DNA droplets, the assay was not specific or sensitive enough or the settings of the ddPCR program had to be altered.

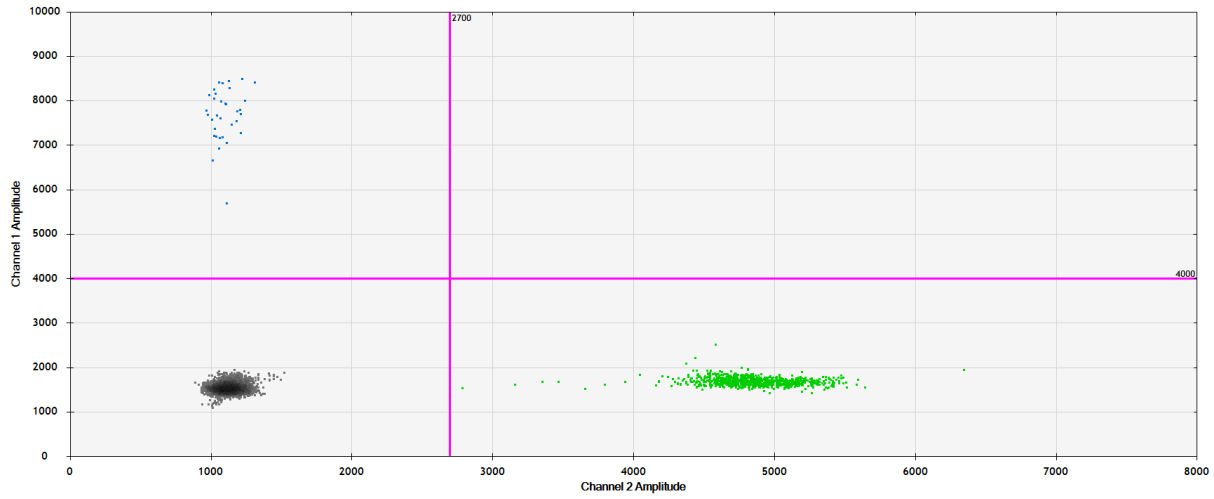

Figure 19 Example positive control containing empty (black), WT (green) and mutated (blue) droplets

3.3.4 *PIK3CA* ddPCR assay optimization

The used Bio-Rad ddPCR assays are commercially available and therefore the validated standard protocol was first tested. Due to a lack of sufficient cfDNA material (for 4 assays in duplo), the amount of components in the ddPCR master mix had to be adjusted. Therefore, 16 µl of ddPCR mix and 6 µl of cfDNA extract was used per reaction instead of 14 µl of ddPCR mix and 8 µl of cfDNA extract. To obtain the 16 µl ddPCR mix, 4 µl of Nuclease-free water was used instead of 2 µl. The assays performance was checked when using these new conditions. The assay was accounted as optimal when no false positive and false negative results (droplets) were observed in the selected control samples and if the low positive control was positive.

It was also important that no inhibition of the PCR amplification reaction was observed, caused by the addition of too much cfDNA input (cfr. previously observed at UZ Leuven as shown in Figure 29 in the result section). Therefore, the data were checked on inhibition when using Maxwell 4ml cfDNA extracts.

3.3.5 *PIK3CA* ddPCR assay validation

After optimization, the *PIK3CA* ddPCR assays were validated whereby they were checked on several parameters as described in the paper of Mattocks et al, see Figure 20 [42]. The ddPCR assays are considered qualitative binary assays, group E. The precision, composed by the intra- and inter-run variation, robustness and correctness were examined.

Naast de parameters die hieronder beschreven worden, moet voor alle soorten testen/methoden ook de **robuustheid** getest worden.

	Omschrijving	Voorbeelden	Sensitiviteit	Specificiteit	Juistheid	Precisie	Detectie-limieten	Probabiliteit[b]
A	**Kwantitatieve testen.** Het resultaat kan elke waarde zijn tussen twee limieten (waaronder decimalen)	Bepalen van de hoeveelheid methylatie (%); karakterisatie van een mosaïsche mutatie; heterplasmy van mitochondriale varianten, genexpressie.			++	++	++	
B	**Categorische testen** waarvan het kwantitatieve signaal wordt omgezet naar een ordinale reeks die het finale resultaat geeft.	Lengtebepaling van een PCR produkt; bepaling van de lengte van een "triplet repeat" (FRAXA, Huntington, ...).			++	++	++	+
C	**Categorische testen** waarvan het kwantitatieve signaal wordt omgezet naar één van een beperkte serie van vooraf gedefinieerde categoriën dewelke het finale resultaat geeft.	Bepaling van het aantal kopies met PCR of MLPA: exon deletie/duplicatie in *BRCA1*; *PMP22* gen dosage in CMT en HNPP			Om correctiefactoren en/of "cut-offs" te kunnen bepalen			++
D	**Kwalitatieve testen** waarvan het echte kwantitatieve signaal één van vele mogelijke waarden kan hebben, maar het vereiste resultaat kan enkel één van twee mogelijke waarden zijn.	Mutatie screening voor ongekende mutaties door middel van sequenering of smeltcurves	++	++	Om correctiefactoren en/of "cut-offs" te kunnen bepalen		++[a]	
E	**Kwalitatieve testen (binair)** waarvan het echte kwantitatieve signaal één van twee mogelijke waarden kan hebben	Genotypering voor een specifieke mutatie, bijvoorbeeld *HFE* Cys282Tyr in hemochromatose.	++	++	Om correctiefactoren en/of "cut-offs" te kunnen bepalen		++[a]	+

a Moet getest worden bij testen waar genotypering gebeurt van varianten die in een kleine fractie van het testmateriaal voorkomen (bijvoorbeeld mitochondrial DNA)
b De term "probabiliteit" wordt gebruikt om situaties te beschrijven waarin de probabiliteit dat het testresultaat correct is kan worden bepaald- voornamelijk bij continue validatie.

Legende

++	vereiste parameter
+	toepasbara parameter
	Gebruikt bij het implementeren van een test
	Gebruikt bij het implementeren van een test of bij continue validatie
	Gebruikt bij continue validatie

Figure 20 Validation criteria depending on type of method [42, p. 1280]

3.3.5.1 Intra-run variation

The intra-run variation or repeatability is defined as the degree of correspondence between successive results obtained with the same method on an identical sample under the same conditions. Therefore, in each experiment the controls and patient materials were examined in duplo by the same person using the same device at the same time. Because measurements where performed in duplo, only two results are obtained and no decent statistic calculations could be performed. Therefore, the concordance between both measurements of the mutated *PIK3CA* cfDNA is checked. This means that both measurements (one from each well) should give the same binary end result (positive/negative).

3.3.5.2 Inter-run variation

The inter-run variation is defined as the degree of correspondence between results obtained with the same method on an identical sample under different conditions. Therefore, the positive and negative controls were examined by the same person using the same device at a different time. Because these controls were only measured a few times, no decent statistic calculations could be performed. Therefore, the main criteria for the sample examinations is the concordance between the expected results and the experimental results. However, the controls were examined 3-5 times and therefore, a deviation of the average ±3.5x standard deviation (SD) was calculated and allowed. Additionally, two patient samples were examined per mutation wherefore the average, statistical distribution (R) and ½ R were calculated.

3.3.5.3 Robustness

The robustness is defined as the degree of insensitivity for various circumstances such as sample type, concentration of DNA, executor, device type, …. To examine the robustness in this work, one experiment was executed by two different people at a different time on an identical sample. The obtained results from both experiments were compared whereby the concordance of the binary result (positive/negative) between the two executors was examined.

3.3.5.4 Correctness

The correctness is defined as how close the experimental value is situated to the reference value and as the possibility to correctly detect the genotype. In this work, the correctness was examined by checking the controls on correspondence between experimental results and expected results. In the near future, the lab will also participate in the external quality assessment (EQA) program.

4 RESULTS

4.1 Cobas vs Maxwell cfDNA plasma extraction

The corrected raw data obtained after the Qubit concentration measurements for the 3 extraction procedures are listed in Table 4 below. As mentioned before, the elution volume of the Cobas extraction kit (80 µl) is larger than the elution volume of the Maxwell extraction kit (60 µl). Therefore, the Cobas concentrations are corrected with a factor *1.33 (80µl/60µl) to obtain a result analogous to the Maxwell concentrations.

Table 4 Corrected concentrations cfDNA plasma extractions

Patient	Total available sample amount (ml)	Concentration Cobas 2 ml cfDNA extract (ng/µl)	Concentration Maxwell 2 ml cfDNA extract (ng/µl)	Concentration Maxwell 4 ml cfDNA extract (ng/µl)
1	4	0.736	0.636	**
2	4	1.36	0.936	
3	4	3.81	3.30	
4	4	2.35	2.70	
5	8	6.21	5.70	10.5
6	8	3.73	3.40	8.10
7	8	3.17	2.28	3.54
8	8	0.477	0.418	0.696
9	8	0.237	0.368	0.456
10	8	0.451	0.232	0.340
11	8	0.435	0.348	0.510
12	8	0.259	0.180	0.286
13	8	0.416	0.246	0.358
14	8	0.232	0.132	0.192
15	8	<0.50 ng/ml*	<0.50 ng/ml*	0.152
16	2	0.491	**	
17	8	0.309	0.596	1.09
18	8	<0.50 ng/ml*	<0.50 ng/ml*	0.148
19	8	<0.50 ng/ml*	<0.50 ng/ml*	0.140
20	8	0.328	0.760	1.55
21	4	**		0.756

*The measurement of the sample gave the message 'too low' and could not be measured. Therefore, it is not included in the graphs below.

** The marked cells indicate that the extraction could not be performed due to the lack of material.

4.1.1 Cobas 2 ml vs Maxwell 2 ml cfDNA plasma extractions

The results from the manual Cobas extraction and the semi-automatic Maxwell 2 ml extraction are shown in Figure 21.

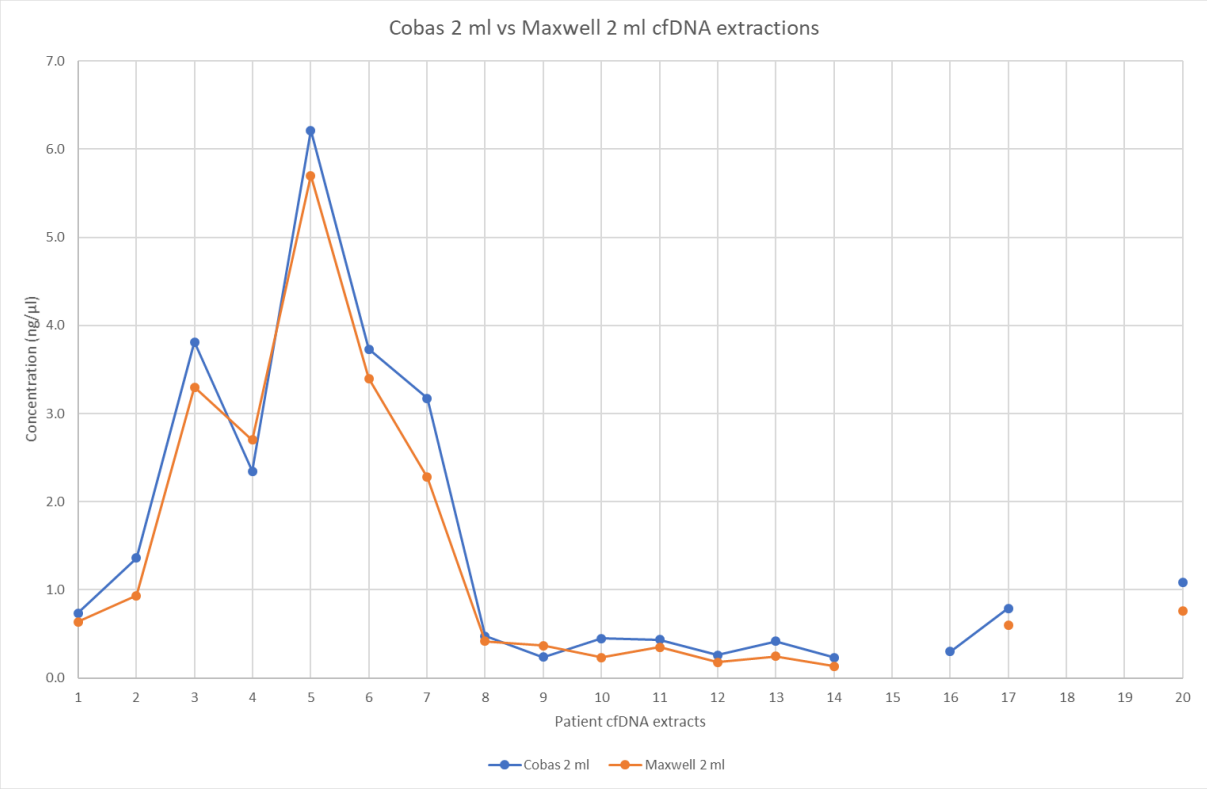

Figure 21 Concentration of Cobas 2 ml and Maxwell 2 ml extractions in function of the patient cfDNA extracts

Figure 21 is composed by putting the concentration (ng/µl) in function of the patient cfDNA extract for both extraction methods. It shows comparable trends for both the Cobas and Maxwell extraction method which indicates that the results can be assumed as correct. Although a small difference is observed showing that the Cobas extraction method results in slightly higher concentrations for almost all the patient cfDNA extracts. However, two extracts (4 and 9) show an inversed result.

