Hamburg University of Applied Sciences

Faculty of Life Sciences

Department of Biotechnology

Ultrasensitive Detection of Lung Cancer Mutation Pattern Favoring Minimal Invasive Liquid Biopsy – An Experimental Approach

Bachelor thesis

For the degree of

B. Sc. Biotechnology

by

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Hamburg

22. January 2020

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Acknowledgments

I would like to express my sincere appreciation to my supervisor, Dr. Caren Vollmert, for providing me with the opportunity to pursue my bachelor thesis at the Agena Bioscience GmbH, Hamburg. I am grateful for her constant guidance and encouragement, without which this work would not have been possible.

I would like to thank my first supervisor, Prof. Dr. Oliver Ullrich, for his invaluable insights and suggestions for my bachelor thesis.

My gratitude also extends to Dr. Alexander Sartori and Maroje Krajina, for their support, guidance and useful feedback during my work.

And a special thanks to my spouse, family and friends for always supporting and encouraging me during my years of study.

Abstract

Lung cancer is the most commonly diagnosed cancer type and the leading cause of cancer death in men and women worldwide. Early detection of lung cancer is problematic as it requires surgery to extract tissue specimens. In contrast, liquid biopsy is a minimally invasive method requiring only a blood sample to perform early diagnosis and treatment monitoring of cancer. Analyzing tumor-derived biomarkers, such as circulating tumor cells (CTCs) and/or circulating tumor DNA (ctDNA), require ultrasensitive detection methods for profiling lung cancer. The purpose of this study is to analyze the ultrasensitive detection of lung cancer mutations (ctDNAs) in patients' plasma samples using the UltraSEEK Lung Panel. Results of this study will be integrated in the collaboration project "The Brain Metastases" between Universitätsklinikum Hamburg-Eppendorf and Agena Bioscience.

Plasma samples of seventeen lung cancer patients have been collected for this study. The quality analysis of cfDNA and preliminary on-site tests, using reference material, were used to verify the performance of the UltraSEEK Lung Panel. In addition, participating in a ring trial was an appropriate opportunity to evaluate the performance of the UltraSEEK Lung Panel. Silica membrane adsorption technique and magnetic beads binding technology were the two different methods used to extract cfDNA. NanoDrop, Qubit, LabChip, and LiquID IQ Panel were used to quantify the cfDNA concentration. Finally, the MassARRAY System technology combined with the UltraSEEK Lung Panel was used to detect the mutation in plasma samples of the seventeen lung cancer patients. During verification and validation of the UltraSEEK Lung Panel, differences in cfDNA fragment lengths were observed in synthetically manufactured cfDNA versus human cfDNA. DNA fragmentation has an impact on the ability of the analysis panel to detect low mutation frequencies. The verification test, using reference materials, showed a high sensitivity (91-100%) of mutation detection for mutation with allele frequencies (AF) between 0.5% to 2.0%. The sensitivity of mutation detection decreased to 44%-70% for AF in the range of 0.1% to 0.3%. The high performance of the UltraSEEK Lung Panel was confirmed by the Reference Institute for Bioanalytics, a ring trial supervising laboratory. Finally, in the UltraSEEK Lung Panel clinical study, the assay detected mutations (on genes such as *KRAS, EGFR,* and *PIK3CA*) in 9 out of 17 lung cancer patient samples.

As a conclusion, the UltraSEEK Lung Panel was shown to be able to detect mutations (ctDNA) of lung cancer patients in blood samples with AF as small as 0.1%, and could therefore be used for early detection and treatment monitoring of lung cancer using minimal invasive liquid biopsies.

Contents

List of Abbreviations

+AM	positivity charged analyte-molecules
C	Cytosine
CCD	charge-coupled device
cfDNA	cell free DNA
cfNAs	cell-free nucleic acids
CPM	Chip Prep Module
CTCs	circulating tumor cells
ctDNA	circulating tumor DNA
dNTP	deoxynucleotide triphosphate
dsDNA	double strand DNA
G	Guanine
gDNA	genomic DNA
HapMap	haplotype map
LDCT	low-dose computed tomography
MA 4	MassARRAY Analyzer 4
MALDI-TOF	Matrix-assisted Laser Desorption/Ionization - Time of Flight
NSCLC	non-small cell lung carcinomas
NTC	negative template control
pb	base pairs
PCR	polymerase chain reaction
RfB	Referenzinstitut fuer Bioanalytik
rpm	rounds per minute
SAP	Shrimp alkaline Phosphatase
SC	SeraCare
SCLC	small-cell lung carcinomas
ssDNA	single strand DNA
UKE	Universitätsklinikum Hamburg-Eppendorf
WT	wildtype

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1. Introduction

1.1 Lung cancer

After cardiovascular diseases, cancer is the second most common cause of death globally. According to the International Agency for Research on Cancer (IARC) report, approximately 18.1 million cancer-related cases and approximately 9.6 million deaths due to cancer were estimated in 2018 worldwide. The major types of cancers are lung and female breast cancers (for each of these types, approximately 2.1 million cases) followed by colon cancer, prostate cancer, and other cancers (World Health Organization, 2019).