When there is only 2 ml of blood plasm available, the manual Cobas extraction method provides a higher concentration and is preferred over the Maxwell 2 ml extraction method. This result is expected since it is a common trend at UZ Leuven that manual performances result in higher yields compared to (semi-)automated procedures when using the same starting material.

4.1.2 Maxwell 2 ml vs 4 ml cfDNA plasma extractions

The results from the Maxwell 2 ml vs 4 ml cfDNA plasma extractions are shown in Figure 22. Hereby, the difference in volume of the starting material is examined.

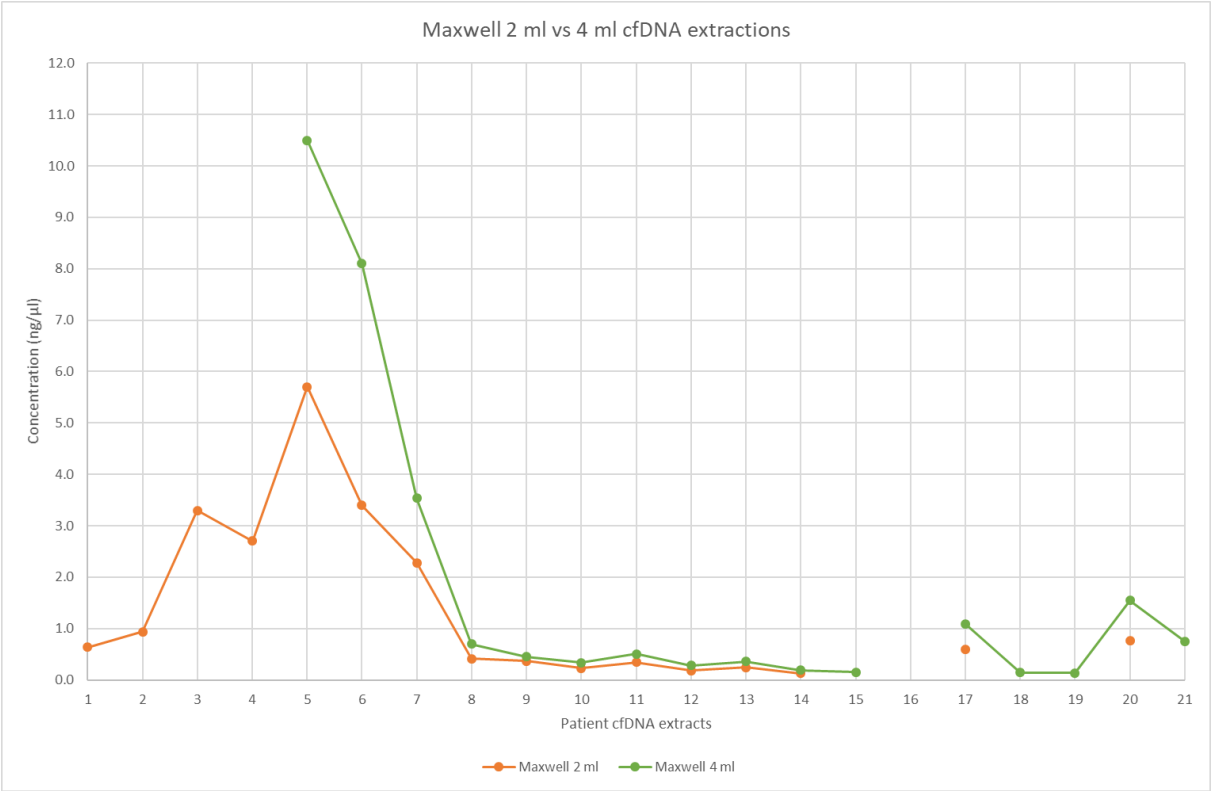

Figure 22 Concentration of Maxwell 2 ml and Maxwell 4 ml extractions in function of the patient cfDNA extracts

Figure 22 is composed by putting the concentration (ng/µl) in function of the patient cfDNA extract for both volumes. It shows comparable trends for both extractions which indicates that these results can also be assumed as correct. All the Maxwell 4 ml extractions result in a higher concentration yield than the Maxwell 2 ml extractions. Even the patient cfDNA extracts that were indicated as 'too low' when using 2 ml starting material during the concentration determination now result in a measurable concentration which shows the benefit of the Maxwell 4 ml extraction. However, the difference in concentration yield is greatest when the blood plasm contains a high concentration in cfDNA.

When there is 4 ml of blood plasm available, the use of this volume in starting material provides a higher concentration and is preferred over the Maxwell 2 ml extraction method.

4.1.3 Comparison three cfDNA plasma extraction methods

The yield of all three extraction procedures are shown in Figure 23.

Figure 23 Concentration of Cobas 2 ml, Maxwell 2 ml and Maxwell 4 ml extractions in function of the patient cfDNA extracts

Figure 23 is composed by putting the concentration (ng/µl) in function of the patient cfDNA extract. This graph shows that the concentration is highest for 12/15 patient cfDNA extracts (excluding 1-4, 16 and 21) when using the Maxwell 4 ml extraction. In three cases (10, 13 and 14), the Cobas extraction obtained a higher concentration. However, the Maxwell 4 ml extraction is still favored because of its capability to obtain a measurable concentration (15, 18 and 19) while the other two extraction methods cannot generate these results.

The results from Figure 23 indicate that when 2 ml of blood plasm is available, the Cobas extraction method is preferred over the Maxwell 2 ml extraction method. When 4 ml of blood plasm is available, the Maxwell 4 ml extraction method is preferred.

4.2 *PIK3CA* ddPCR analysis

To process all of the generated raw data in QuantaSoft™, thresholds are defined for each *PIK3CA* ddPCR assay based on the generated 2D-scatterplots of all the experiments. The thresholds used in this work are listed in Table 5 below.

Table 5 Defined thresholds for the QuantaSoft™ software

	Glu542Lys	Glu545Lys	His1047Leu	His1047Arg
Channel 1 = FAM	4250	4250	4000	4000
Channel 2 = HEX	2700	2700	1600	1600

4.2.1 Preparation ddPCR controls

The DLD-1 and PA-1 cell line were first screened on the absence of *PIK3CA* mutations as described in section 3.3.1. The results obtained during this screening are listed in Table 6 below.

Table 6 Results obtained during ddPCR cell line screening

	Glu542Lys	Glu545Lys	His1047Leu	His1047Arg
DLD-1	-	+	-	-
PA-1	-	-	-	-

The 2D-scatterplots for mutation Glu545Lys of both the DLD-1 and PA-1 cell line are shown below, the 2D-scatterplots from the other mutations are not shown since they are all negative.

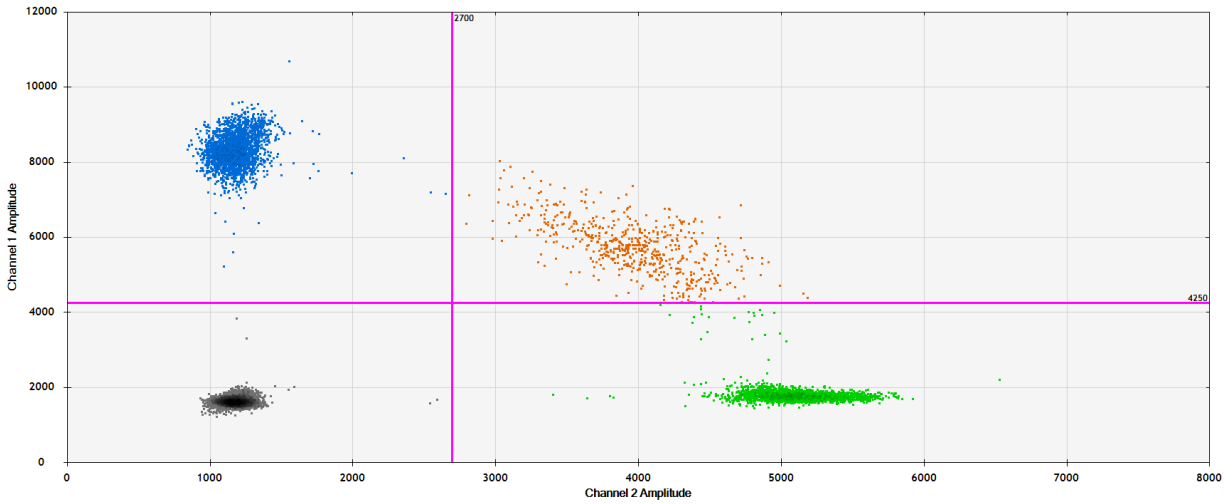

Figure 24 2D-scatterplot DLD-1 cell line for mutation Glu545Lys

Figure 24 shows a strongly positive profile which indicates that de DLD-1 cell line carries the Glu545Lys hotspot mutation and thus cannot be used as negative control/WT DNA to add to the positive controls. After a thorough literature search, these results are confirmed by a study performed by Samuels *et al.* [43].

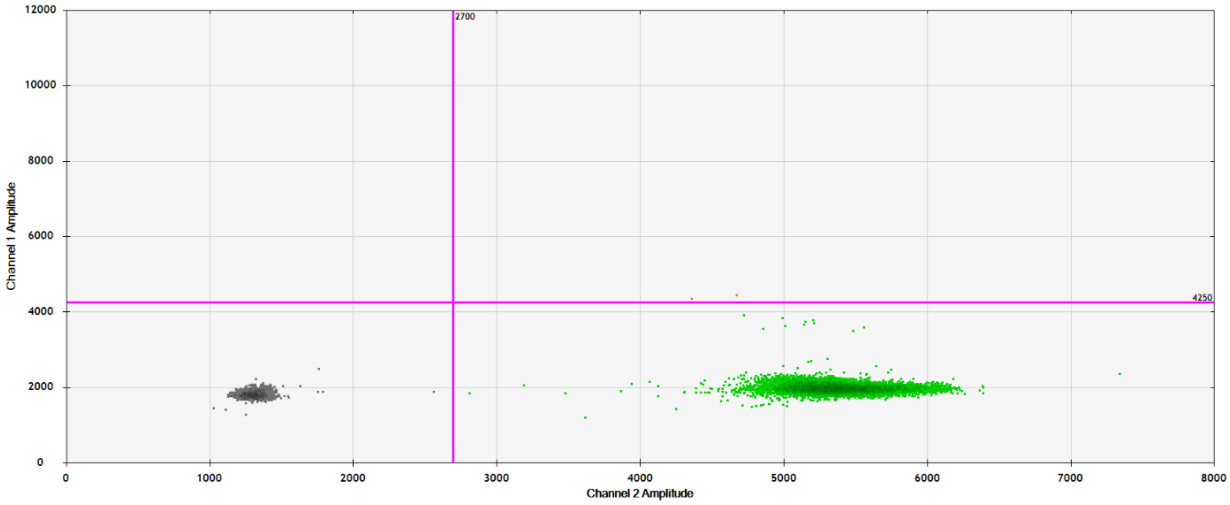

Figure 25 2D-scatterplot PA-1 cell line for mutation Glu545Lys

Figure 25 shows a negative profile which indicates that the PA-1 cell line does not contain the Glu545Lys hotspot mutation. However, two double positive orange droplets are spotted right above the horizontal threshold line. They are not accounted as positive because they probably belong to the green WT DNA cluster. If a low concentration of mutated DNA would be present in the PA-1 cell line, few blue positive droplets would be spotted instead of orange double positive droplets because the chance is very small that both DNA types (mutated and WT) would end up in the same droplet. The PA-1 cell line is thus used as negative control and as WT DNA to add to the positive controls. One of the advantages of the cell line is that it has a near-diploid character [44] which corresponds the most to the normal human diploid character.

After screening, two different low positive controls were prepared and ddPCR screened. The results from this screening are listed in Table 7 below.

Table 7 Concentration determination for the low positive control

	Glu542Lys	Glu545Lys	His1047Leu	His1047Arg
gBlock 0.6 copies/µl	-	+	+	+
gBlock 0.8 copies/µl	+	+	+	+

The criteria for a positive control to be accounted as positive is that at least two blue positive droplets must be present in one duplo experiment (two wells). The merged 2D-scatterplots from the duplo 0.6 copies/µl and duplo 0.8 copies/µl concentration experiments for hotspot mutation Glu542Lys are shown as an example in the figures below. The 2D-scatterplots from the other mutations are not shown since they are all positive.

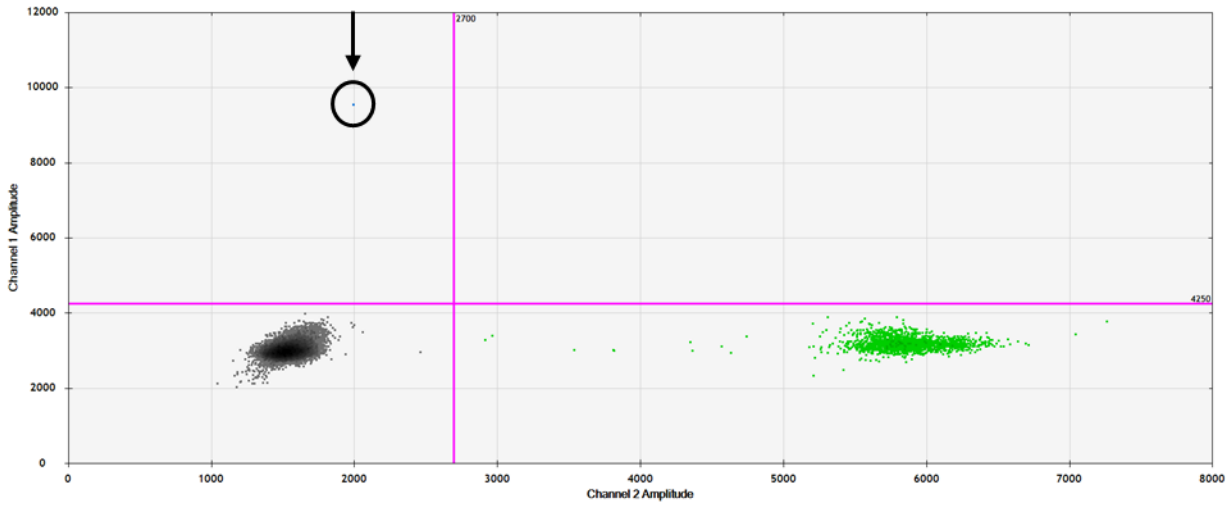

Figure 26 2D-scatterplot 0.6 copies/µl low positive control for hotspot mutation Glu542Lys

Figure 26 shows the result from the 0.6 copies/µl low positive control. Only one blue droplet is present in the merged plot which means it does not meet the predetermined criteria. Therefore the 0.6 copies/µl concentration cannot be used as low positive control.

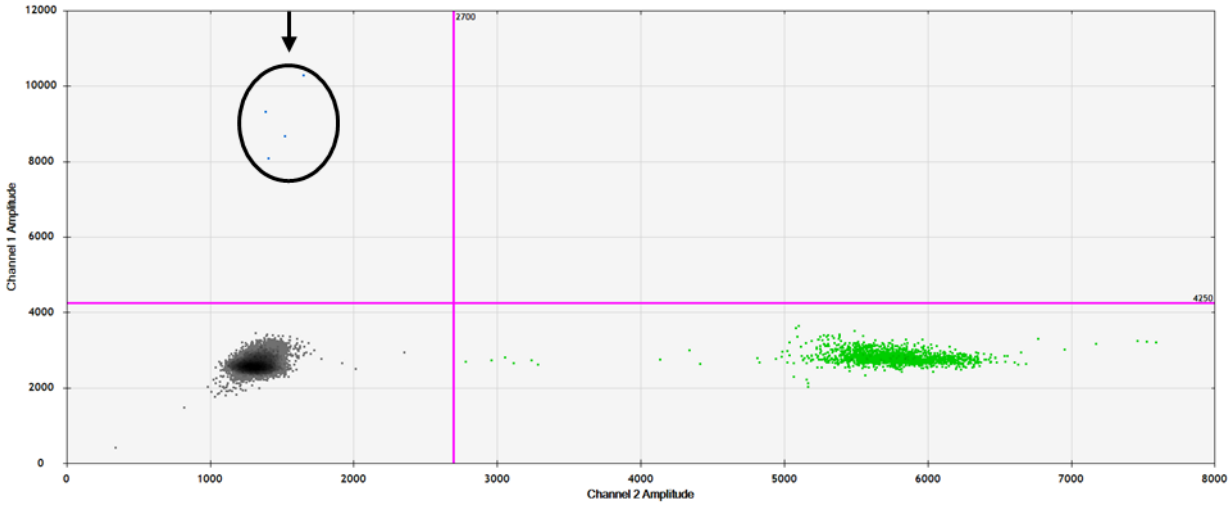

Figure 27 2D-scatterplot 0.8 copies/µl low positive control for hotspot mutation Glu542Lys

Figure 27 shows the result from the 0.8 copies/µl low positive control. This concentration however shows more than 2 blue positive droplets per 2 wells and meets the predetermined criteria. Therefore the 0.8 copies/µl concentration is used as low positive control.

4.2.2 Optimization *PIK3CA* ddPCR assays

First a screening experiment was performed using a Maxwell cfDNA extract to investigate the presence of possible PCR inhibition. Previously, inhibition was observed with cfDNA extracted, by another group of the lab, with a Promega method on a Hamilton robot. Figure 28 shows the 2D-scatterplot from the screening experiment.

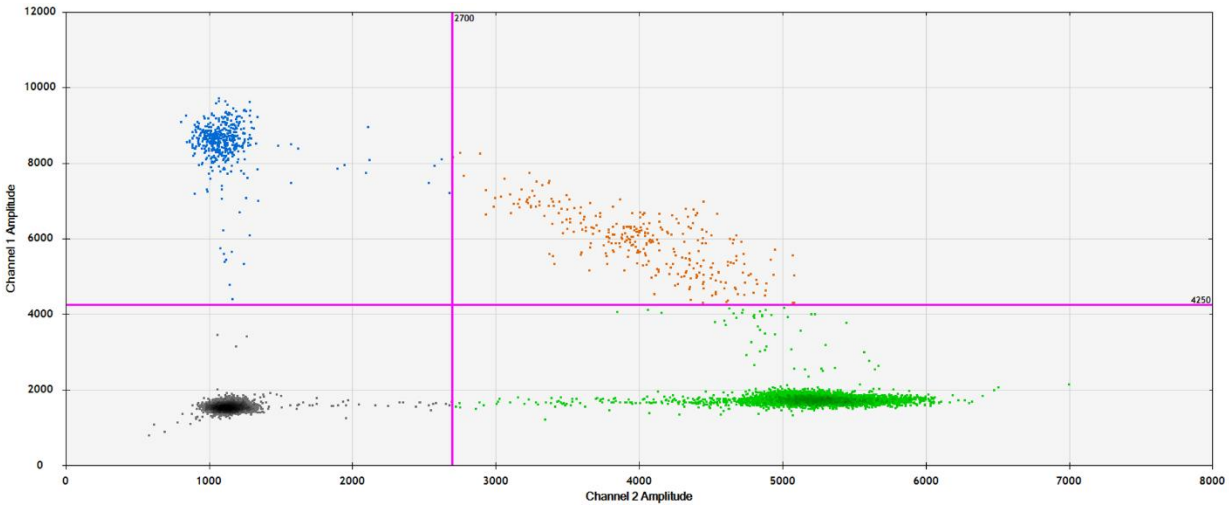

Figure 28 2D-scatterplot from the screening experiment of a positive patient with the use of Maxwell cfDNA extracts

Figure 28 shows that no inhibition is present because analogue droplet clusters are obtained as when using Cobas extracts. If inhibition would be present, it would result in a 2D-scatterplot as shown in Figure 29.

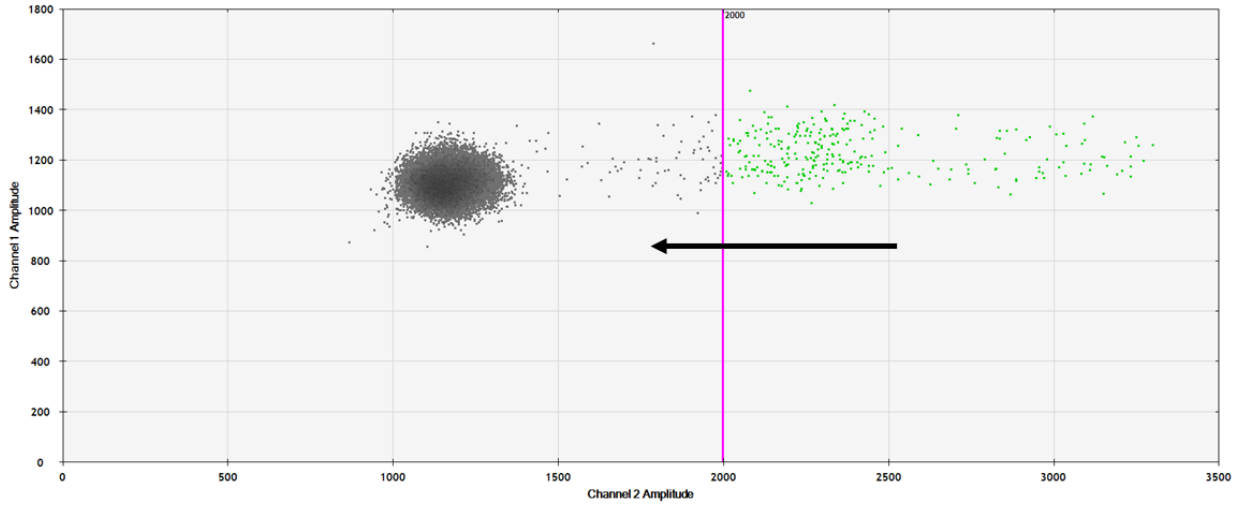

Figure 29 Example of a 2D-scatterplot from a sample that shows inhibition

Visually, inhibition leads to a shift from green WT droplets toward the black empty droplet cluster as illustrated in 2D-scatterplot above. This effect was the strongest when working with a high cfDNA input.

Next, the use of 6 µl cfDNA was tested instead of 8 µl due to a lack of sufficient cfDNA material (for 4 assays in duplo). Hereby, no difference in cluster position was noted and the blue positive droplets where still present when using 6 µl of cfDNA. Based on these results it can be concluded that the protocol can also be performed using 6 µl of cfDNA as input.

4.2.3 Validation *PIK3CA* ddPCR assays

The correctness is checked during all experiments and shows correspondence between the experimental results and the expected results.

4.2.3.1 Intra-run variation

The intra-run variation is evaluated by comparing the duplo measurements from each performed ddPCR experiment and is illustrated using the Glu545Lys mutated *PIK3CA* cfDNA ddPCR results, shown in Table 8. The raw data from the other mutations can be found in ANNEX 6.

Table 8 Intra-run variation of the mutated PIK3CA cfDNA results for hotspot mutation Glu545Lys

	Concentration mutated *PIK3CA* cfDNA (copies/µl)	
Patient	**Well 1**	**Well 2**
1	0	0
2	9.40	8.50
3	0.250	0.520
4	0	0.130*
5	262	249
6	56.7	52.4
7	71.0	71.8
8	0.690	1.10
9	0	0
10	0	0
11	0	0
12	0	0
13	0	0
14	0	0
15	0	0
16		
17	0.0600*	0
18	0	0
19	0	0.0700*
20	0.0600*	0
21	0	0.0600*

Table 8 shows concordant results for 15/20 measurements. Discordant results are obtained for patient 4, 17, 19, 20 and 21. All these patients show one negative measurement and one slightly positive result (*). However, the same reasoning is followed as in the description of Figure 25 being that the positive results come from an orange double positive droplet. These are not real positive results and therefore they are accounted as negative.

When comparing the duplo measurements per positive patient, the obtained results are situated in the same magnitude. Hereby a good degree of correspondence between successive results, obtained with the same method on an identical sample under the same conditions, is obtained.

4.2.3.2 Inter-run variation

The inter-run variation is evaluated and illustrated using the Glu545Lys mutation ddPCR results because it accounts the most repeats. Therefore, it is the best representation for the inter-run

variation. The raw data results from the other mutations can be found in ANNEX 7 since they are all comparable. To evaluate the inter-run variation, the ddPCR results from the controls are listed. Some experiments are performed using 8 µl of DNA, others using 6 µl of DNA. Therefore, the obtained results from the 8 µl input experiments are corrected with a factor *0.75 (6µl/8µl) to obtain a result analogous to the 6 µl input experiments. These corrected raw data are shown in Table 9 and Table 10 below.

Table 9 Corrected control raw data mutated PIK3CA cfDNA for hotspot mutation Glu545Lys

	Concentration mutated *PIK3CA* cfDNA (copies/µl)					
	Repeat 1	**Repeat 2**	**Repeat 3**	**Repeat 4**	**Repeat 5**	**Repeat 6**
gBlock 10 copies/µl	Not performed	2.06	2.15	1.80	2.60	2.20
gBlock 0.8 copies/µl	0.165	0.218	0.0700	0.248	0.290	0
PA-1 cell line	0.0300*	0	0	0	0	0
A.D.	0	0	0	0	0	0

*The concentration of the PA-1 cell line should be 0, however one orange double positive droplet is present which is not accounted as positive.

Table 10 Corrected control raw data wildtype cfDNA for hotspot mutation Glu545Lys

	Concentration wild type cfDNA (copies/µl)					
	Repeat 1	**Repeat 2**	**Repeat 3**	**Repeat 4**	**Repeat 5**	**Repeat 6**
gBlock 10 copies/µl	Not performed	49.9	52.2	48.2	54.2	49.2
gBlock 0.8 copies/µl	44.4	45.3	44.8	42.2	48.0	44.9
PA-1 cell line	47.3	50.5	52.1	54.6	55.0	47.9
A.D.	0	0	0	0	0	0

Based on these data, it seems that all the results are similar and no outliers are present. To confirm this assumption, the averages ±3.5x standard deviation (SD) are calculated as shown in Table 11 and Table 12. The calculations for the other mutations can be found in ANNEX 8 since they are all comparable.