Lung cancer is the most commonly diagnosed cancer type and the leading cause of cancer death in men. The most significant risk factor for lung cancer is the use of tobacco, which is responsible for approximately 1.8 million deaths in 2018 worldwide (World Health Organization, 2019). Other risk factors such as air pollution exposures, radon gas, asbestos, and chronic infection can cause lung cancer (Lemjabbar-Alaoui *et al*, 2016; Molina *et al*, 2008).

Surgery, radiation therapy, chemotherapy, and targeted therapy are possible lung cancer treatments. The selection of therapy type depends on the type and stage of cancer (Molina *et al*, 2008).

50 percent of patients diagnosed with lung cancer die during the first year. The five-year survival rate of patients with a lung cancer diagnosis at stage I-II is 56 percent, and for those with metastatic tumors at stage IV, the survival rate is only five percent (Revelo *et al*, 2019).

There are two major types of lung cancer, small-cell lung carcinomas (SCLC) and non-small cell lung carcinomas (NSCLC). The growth and spread of these types of cancer are different. SCLC tumors tend to grow faster than NSCLC tumors. The NSCLC accounts for approximately 85 percent of lung cancer, and the SCLC accounts for the remaining 15 percent of all cases of lung cancer (Lemjabbar-Alaoui *et al*, 2016; Molina *et al*, 2008).

Early diagnosis of lung cancer is a good opportunity to decrease mortality. The recommended screening test for lung cancer is low-dose computed tomography (LDCT). Over 10 years, LDCT scanning decreased mortality by 26 percent in men and 61 percent in women. However, as the only screening device for lung cancer, LDCT has at least three risks; false-positive results, overdiagnosis, and radiation exposure. Early detection of lung cancer is problematic as it requires surgery to extract tissue specimens or tissue biopsy (Revelo *et al*, 2019).

1.2 Liquid Biopsy

Liquid biopsy is a diagnostic tool to detect tumor markers such as circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), circulating miRNAs, proteins, tumor-derived extracellular vesicles (EVs) and methylation changes in the body fluid. Body fluids include saliva, urine, cerebrospinal fluid, sputum, stool samples, seminal plasma, pleural effusions, but mainly blood (serum and plasma) (Lousada-Fernandez *et al*, 2018). In contrast to traditional biopsy, liquid biopsy has the major advantage of being minimally invasive, can be serially repeated, and delivers information from the tumor in real-time (Evi Lianidou *et al*, 2018). Moreover, liquid biopsy has broad application in the diagnosis and therapy of cancer. It includes diagnosis of cancer in the early stage, assessment of tumor heterogeneity and dynamics, monitoring of therapy response in real-time, and the prospect to perform continuous follow-up analysis (Bai *et al*, 2018).

History of Liquid Biopsy

The presence of circulating, cell-free nucleic acids (cfNAs) in human blood was discovered by Mandel and Metais in 1948. Unfortunately, this observation was forgotten for a long time until 1977. Leon and colleagues proved that the concentration of circulating DNA in plasma of cancer patients was higher than in healthy controls. Stroun and colleagues showed that circulating DNA and tumor DNA were similar, in that both showed decreased strand stability. It was suggested that the circulating DNA originated from the tumor, so the concept of “liquid biopsy” was born in 1989. Five years later in 1994, scientists found mutated RAS gene fragments in the blood of cancer patients, which turned the focus back on cfDNA. In 1996, scientists showed microsatellite alterations in serum DNA of cancer patients. In 2009, EGFR T790M mutations in ctDNA of non-small lung cancer patients were detected (Kuang *et al*, 2009). All these discoveries indicated that cancerous tumors release cfDNA into the blood, which makes the circulating tumor DNA (ctDNA) an ideal biomarker to detect cancer. Due to technological progress in recent years, the ability to analyze ctDNA has greatly improved (Siravegna *et al*, 2014).

ctDNA fragment size

Isolation and analysis of ctDNAs are challenging because of their low concentration and heavy fragmentation. ctDNA (from 0.01% to slightly over 50%) is a small variable fraction of cfDNA. Studies showed that during apoptosis, ctDNAs from tumor cells are released into the bloodstream (Marrugo-Ramírez *et al*, 2018). The length of cfDNA in healthy controls is between 70- 200 bp and concentration ranges between 0-100 ng/ml. In the blood of cancer

patients, the length of ctDNA is between 200 pb and more than 1 kb, with concentration between 7-18 ng/ml of whole blood. The half-life of these ctDNAs is very short (15 minutes to 120 minutes) before being eliminated by kidney and liver (Marrugo-Ramírez *et al*, 2018; Sartori, 2017).

Molecular techniques

In recent years, technological advances enabled the analysis of small amounts of ctDNA in the genome. These technologies include (see Figure 1):

- Sanger Sequencing, with a detection limit ranging between 10-100% of ctDNA fraction.
- Real-time PCR and NGS (next-generation sequencing), with ctDNA fraction detection ranging between 1% and 10%.
- ARMS (amplification refractory mutation system), PNA-LNA (peptide nucleic acid locked nucleic acid), ddPCR (Droplet Digital PCR), BEAMing (beads, emulsions, amplification, and magnetics) and MassARRAY UltraSEEK are allele-specific detection techniques. The detection limit of ctDNA fraction ranges between less than 0.01- 1%.