Table 11 Calculations control average, SD and average ±3.5xSD mutated PIK3CA cfDNA for hotspot mutation Glu545Lys

	Concentration mutated *PIK3CA* cfDNA (copies/µl)		
	Average	**SD**	**Average ± 3.5xSD**
gBlock 10 copies/µl	2.16	0.289	1.15 – 3.17
gBlock 0.8 copies/µl	0.165	0.111	0 – 0.554
PA1-cell line	0.00500	0.0122	0 – 0.0477
A.D.	0	0	0

Table 12 Calculations control average, SD and average ±3.5xSD wild type cfDNA for hotspot mutation Glu545Lys

	Concentration wild type cfDNA (copies/µl)		
	Average	**SD**	**Average ± 3.5xSD**
gBlock 10 copies/µl	50.7	2.43	42.2 – 59.2
gBlock 0.8 copies/µl	44.9	1.88	38.3 – 51.5
PA-1 cell line	51.2	3.29	39.7 – 62.7
A.D.	0	0	0

After the calculations, graphs are drawn for each control. Only the graphs from the gBlock 10 copies/µl will be shown since the other ones are analogous.

Figure 30 Inter-run variation of mutation p.Glu545Lys for the gBlock 10 copies/µl control PIK3CA mutated cfDNA

Figure 30 is composed by putting the concentration (copies/µl) of mutated *PIK3CA* cfDNA, obtained in the QuantaSoft™ software, in function of the repeated measurement. Three data sets are shown being the lower limit (average – 3.5xSD) and upper limit (average + 3.5xSD) indicated in blue and the experimental results indicated in orange. The graph shows that all the experimental data are situated between the defined limits which indicates that no outliers are present. Hereby, a good degree of correspondence between the results, obtained with the same method on an identical sample under different conditions, is obtained.

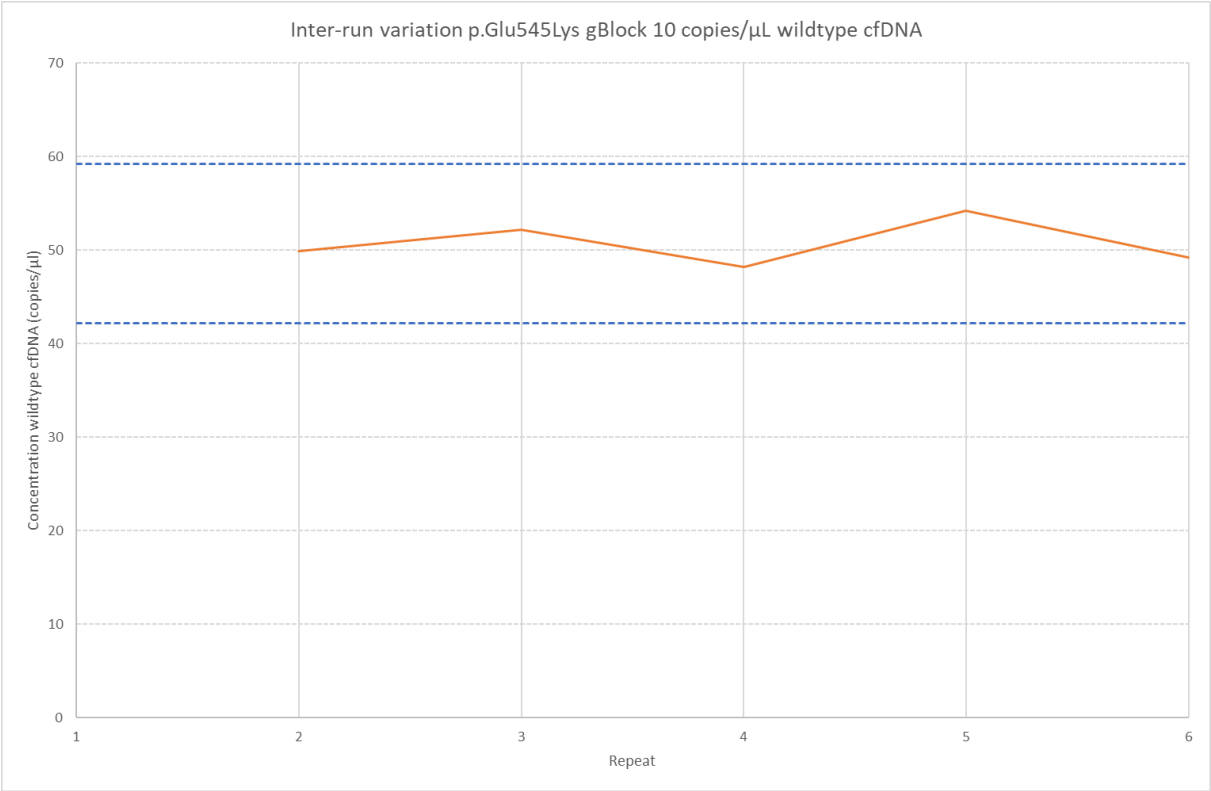

Figure 31 Inter-run variation of mutation p.Glu545Lys for the gBlock 10 copies/µl control PIK3CA wild type cfDNA

Figure 31 is composed by putting the concentration (copies/µl) of wild type cfDNA, obtained in the QuantaSoft™ software, in function of the repeated measurement. Analogue data sets are shown as in Figure 30 and the graph shows comparable results.

Besides the statistic calculations, the concordance between the expected result and experimental result is checked. Meaning that the positive controls should have a positive outcome in the ddPCR experiments and that the negative control and the no template control should have a negative outcome in the ddPCR experiments. Checking theses data indeed shows concordance between the expectations and the experimental results (=correctness).

The controls were not the only samples used to examine the inter-run variation. Per hotspot mutation, two patient samples (one positive and one negative) were tested in two different experiments to check the inter-run variation. The raw data results from hotspot mutation Glu545Lys are shown in Table 13 below. The raw data results from the other hotspot mutations can be found in ANNEX 9.

Table 13 Raw data patient samples for hotspot mutation Glu545Lys

	Concentration mutated *PIK3CA* cfDNA (copies/µl)		**Concentration wild type cfDNA (copies/µl)**	
Patient	**Repeat 1**	**Repeat 2**	**Repeat 1**	**Repeat 2**
6	19.3	17.9	221	199
14	0	0	6.30	6.90

To evaluate the inter-run variation of these results, the average, statistical distribution (R) and ½ R were calculated as shown in Table 14. The calculations for the other hotspot mutations can be found in ANNEX 10.

Table 14 Calculations patient 6 and 14 average, distribution (R) and 1/2 R for hotspot mutation Glu545Lys

	Concentration mutated *PIK3CA* cfDNA (copies/µl)			Concentration wild type cfDNA (copies/µl)		
Patient	**Average**	**Distribution (R)**	**½ R**	**Average**	**Distribution (R)**	**½ R**
6	18.6	1.40	0.700	210	22.0	11.0
14	0	0	0	6.60	0.600	0.300

These results are plotted into bar charts shown in Figure 32.

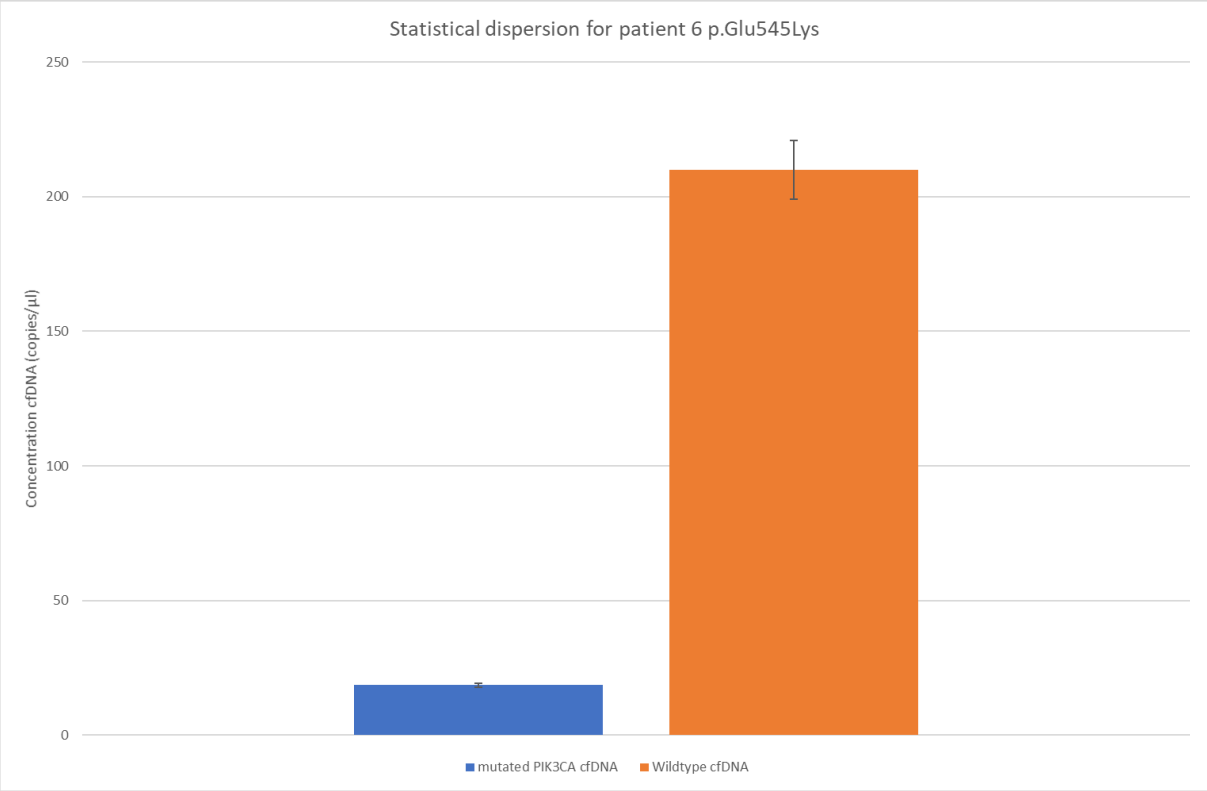

Figure 32 Statistical dispersion for patient number 6 from hotspot mutation Glu545Lys

Figure 32 is composed by putting the concentration (copies/µl) obtained in the QuantaSoft™ software in function of the type of cfDNA for patient number 6 (positive). Error bars are added which indicate ±½R. The graph shows the statistical dispersion pertaining to the average and indicates that it is relatively small compared to the average value.

The same chart is composed for patient number 14 and is shown in Figure 33.

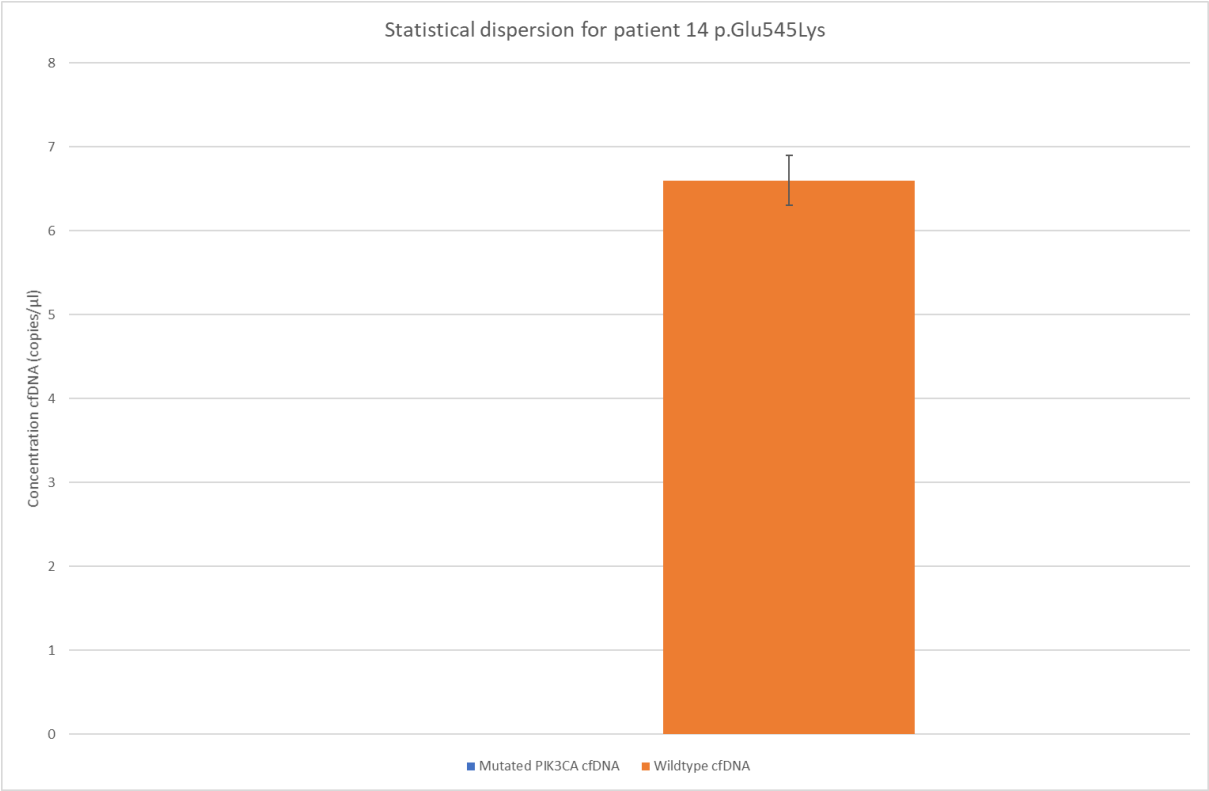

Figure 33 Statistical dispersion for patient number 14 from hotspot mutation Glu545Lys

Figure 33 shows analogous results for patient 14 as for patient 6, however no bar is shown for the mutated *PIK3CA* cfDNA result. This is because patient 14 is a negative patient for ddPCR and thus does not contain any mutated *PIK3CA* cfDNA.

When looking at the concordance between the expected result and experimental result, a good result is obtained. Patient number 6 (positive on tissue) shows positive results in the ddPCR experiments, patient number 14 (negative on tissue) shows negative results in the ddPCR experiments. This means that the data indeed show concordance between the expectations and the experimental results (=correctness)

4.2.3.3 Robustness

The robustness is evaluated and illustrated using the raw data from identical experiments performed by two different executors shown in Table 15 and Table 16.

Table 15 Examined robustness for hotspot mutation Glu545Lys

		Concentration mutated *PIK3CA* cfDNA (copies/µl)	Concentration wild type cfDNA (copies/µl)
Demi	gBlock 10 copies/µl	2.60	54.2
	gBlock 0.8 copies/µl	0.290	48.0
	PA-1 cell line	0	55.0
	A.D.	0	0
	patient 14	0	6.30
Karen	gBlock 10 copies/µl	2.20	49.2
	gBlock 0.8 copies/µl	0*	44.9
	PA-1 cell line	0	47.9
	A.D.	0	0
	patient 14	0	6.90

The experimental results obtained by both executors correspond with the expectations except for the indicated result (*). The gBlock is a positive control that contains the mutated DNA template and should therefore be measured as positive.

Table 16 Examined robustness for hotspot mutation His1047Arg

		Concentration mutated *PIK3CA* cfDNA (copies/µl)	Concentration wild type cfDNA (copies/µl)
Demi	gBlock 10 copies/µl	3.30	53.0
	gBlock 0.8 copies/µl	0.315	44.8
	PA-1 cell line	0	53.5
	A.D.	0	0
	patient 14	0	6.90
Karen	gBlock 10 copies/µl	3.40	52.4
	gBlock 0.8 copies/µl	0.280	44.3
	PA-1 cell line	0	52.2
	A.D.	0	0
	patient 14	0	7.10

The experimental results obtained by both executors correspond with the expected results. The positive controls show positive ddPCR results, the negative control and no template control show negative ddPCR results.

Overall when comparing the results from both executors, results from the same magnitude are obtained except for the indicated result (*) from executor 2. However, the same results are obtained by both executors for the patient sample.

4.2.4 Concordance experimental ddPCR results vs tissue NGS results

As last, the concordance between the obtained ddPCR results using blood plasm are compared with the results of the next generation sequencing (NGS) on tissue. Therefore, Table 17 is composed which shows the binary result (positive/negative) results from both methods for every assay.

Table 17 Comparison NGS tissue results VS ddPCR plasma results

	ddPCR plasma results				NGS tissue results			
Patient	**Glu542Lys**	**Glu545Lys**	**His1047Leu**	**His1047Arg**	**Glu542Lys**	**Glu545Lys**	**His1047Leu**	**His1047Arg**
1	+	-	-	-	+	-	-	-
2	-	+	-	-	-	+	-	-
3	+	+	-	+	-	-	-	+
4	+	-	-	-	-	+	-	-
5	-	+	-	-	-	+	-	-
6	-	+	-	-	-	+	-	-
7	-	+	-	-	-	+	-	-
8	-	+	-	-	-	+	-	-
9	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-	-
15	-	-	-	-	-	-	-	-
16					-	-	-	-
17	-	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-	-
19	-	-	-	-	-	-	-	-
20	-	-	-	-	-	-	-	-
21	-	-	-	-	-	-	-	-

All the ddPCR experiments are performed using Maxwell 4 ml extracts, except for sample 1-4 which are performed using Cobas extracts. Based on the results, a concordance for 18 of 20 samples is obtained. Patient 3 and 4 show different results for hotspot mutation Glu542Lys and Glu545Lys. Therefore, an additional ddPCR experiment is performed using the DNA extracted from the tissue sample. The obtained results are shown in Table 18.

Table 18 Comparison NGS tissue results, initial ddPCR plasma results and additional ddPCR tissue results

	Patient 3		
	Tissue NGS	**Additional tissue ddPCR**	**Initial plasma ddPCR**
Glu542Lys	-	+	+
Glu545Lys	-	-	+
His1047Leu	-	+	-
His1047Arg	+	+	+
	Patient 4		
	Tissue NGS	**Additional tissue ddPCR**	**Initial plasma ddPCR**
Glu542Lys	-	+	+
Glu545Lys	+	+	-
His1047Leu	-	Not performed	-
His1047Arg	-	Not performed	-

Table 18 shows that the results from the additional ddPCR for the Glu545Lys hotspot mutation corresponds to the tissue results generated with NGS for both patients. This probably is the result of a sample swap in the initial ddPCR experiment since both samples were examined in the same experiment, at consecutive positions in the 96 well plate.

However, the Glu542Lys hotspot mutation for patient 3 is still present and an additional mutation in hotspot His1047Leu is noted. For patient 4, the Glu542Lys hotspot mutation is also present in the additional ddPCR experiment using tissue DNA.

5 DISCUSSION

Every year in Belgium 10,500 patients are diagnosed with breast cancer of which 99% are women [2]. About 40% of estrogen receptor (ER+) metastatic breast cancer patients harbor *PIK3CA* hotspot mutations which lead to an overactivated PI3K pathway. If an activating *PIK3CA* mutation is confirmed, the patient can be treated with alpelisib, a PI3Kα-selective inhibitor [8]. Therefore, the *PIK3CA* hotspot mutations (Glu542Lys, Glu545Lys, His1047Leu and His1047Arg) need to be detected and currently, the material used to obtain information about a tumor is in first instance the core needle biopsy (CNB) or surgical biopsy which are fairly serious procedures [4]. Therefore, the use of less invasive liquid biopsies is examined. One of the components present in the blood plasm is ctDNA originating from and mimicking the (metastatic) tumor tissue [4]. However, this fraction of ctDNA is limited. The fragment length of the ctDNA is also very short and therefore, several extraction methods especially designed for this purpose are examined.

Currently, the Cobas® cfDNA Sample Preparation kit (Roche) is used in the routine at UZ Leuven which uses 2 ml of blood plasm. To obtain a higher concentration, it would be favorable if a higher input of starting material could be used. Therefore, the Maxwell® RSC LV ccfDNA Custom Kit (Promega) is tested which can process 2 or 4 ml of blood plasm and is performed on a Maxwell RSC device which can process up to 48 samples at once. The three different methods, Cobas 2 ml and Maxwell 2 ml and 4 ml, are tested and overall, the Maxwell® RSC LV ccfDNA Custom Kit using 4 ml of blood plasm comes out as the preferred extraction method. It achieves the highest cfDNA concentrations and can generate measurable results for low concentration patient samples while the other methods cannot generate these results. Previously, a study performed by Sorber *et al.* compared the use of four different extraction kits for the extraction of cfDNA in plasma to the QIAamp circulating nucleic acid kit [45]. The extraction kit that obtained the best results was the Maxwell RSC ccfDNA Plasma Kit together with the QIAamp circulating nucleic acid kit. This study confirms that the use of the Maxwell extraction kit is assigned.

One of the disadvantages of the Maxwell extraction kit is that the obtained elution volume amounts only 60 µl. In this work, four singular *PIK3CA* ddPCR assays are optimized and validated with the consequence that a sample needs to be examined by four separate assays. Since each assay must be performed in duplo to meet the predetermined criteria, 64 µl of cfDNA is required (16 µl per duplo x 4 assays). However, only 60 µl of cfDNA extract is available and thus the amount of input material must be adjusted. Optimization experiments are performed to check the use of 6 µl cfDNA input and no divergent results are obtained. Additionally, the ddPCR assays are checked on inhibition when using Maxwell extracts because this phenomenon was previously observed with other extracts in other ddPCR assays at UZ Leuven. The results of the experiments showed no inhibitory effects which conclude that the Maxwell cfDNA extracts can be used to perform the Bio-Rad ddPCR *PIK3CA* assay experiments.

To validate the four ddPCR assays, positive and negative controls are used. As positive controls, four commercially available gBlocks (one per assay) are used which are DNA sequences that contain the examined hotspot mutation. The positive control is diluted into TE buffer and a fraction of wild-type DNA to obtain a control that is as similar as possible to a patient blood sample. The negative control wild-type DNA sequence does not contain the studied mutations and is used to determine the false positive rate (FPR). Ideally it has a high ploidy correspondence to a normal human DNA sequence and is available in large amounts and therefore, the use of a cell line is chosen. Two cell lines are ddPCR screened being the DLD-1 cell line, derived from a colorectal adenocarcinoma [40], and the PA-1 cell line, derived from an ovary teratocarcinoma [41]. This screening shows that the DLD-1 cell line contains the Glu545Lys hotspot mutation which is also confirmed in a study performed by Samuels *et al.* [43].

The PA-1 cell line does not contain a hotspot mutation and is therefore an appropriate negative control cell line. Additionally, it has the advantage that it contains a near-diploid character [44] which corresponds the most to the normal human diploid character.

After preparation of the controls, the assays are validated by examining the intra-run variation, inter-run variation, robustness and correctness. All validation results met the predetermined criteria. Therefore it can be concluded that the optimized *PIK3CA* ddPCR assays can be used to detect the Glu542Lys, Glu545Lys, His1047Leu and His1047Arg hotspot mutations in liquid biopsies. The usage of singular ddPCR assays is interesting for the follow-up of patients with known mutation status however, when the blood of a patient with unknown mutation status is screened, all mutations are examined whereby it would be interesting if a multiplex assay could be used. In future work, multiplexing of the assays will be examined. When the ddPCR Bio-Rad assays are multiplexed, a mixture of two assays or all assays would be prepared whereby the four hotspot mutations would be detected in two or one experiment respectively. The experiment would then only require 36 µl or 16 µl of cfDNA extract (duplo experiment) respectively instead of 64 µl now. However, this concept could not be performed within the allocated time period of this work but can definitely be a next step to further improve the optimized and validated assays. This multiplexing will have an impact on the interpretation because different clusters will be present in one quadrant of the 2D-scatterplot and therefore, additional interpretation criteria probably must be determined. An extra reason why multiplexing has to be considered is that there is evidence from the recently published SOLAR-1 study that also the presence of mutations in the C2 domain (exon 8, coding exon 7) of PI3K has to be investigated [46].

Subsequently, plasma of 20 patients with known mutation status on tumor tissue (n=12 *PIK3CA* negative, n=8 *PIK3CA* positive) is tested using the optimized and validated ddPCR assays. Hereby a concordance for 18 of 20 samples is obtained. However, for 2 patients (patient 3 and patient 4) a discordant result is obtained.

For patient 3, a His1047Arg mutation is detected with NGS on tissue. An additional ddPCR experiment was performed on the previously NGS tested tissue DNA (but different eluate) and now shows an additional hotspot mutation on position Glu542Lys and His1047Leu. On plasma of this patient the Glu542Lys, Glu545Lys and His1047Arg mutations are detected. A possible hypothesis for the detection of similar but also different mutations between tissue and plasma could be tumor heterogeneity. This in a phenomenon in which heterogeneity occurs in the tumor by evolution and differentiation of the tumor cells whereby subclones arise from the initial tumor cells which have slightly altered characteristics [47], [48]. Also here, smaller subclones were maybe initially not picked up by NGS due to a lower sensitivity of NGS. However, the hypothesis cannot be further examined since the patient passed away and no additional samples (blood plasm or tissue) can be taken.

For patient 4, a Glu545Lys mutation is detected with NGS on tissue while a Glu542Lys mutation is detected in 25.5% with ddPCR in plasma. The additional ddPCR experiment performed on the previously NGS tested tissue DNA (same eluate) shows correspondence for the Glu545Lys hotspot mutation but on the other hand also demonstrates the presence of the Glu542Lys mutation in 26.1% and 0.17%, respectively. Probably, the presence of the smaller Glu542Lys subclone is not detected on the tissue due to the lower sensitivity of the NGS technique. However, the hypothesis cannot be further examined since the patient passed away and no additional samples (blood plasm or tissue) can be taken. An overview and comparison of the three experiments (NGS, initial ddPCR and additional ddPCR) can be found in Table 18.

In general the sensitivity and specificity of the ddPCR assay are 87.5% and 95.8% respectively. The examination of blood plasm for the detection of *PIK3CA* mutations in patients with invasive breast tumor is not refunded at this moment by the Belgian RIZIV (Rijksinstituut voor Ziekte- en Invaliditeitsverzekering). By performing the *PIK3CA* assays in routine, more evidence can be gathered to substantiate a request for reimbursement of the test by RIZIV. In routine, the test will be performed on patients belonging to the target population and so the efficacy of Alpelisib, a PI3Kα-specific inhibitor, can be further elucidated.

6 CONCLUSION

Examination showed that the 4 ml Maxwell extraction method is preferred because this extraction method generates the highest cfDNA concentrations. Additionally it generates measurable results for low concentration samples while the other extraction methods can't.

The optimization of the *PIK3CA* ddPCR shows that the validated Bio-Rad protocol can be performed using 6 µl of cfDNA whereby the assays shows no inhibition when using Maxwell cfDNA extracts. The validation of the *PIK3CA* ddPCR assays is performed by examining the intra-run variation, inter-run variation and robustness using positive and negative controls. The experimental results met all the predefined criteria which shows the ddPCR assays can be used to detect the *PIK3CA* hotspot mutations in daily practice.

Subsequently, plasma of 20 patients with known mutation status on tumor tissue (n=12 *PIK3CA* negative, n=8 *PIK3CA* positive) are tested. For 18 of 20 samples, concordant results are observed between tissue and plasma. For the 2 samples with discordant results, a hypothesis is formed. In general, the sensitivity and specificity are 87.5% and 95.8% respectively. A good correlation is observed between the detection of *PIK3CA* mutations in tissue and plasma. The validated test will be included in the routine diagnostics and provides an opportunity for the follow-up of the mutation status and for the support of the treatment strategy.

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ANNEX

ANNEX 1: Cobas® cfDNA Sample Preparation Kit (Roche) protocol

ANNEX 2: Maxwell® RSC LV ccfDNA Kit, Custom (Promega) protocol

ANNEX 3: Qubit® dsDNA HS Assay Kit Q32851 (Invitrogen) protocol

ANNEX 4: Characteristics gBlocks

ANNEX 5: Positive control calculations

ANNEX 6: Raw data intra-run variation for hotspots Glu542Lys, His1047Leu and His1047Arg

ANNEX 7: Raw data controls inter-run variation for hotspots Glu542Lys, His1047Leu and His1047Arg

ANNEX 8: Calculations control average, SD and average ±3.5xSD for hotspots Glu542Lys, His1047Leu and His1047Arg

ANNEX 9: Raw data patients inter-run variation for hotspots Glu542Lys, His1047Leu and His1047Arg

ANNEX 10: Calculations patient average, distribution (R) and 1/2R for hotspots Glu542Lys, His1047Leu and His1047Arg

ANNEX 11: Poster presented on the Belgian oncoforum by Apr. PhD Laurence Slembrouck

ANNEX 1: Cobas® cfDNA Sample Preparation Kit (Roche) protocol

UZ LEUVEN | ACTIVITEITENCENTRUM MOLECULAIRE DIAGNOSTIEK

WERKWIJZE	CEMOL-MT264-WW01	M01

COBAS cfDNA Sample Preparation Kit

Product No: 07247737190 (Roche)

BIJ OPENEN NIEUWE KIT:

- **Proteïnase K**:
  - Voeg 4,5ml H2O,steriel toe, meng door 10X omdraaien en verdeel 1,1ml per epje.
  - Noteer nieuwe houdbaarheidsdatum op het epje. Bewaar op -20°C.

  Houdbaarheid (opgelost in H2O,steriel): 90 dagen op -20°C, tenzij de houdbaarheid van het potje eerder is.

- **Wash Buffer I (WBI)**:
  - Voeg 15ml absolute ethanol toe en meng door 10X omdraaien
  - Noteer "ethanol toegevoegd" en de nieuwe houdbaarheidsdatum op het potje (Uit:...)

  Houdbaarheid (ethanol toegevoegd): 90 dagen op 15-30°C, tenzij de houdbaarheid op het potje eerder is.

- **Wash Buffer II (WBII)**:
  - Voeg 50ml absolute ethanol toe en meng door 10X omdraaien
  - Noteer "ethanol toegevoegd" en de nieuwe houdbaarheidsdatum op het potje (Uit:...)

  Houdbaarheid (ethanol toegevoegd): 90 dagen op 15-30°C, tenzij de houdbaarheid op het potje eerder is.

➔ HOUDBAARHEID OP KIT NOTEREN: NA OPENING 90 DAGEN

Opmerking: alle oplossingen van de kit moeten helder zijn. Indien er precipitaten gevormd zijn, verwarm op 37°C tot de precipitaten opgelost zijn.

PROCEDURE EXTRACTIE:

1. Vortex **2 mL plasma** (of 2ml supernatans lumbaal vocht*) en breng over naar een gelabelde **15ml tube**
2. Voeg **250 µl PK** toe
3. Voeg **2 ml DNA PBB** toe
4. **Meng** door 5 keer om te draaien
5. Incubeer op **kamertemperatuur – 30 minuten**
6. Tijdens de incubatie label per staal: 1 HPEA FT, 3 collection tubes en 2 elutie tubes (1,5ml epjes)
7. Voeg **500 µl isopropanol** toe en **meng** door 5 keer om te draaien
8. Breng over naar een **HPEA FT** (met een pipetteerapparaat en steriele pipet)
9. Centrifugeer op **4.000 x g – 5 minuten**

10. Verwijder de HPEA-FT van de 50mL tube en plaats op een collection tube
11. Label de filter cap
12. Voeg **500 µl WBI** toe
13. Centrifugeer **8000 x g – 1 minuut**
14. Plaats de FT op een nieuwe collection tube
15. Voeg **500 µl WB II** toe
16. Centrifugeer **8000 x g – 1 minuut**
17. Plaats de FT op een nieuwe collection tube
18. Centrifugeer **16000 – 20000 x g – 1 minuut** (drogen filter membraan)
19. Plaats de FT op een gelabelde elutie tube
20. Voeg **100 µl DNA EB** toe aan het midden van de FT membraan (zonder membraan aan te raken)
21. Incubeer op **kamertemperatuur – 5 minuten**
22. Plaats de tubes in de centrifuge en breng een orientatie streepje aan op elke tube
23. Centrifugeer **8000 x g – 1 minuut**
24. Breng heel voorzichtig **80 µl** over naar een nieuwe elutie tube **zonder de pellet te verstoren** (met pipettip NIET tot de bodem gaan).

    Indien de pellet toch verstoord wordt, breng alles terug naar de eerste elutietube en herhaal stap 23 en 24

ANNEX 2: Maxwell® RSC LV ccfDNA Kit, Custom (Promega) protocol

Promega Corporation
2800 Woods Hollow Road
Madison, WI 53711-5399
United States

tel: 608.274.4330 • fax: 608.277.2516 • www.promega.com

For Research Use Only. Not for use in Diagnostic Purposes.

Promega Maxwell® RSC Circulating DNA Purification kit, Large Volume Protocol (AX1115)

Introduction:

The Maxwell RSC Circulating DNA Purification kit is designed to provide a fast, reliable method to purify fragmented DNA from human plasma. The Kit uses a unique, non-silica chemistry that enables purification without a protease digestion step, allowing the entire procedure to be done at room temperature. Each prep can process 2-4 ml of plasma.

Kit Components and Storage Conditions:

The kit contains cartridges and reagents for 48 automated isolations from 1ml plasma samples. Includes:

- 4ml Elution Buffer
- 48 pre-filled Maxwell Cartridges (Please Note: magnetic resin is NOT dispensed in Well #2 of the Maxwell cartridge)
- 50 Maxwell Plungers
- 200ml Binding Buffer
- Concentrated Magnetic Resin
- Storage Conditions: Store the Maxwell Circulating DNA Alpha Kit at **15-30C**.
- Maxwell RSC Protocol for Large Volume Method

Maxwell RSC method file:

Please ensure that you are using the latest method file for this kit. The latest version is Maxwell RSC LV ccfDNA Custom method, v1.0.1

Instructions for method installation are found on page 30 of the Maxwell® RSC Instrument Operating Manual, TM411

Safety Information:

The solutions contain alcohols, which are flammable, and chaotropes, which are irritants. This Maxwell kit is designed to be used with potentially infectious substances. Users should wear appropriate personal protective equipment (e.g., gloves and goggles) when handling infectious substances.
Users should adhere to their institutional guidelines for the handling and disposal of all infectious substances when used with this system.

Materials to Be Provided by the User:

Whole blood or plasma

Optional, table top centrifuge

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Pre Processing:

The pre-processing involves the binding of circulating nucleic acid to the magnetic resin. This takes place in either a rotisserie shaker or Promega's Heater Shaker Magnet (HSM). **Follow ONE of the two pre-processing protocols listed below.**

Once the pre-processing step is completed, the magnetic resin is transferred to well 1 of the Maxwell cartridge, and the protocol is completed in the Maxwell instrument.

Rotisserie Shaker Protocol

1. Add 1-4 ml plasma to a 15ml tube. Add an equal amount of Binding Buffer.
2. Shake the bottle containing magnetic resin until it is COMPLETELY resuspended.
3. Add 140ul of Magnetic Resin.
4. Incubate for 45 minutes while shaking. We recommend a rotisserie shaker; the resin must be kept in suspension for the entire incubation.
5. Centrifuge the tubes at 1000g for 2 minutes to pellet the resin.
6. Carefully decant the supernate. Placing a magnet alongside the resin pellet to fix it in place is recommended. Continue with step 7 below.

Heater Shaker Magnet Protocol

1. Add 2-4 ml plasma to a 50ml tube. Add an equal amount of Binding Buffer.
2. Shake the bottle containing magnetic resin until it is COMPLETELY resuspended.
3. Add 140ul magnetic resin to each tube.
4. Place the tube(s) in the HSM and load the protocol "MaxLV". When prompted, press "Start".
5. The HSM will shake for 45 minutes and then stop. The magnets will engage, drawing the resin to the side of the tubes.
6. Once the resin is completely magnetized, use a pipette to remove the supernatant. Remove the tubes from the HSM. Continue with step 7 below.

Instructions for pre-processing continue in the next section.

Preparation of Maxwell Cartridges:

Change gloves before handling cartridges, Plungers and Elution Tubes. Place the cartridges to be used in the Maxwell Deck Tray. Place each cartridge in the rack with well #1 (the largest well in the cartridge) farthest away from the Elution Tubes. Press down on the cartridge to snap it into position. Ensure both cartridge ends are fully seated in the cartridge rack. Carefully peel back the seal so that all plastic comes off the top of the cartridge. Ensure that all sealing tape and any residual adhesive are removed before placing cartridges in the instrument.

If you are processing fewer than 16 samples, center the cartridges on the deck tray.

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7. Using a pipetman, transfer the contents of well 1 (the large well) into the tube containing the magnetic resin pellet.
8. Resuspend the resin in the reduced volume. It is recommended that a pipette is used for resuspension, as vortexing might cause resin to adhere to the upper sides of the tube.
9. Transfer the resin and liquid back to well 1 of the Maxwell cartridge.
10. Place one plunger into well #8 of each cartridge.
11. Place an empty Elution Tube into the Elution Tube position for each cartridge in the Maxwell Deck Tray.
12. Add 75ul of Elution Buffer to the bottom of each Elution Tube.
    a. Note: For the Large Volume Protocol the void volume of the resin will be between 10 and 15ul; if you elute in 75ul you will typically recover 60ul in the elution tube.
    b. Eluting in less than 60ul will not improve concentration and may negatively impact yield.

Instrument Run:

Refer to the Maxwell® Instrument Operating Manual (TM411) for more detailed information.

Steps:

1. Turn on the Maxwell® Instrument and Tablet PC. The instrument user interface will start automatically, and the instrument will proceed through a self-check and home all moving parts.

2. Select "Start" on the Home screen.

3. On the extraction method selection screen, select a method using one of the two options below:

- Manually touch the RSC ccfDNA Plasma method. If selecting the method manually proceed to step 3a.
- If slecting method manually proceed to step 3a. If using a 2 D bar code reader proceed to step 3b.

3a. Manually touch the RSC method

3b. Select the RSC method using the bar code scanner. scan the barcode below:

X100511234562016-12

4. Select cartridge positions to be run.

5. Verify that the samples were added to well #1 of the cartridges, the cartridges are loaded on the instrument, the Elution Tubes containing 75uL of Elution Buffer are present and the plungers are placed in well #8.

6. Transfer the Maxwell® Deck Tray containing the prepared cartridges to the Maxwell® Instrument deck. Ensure that the deck tray is placed in the Maxwell® RSC Instrument with the Elution Tubes closest to the door. The deck tray will only fit in the instrument in this orientation. If you have difficulty fitting the deck tray on the platform, check that it is in the correct orientation.

Ensure that the deck tray is level on the instrument deck.

Note: Hold the Maxwell® Deck Tray by the sides to avoid dislodging cartridges from the rack.

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7. Confirm all indicated pre-processing has been performed, and touch "Start" to close the instrument door and start processing.

Warning: Pinch point hazard.

8. The Maxwell® Instrument will immediately begin the purification run. The screen will display the steps performed and the approximate time remaining in the run. The Maxwell® has adaptive timing so the approximate time remaining in the run will be inaccurate for the first 3 runs before listing an accurate run time.

Note: If the run is aborted before completion, the instrument will wash the particles off the plungers and eject the plungers into well #8 of the cartridge. The samples will be lost. Do not attempt to re-purify samples for which an instrument run was aborted.

9. When the automated purification run is complete, the Tablet PC screen will display a message that the method has ended.

End of Run:

10. Follow on-screen instructions at the end of the method to open door. Verify that plungers are located in well #8 of the cartridge at the end of the run. Remove the deck tray from the instrument and remove eluted samples from the deck tray. If plungers have not all been removed from the plunger bar: follow the on-screen prompts to perform the Clean Up method for an aborted run; or select the Clean Up method from the Settings Screen for a successfully completed run to remove the remaining plungers.

11. Remove the Maxwell® Deck Tray from the instrument immediately following the run to prevent evaporation of the eluates. Remove Elution Tubes containing DNA, and close the tubes.

12. Remove the cartridges and plungers from the Maxwell® Deck Tray, and discard as hazardous waste according to your institution's procedures. Do not reuse Maxwell® Cartridges, CSC/RSC Plungers or Elution Tubes.

Post Purification:

We strongly recommend quantitating the amount of DNA purified by PCR, as concentrations of nucleic acid found in normal human plasma are too dilute to be accurately measured by spectrophotometric methods.

ANNEX 3: Qubit® dsDNA HS Assay Kit Q32851 (Invitrogen) protocol

WERKBLAD voor cfDNA concentratie bepaling met Qubit kit

Meting met Qubit

1. Schrijf volgnummer + initialen op de Qubit assay tube voor ieder staal + 2 standaarden
2. Verdun het Qubit reagent 1 op 200 (= Qubit Working Solution) in een tube.
   Vortex het Qubit reagent en draai kort af.

	#µL/staal	#µL/.... stalen
Qubit® dsDNA HS Assay Kit	1	
Qubit buffer	199	
	200	

3. Vortex de Qubit Working Solution + standaarden en pipetteer het aantal µL Qubit Working Solution in e assay tubes en het aantal µL Standaard/DNA zoals in onderstaande tabel:

	#µL/stand.	#µL/staal
Working solution (zie punt 2)	190	199
Standard	10	-
Staal	-	1
	200	200

4. Vortex alle tubes 2-3 seconden
5. Incubeer de tubes 2 minuten op kamertemperatuur
6. Steek de USB stick in de Qubit 2.0 Fluorometer
7. Kies op de Qubit 2.0 Fluorometer de assay: 'DNA' -> 'dsDNA Hight Sensitivity'
8. Nieuwe standaard lezen? 'yes'
9. Plaats standaard 1 in het toestel en meet -> 'Read'. Herhaal voor standaard 2.
10. Plaats een staal in het toestel en meet → 'Read (next sample)'.
11. Bepaal de stock concentratie -> 'Calculate stock conc.' -> 'Volume of sample used' instellen op 1 µl -> 'ng/µl' instellen als conentratie eenheid
12. Bewaar de meting -> 'Save'. Schrijf de meting ook op het werkblad.
13. Herhaal stap 10-12 voor de andere stalen en sla de data op op de USB stick.
14. Verwijder de data op het Qubit toestel → 'clear data'.

ANNEX 4: Characteristics gBlocks

The black framework indicates the entire exon (Glu542Lys and Glu545Lys) or a part of the exon (His1047Leu and His1047Arg) corresponding to this on Alamut. The red mark indicates the altered base.

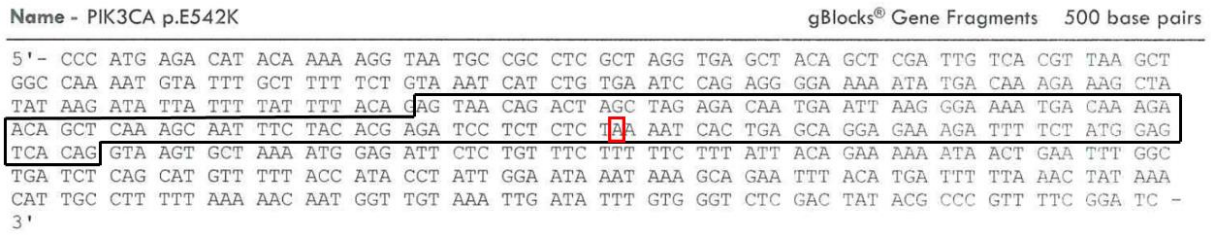

Figure 34 PIK3CA c.1624G>A (p.(Glu542Lys)) gBlock [49]

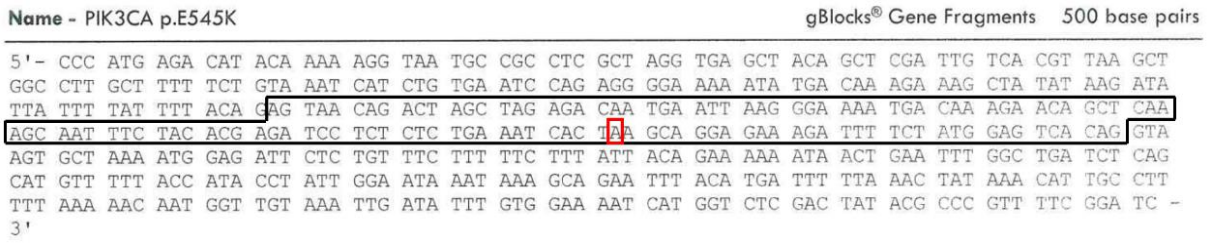

Figure 35 PIK3CA c.1633G>A (p.(Glu545Lys)) gBlock [50]

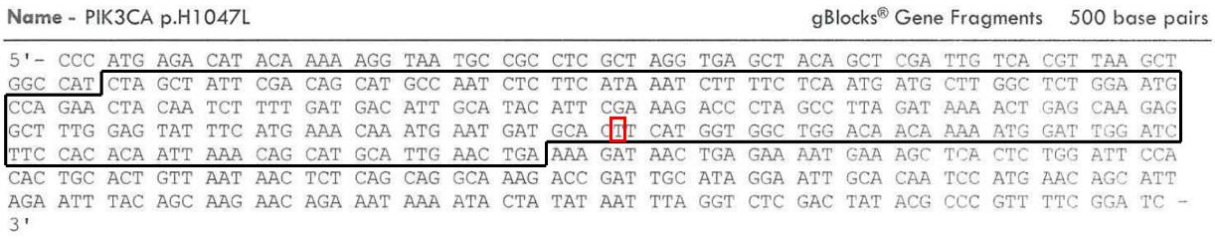

Figure 36 PIK3CA c.3140A>T (p.(His1047Leu)) gBlock [51]

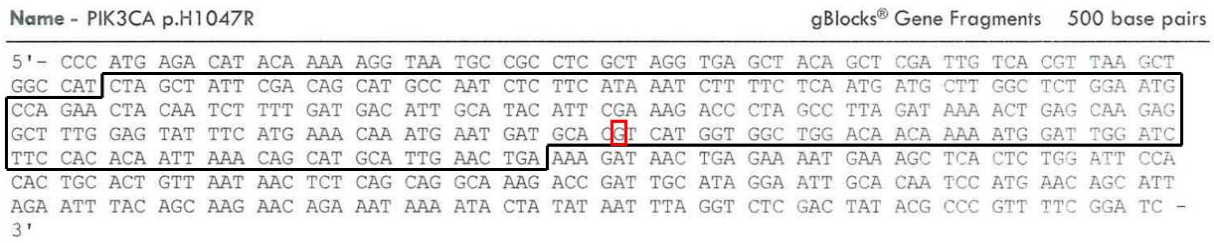

Figure 37 PIK3CA c.3140A>G (p.(His1047Arg)) gBlock [52]

Table 19 Overview physicochemical characteristics gBlocks [49], [50], [51], [52]

	Glu542Lys	Glu545Lys	His1047Leu	His1047Arg
Length (bp)	500	500	500	500
Quantity (ng)	500	500	500	500
GC content (%)	34.8	35	40.4	40.6
Molecular weight (D)	308750.3	308751.3	308777.9	308778.9
fmoles/ng	3.24	3.24	3.24	3.24
µg/OD_{260}	50	50	50	50
Reference Number	222057489	222057488	222057486	222057487

ANNEX 5: Positive control calculations

Preparation positive control containing 10 copies/µl gBlock and 7.5 copies/µl WT DNA

gBlock 10 copies/µl

Stock concentration	10 ng/µl		
Stock concentration	1.95E+10 copies/µl	Desired concentration	10 copies/µl
Dilution 1	1.95E+07 copies/µl	Desired volume	260 µl
Dilution 2	19505 copies/µl		
Dilution 3	19.5 copies/µl		

Dilutions are made by pipetting 1.5 µl concentrated solution into 1.5 ml buffer

Required quantity of dilution 3 to obtain a total volume of 260 µl with 10 copies/µl gBlock

133 µl

Wild type cell line Conversion: 4 ng DNA/µl = 60 copies/µl

Stock concentration	142 ng/µl	Desired concentration	7.5 copies/µl
Stock concentration	2130 copies/µl	Desired volume	260 µl
Dilution 1	213 copies/µl		

Required quantity of dilution 1 to obtain a total volume of 260 µl with 7.5 copies/µl wild type DNA

9.15 µl

Buffer TE low 10/0.1 pH 8.0 + POLY(A)

118 µl

Preparation positive control containing 0.8 copies/µl gBlock and 7.5 copies/µl WT DNA

gBlock 0.8 copies/µl

Stock concentration	10 ng/µl		
Stock concentration	1.95E+10 copies/µl	Desired concentration	0.8 copies/µl
Dilution 1	1.95E+07 copies/µl	Desired volume	130 µl
Dilution 2	19505 copies/µl		
Dilution 3	19.5 copies/µl		
Dilution 4	1.95 copies/µl		

Dilutions are made by pipetting 1.5 µl concentrated solution into 1.5 ml buffer

Required quantity of dilution 4 to obtain a total volume of 130 µl with 0.8 copies/µl gBlock

53.3 µl

Wild type cell line Conversion: 4 ng DNA/µl = 60 copies/µl

Stock concentration	142 ng/µl	Desired concentration	7.5 copies/µl
Stock concentration	2130 copies/µl	Desired volume	130 µl
Dilution 1	213 copies/µl		

Required quantity of dilution 1 to obtain a total volume of 130 µl with 7.5 copies/µl wild type DNA

4.58 µl

Buffer TE low 10/0.1 pH 8.0 + POLY(A)

72.1 µl

ANNEX 6: Raw data intra-run variation for hotspots Glu542Lys, His1047Leu and His1047Arg

Table 20 Intra-run variation of the mutated PIK3CA cfDNA results for hotspot mutation Glu542Lys

	Concentration mutated *PIK3CA* cfDNA (copies/µl)	
Patient	**Well 1**	**Well 2**
1	21.0	18.0
2	0.0700	0
3	2.20	1.70
4	62.6	63.5
5	0	0
6	0	0
7	0.230*	0
8	0	0
9	0	0
10	0.0700*	0
11	0	0
12	0	0.0700*
13	0.0700*	0.130*
14	0.0700*	0
15	0	0.0700*
16		
17	0	0.0900*
18	0	0
19	0	0
20	0	0
21	0	0

Table 21 Intra-run variation of the mutated PIK3CA cfDNA results for hotspot mutation His1047Leu

	Concentration mutated *PIK3CA* cfDNA (copies/µl)	
Patient	**Well 1**	**Well 2**
1	0	0
2	0	0
3	0	0
4	0	0
5	0	0
6	0	0
7	0	0
8	0	0
9	0	0
10	0	0
11	0	0
12	0	0
13	0	0
14	0	0
15	0	0
16		
17	0	0
18	0	0
19	0	0
20	0	0
21	0	0

Table 22 Intra-run variation of the mutated PIK3CA cfDNA results for hotspot mutation His1047Arg

	Concentration mutated *PIK3CA* cfDNA (copies/µl)	
Patient	**Well 1**	**Well 2**
1	0	0
2	0	0
3	162	158
4	0	0
5	0	0
6	0	0
7	0	0
8	0	0
9	0	0
10	0	0
11	0	0
12	0	0
13	0	0
14	0	0
15	0	0
16		
17	0	0
18	0	0
19	0	0
20	0	0
21	0	0

ANNEX 7: Raw data controls inter-run variation for hotspots Glu542Lys, His1047Leu and His1047Arg

Table 23 Corrected control raw data mutated PIK3CA cfDNA for hotspot mutation Glu542Lys

	Concentration mutated *PIK3CA* cfDNA (copies/µl)		
	Repeat 1	**Repeat 2**	**Repeat 3**
gBlock 10 copies/µl	1.34	1.20	1.50
gBlock 0.8 copies/µl	0.105	0.140	0.173
PA-1 cell line	0.0300	0	0
A.D.	0	0	0

Table 24 Corrected control raw data wildtype cfDNA for hotspot mutation Glu542Lys

	Concentration wild type cfDNA (copies/µl)		
	Repeat 1	**Repeat 2**	**Repeat 3**
gBlock 10 copies/µl	51.3	46.7	49.7
gBlock 0.8 copies/µl	45.1	44.2	46.0
PA-1 cell line	49.8	49.4	50.5
A.D.	0.0	0.0400	0

Table 25 Corrected control raw data mutated PIK3CA cfDNA for hotspot mutation His1047Leu

	Concentration mutated *PIK3CA* cfDNA (copies/µl)	
	Repeat 1	**Repeat 2**
gBlock 10 copies/µl	2.70	2.25
gBlock 0.8 copies/µl	0.230	0.120
PA-1 cell line	0	0
A.D.	0	0

Table 26 Corrected control raw data wildtype cfDNA for hotspot mutation His1047Leu

	Concentration wild type cfDNA (copies/µl)	
	Repeat 1	**Repeat 2**
gBlock 10 copies/µl	50.9	51.1
gBlock 0.8 copies/µl	47.0	46.9
PA-1 cell line	50.4	52.3
A.D.	0	0

Table 27 Corrected control raw data mutated PIK3CA cfDNA for hotspot mutation His1047Arg

	Concentration mutated *PIK3CA* cfDNA (copies/µl)			
	Repeat 1	**Repeat 2**	**Repeat 3**	**Repeat 4**
gBlock 10 copies/µl	2.80	2.63	3.30	3.40
gBlock 0.8 copies/µl	0.170	0.165	0.315	0.280
PA-1 cell line	0	0	0	0
A.D.	0	0	0	0

Table 28 Corrected control raw data wildtype cfDNA for hotspot mutation His1047Arg

	Concentration wild type cfDNA (copies/µl)			
	Repeat 1	**Repeat 2**	**Repeat 3**	**Repeat 4**
gBlock 10 copies/µl	51.5	51.6	53.0	52.4
gBlock 0.8 copies/µl	45.5	45.9	44.8	44.3
PA-1 cell line	58.7	50.2	53.5	52.2
A.D.	0	0	0	0

ANNEX 8: Calculations control average, SD and average ±3.5xSD for hotspots Glu542Lys, His1047Leu and His1047Arg

Table 29 Calculations control average, SD and average ±3.5xSD mutated PIK3CA cfDNA for hotspot mutation Glu542Lys

	Concentration mutated *PIK3CA* cfDNA (copies/µl)		
	Average	**SD**	**Average ± 3.5xSD**
gBlock 10 copies/µl	1.35	0.150	0.825 – 1.875
gBlock 0.8 copies/µl	0.139	0.0338	0.0207 – 0.257
PA-1 cell line	0.0100	0.0173	0 – 0.0706
A.D.	0	0	0

Table 30 Calculations control average, SD and average ±3.5xSD wild type cfDNA for hotspot mutation Glu542Lys

	Concentration wild type cfDNA (copies/µl)		
	Average	**SD**	**Average ± 3.5xSD**
gBlock 10 copies/µl	49.2	2.33	41.1 – 57.4
gBlock 0.8 copies/µl	45.1	0.888	42.0 – 48.2
PA-1 cell line	49.9	0.543	48.0 – 51.8
A.D.	0	0.0231	0

For the calculations of the inter-run variations for hotspot mutation His1047Leu, the same calculation method is used as for the patient calculations in Table 14 because only two repeats were measured.

Table 31 Calculations control average, distribution (R) and 1/2 R for hotspot mutation His1047Leu

	Concentration mutated *PIK3CA* cfDNA (copies/µl)			**Concentration wild type cfDNA (copies/µl)**		
Patient	**Average**	**Distribution (R)**	**½ R**	**Average**	**Distribution (R)**	**½ R**
gBlock 10 copies/µl	2.85	0.3	0.15	59.5	17.2	8.6
gBlock 0.8 copies/µl	0.195	0.07	0.035	54.75	15.5	7.75
PA-1 cell line	0	0	0	60.05	19.3	9.65
A.D.	0	0	0	0	0	0

Table 32 Calculations control average, SD and average ±3.5xSD mutated PIK3CA cfDNA for hotspot mutation His1047Arg

	Concentration mutated *PIK3CA* cfDNA (copies/µl)		
	Average	**SD**	**Average ± 3.5xSD**
gBlock 10 copies/µl	3.03	0.377	1.71 – 4.35
gBlock 0.8 copies/µl	0.233	0.0764	0 – 0.500
PA-1 cell line	0	0	0
A.D.	0	0	0

Table 33 Calculations control average, SD and average ±3.5xSD wild type cfDNA for hotspot mutation His1047Arg

	Concentration wild type cfDNA (copies/µl)		
	Average	**SD**	**Average ± 3.5xSD**
gBlock 10 copies/µl	52.1	0.709	49.6 – 54.6
gBlock 0.8 copies/µl	45.1	0.7136	42.6 – 47.6
PA-1 cell line	53.6	3.64	40.9 – 66.3
A.D.	0	0	0

ANNEX 9: Raw data patients inter-run variation for hotspots Glu542Lys, His1047Leu and His1047Arg

Table 34 Raw data patient samples for hotspot mutation Glu542Lys

	Concentration mutated *PIK3CA* cfDNA (copies/µl)		Concentration wild type cfDNA (copies/µl)	
Patient	**Repeat 1**	**Repeat 2**	**Repeat 1**	**Repeat 2**
1	21.1	20.1	41.1	39.1
10	0	0	13.9	14.2

Table 35 Raw data gBlock 0.8 copies/µl His1047Leu and patient sample for hotspot mutation His1047Leu

	Concentration mutated *PIK3CA* cfDNA (copies/µl)		Concentration wild type cfDNA (copies/µl)	
	Repeat 1	**Repeat 2**	**Repeat 1**	**Repeat 2**
gBlock 0.8 copies/µl His1047Leu	0.350	0.210	44.6	45.9
Patient 3	0	0	128	121

Table 36 Raw data patient samples for hotspot mutation His1047Arg

	Concentration mutated *PIK3CA* cfDNA (copies/µl)		Concentration wild type cfDNA (copies/µl)	
Patient	**Repeat 1**	**Repeat 2**	**Repeat 1**	**Repeat 2**
3	165	148	141	117
14	0	0	6.90	7.10

ANNEX 10: Calculations patient average, distribution (R) and ½ R for hotspots Glu542Lys, His1047Leu and His1047Arg

Table 37 Calculations patient 1 and 10 average, distribution (R) and 1/2 R for hotspot mutation Glu542Lys

	Concentration mutated *PIK3CA* cfDNA (copies/µl)			Concentration wild type cfDNA (copies/µl)		
Patient	**Average**	**Distribution (R)**	**½ R**	**Average**	**Distribution (R)**	**½ R**
1	20.6	1.00	0.500	40.1	2.00	1.00
10	0	0	0	14.1	0.300	0.150

Table 38 Calculations gBlock 0.8 copies/µl and patient 3 average, distribution (R) and 1/2 R for hotspot mutation His1047Leu

	Concentration mutated *PIK3CA* cfDNA (copies/µl)			Concentration wild type cfDNA (copies/µl)		
	Average	**Distribution (R)**	**½ R**	**Average**	**Distribution (R)**	**½ R**
gBlock 0.8 copies/µl	0.300	0.140	0.0700	45.3	1.30	0.650
Patient 3	0	0	0	125	7.20	3.60

Table 39 Calculations patient 3 and 14 average, distribution (R) and 1/2 R for hotspot mutation His1047Arg

	Concentration mutated *PIK3CA* cfDNA (copies/µl)			Concentration wild type cfDNA (copies/µl)		
Patient	**Average**	**Distribution (R)**	**½ R**	**Average**	**Distribution (R)**	**½ R**
3	156	16.8	8.40	129	23.4	11.7
14	0	0	0	7.00	0.200	0.100

ANNEX 11: Poster presented on the Belgian oncoforum by Apr. PhD Laurence Slembrouck

Oncoforum 2019

Optimization and Validation of *PIK3CA* Mutation Detection with droplet digital PCR in Liquid Biopsies of Patients with Metastatic Breast Cancer

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Background

- +/- 40% of oestrogen receptor positive (ER+) metastatic breast cancer patients harbor *PIK3CA* mutations causing overactivated PI3K pathway
- Alpelisib, a PI3Kα-selective inhibitor, is currently available in an early access program in patients with metastatic ER+ HER2- breast cancer, if the presence of an activating *PIK3CA* mutation is confirmed

Problems: Tissue procurement in metastatic setting is challenging. Possible solution: mutational analysis on liquid biopsies

Aim: To optimize and validate droplet digital PCR (ddPCR) for the detection of *PIK3CA* hotspot mutations in cell-free DNA (cfDNA) of patients with metastatic breast cancer

Patients and Methods

Type of Study: Prospective

Site: Multidisciplinary Breast Center at University Hospitals Leuven, Belgium

Number of Patients: 20 Patients with metastatic breast cancer at progression (N=12) or during therapy (N=8) with known *PIK3CA* mutation status as previously demonstrated by Next Generation Sequencing (NGS) performed on tumour tissue

Type of samples: 2 Blood samples per patient (cfDNA collection tubes, Roche Diagnostics)

Extraction methods: Manually with Cobas® cfDNA Sample Preparation Kit (2mL plasma) and semi-automatically with Maxwell® RSC ccfDNA Plasma Kit (2mL and 4mL plasma)

Endpoints: Intra- and inter-run variation, precision, robustness, sensitivity and specificity of 4 ddPCR assays (Glu542Lys, Glu545Lys, His1047Leu, His1047Arg)

Concordance between NGS and ddPCR for detection of *PIK3CA* hotspot mutations on tissue and in plasma, respectively

Results

All 20 samples were successfully analysed by ddPCR.

The highest cfDNA yield was obtained by Maxwell 4mL extraction method (median: 0.483 ng/µL; range: 0.140 – 10.500 ng/µL).

The per-mutation sensitivity and specificity between tissue and plasma was 87.5% and 95.8%, respectively.

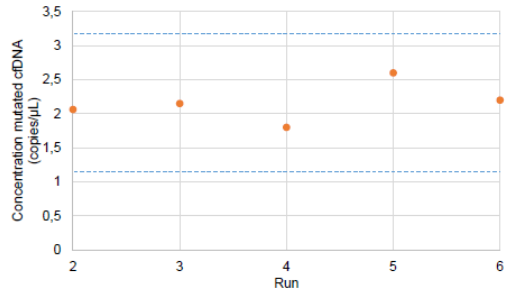


Figure 1. Inter-run variation p.(E545K) gBlock 10 copies/µL *PIK3CA* mutated cfDNA.

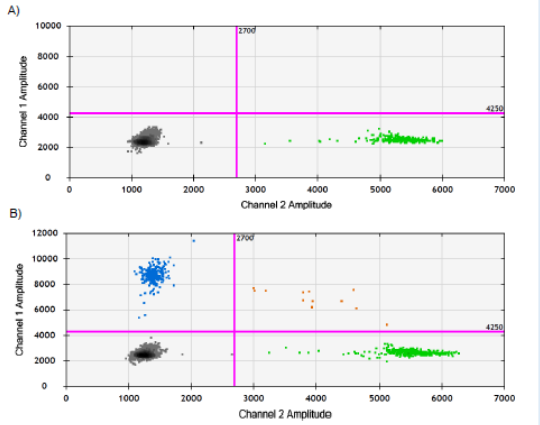
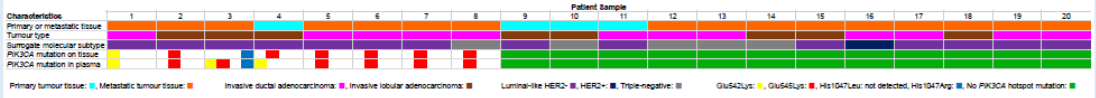


Figure 2. Examples of 2D-scatterplots of A) a sample that tests negative for a *PIK3CA* mutation and B) a sample that tests positive for a *PIK3CA* mutation.
The black cluster are negative droplets that do not contain any DNA, the green cluster are positive droplets that contain only wild type DNA, the blue cluster are positive droplets that contain only mutated DNA and the orange cluster are positive droplets that contain both mutated and wild type DNA.

Table 1. Tumour characteristics and *PIK3CA* mutation status on tumour tissue (NGS) and in plasma (ddPCR).



Conclusion

- Detection of *PIK3CA* hotspot mutations with ddPCR in cfDNA is **feasible**
- **Good concordance** between **NGS and ddPCR** for the detection of *PIK3CA* hotspot mutations on tumour tissue and in plasma, respectively
- **Discordant *PIK3CA* mutation status** in tissue versus plasma (10% of cases) might reflect tumour heterogeneity. **Further investigation** of different metastatic lesions in these cases and/or repetition of plasma samples is ongoing