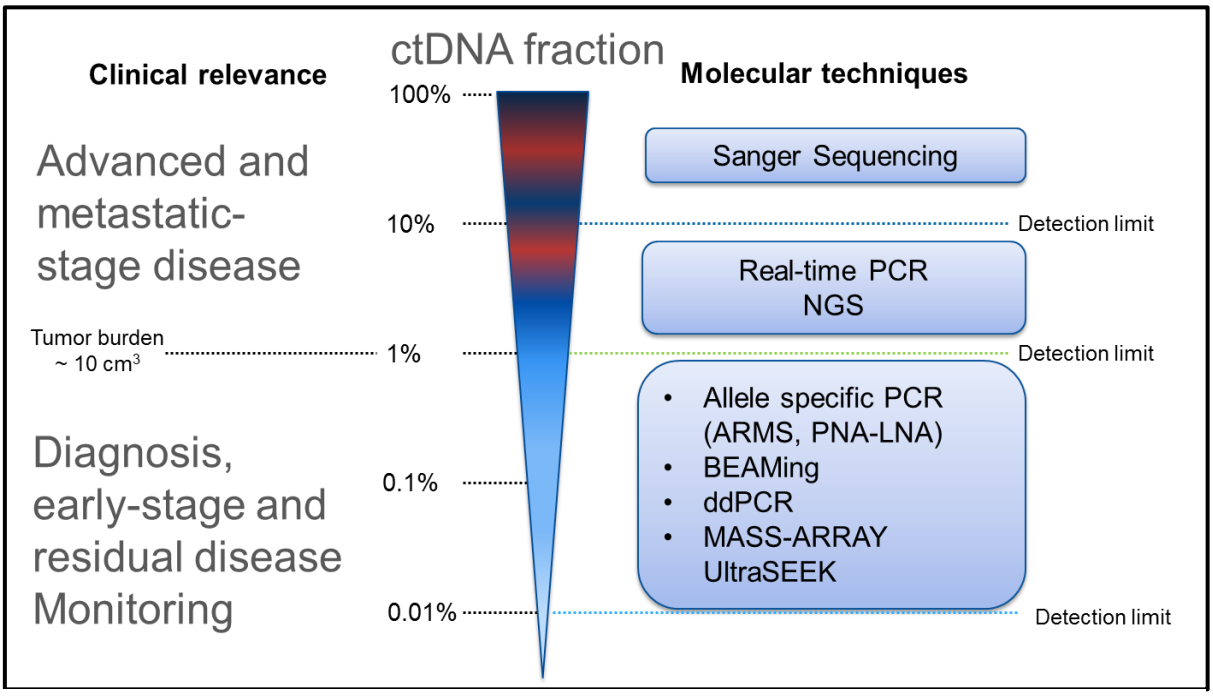

Figure 1. ctDNA fraction (100-0.01%), Clinical relevance of cancer in advanced and early stages, in connection with molecular techniques and detection limit (Sartori, 2017; Saliou *et al*, 2016).

Sanger Sequencing and Real-time PCR techniques were used to detect mutations in advanced and metastatic stages of cancer. To detect mutations in early stages and monitoring of residual diseases, sensitive and target-specific techniques were used to diagnose cancer (Saliou *et al*, 2016; Saleh *et al*, 2019; Michael *et al*, 2016; Fiala *et al*, 2018)

1.3 Aim of the Bachelor Thesis

Lung cancer is one of the most common cancers for both men and women. Worldwide, lung cancer takes more lives than the next three most common cancers combined (colon, breast, prostate). The five-year survival rate of patients diagnosed with lung cancer at an early stage is only fifty percent. When symptoms are appearing, this usually means lung cancer is at an advanced stage, reducing the likelihood of cure. Early detection of lung cancer is problematic as it requires surgery to extract tissue specimens or tissue biopsy.

Liquid biopsy, however, is a minimally invasive method requiring only a blood sample to perform early diagnosis of cancer. The aim of this study is to analyze the ultrasensitive detection of lung cancer mutations in patients' blood samples using the UltraSEEK Lung Panel.

The seventeen plasma samples of lung cancer patients are provided by Universitätsklinikum Hamburg-Eppendorf (UKE) and are part of the Brain Metastases Study. Agena Bioscience GmbH and UKE are members of the Cancer ID Consortium and are collaborating on studies.

Before processing the patient samples, preliminary tests using reference material will be required to baseline the performance of the Lung Panel. The UltraSEEK Lung Panel will be verified on-site, including cfDNA extraction, concentration quantification, and mutation detection method. Participating in a ring trial will be a valuable opportunity to compare the performance of the UltraSEEK Lung Panel with other detection techniques used in other laboratories. Finally, the UltraSEEK Lung Panel will be validated with real patient samples.

2. Materials and Methods

2.1 Sample preparation

Approximately 10 ml of blood sample was centrifuged twice for 10 minutes at 4200 rpm. In the target sample, the plasma was in the supernatant. For the cfDNA isolation, the whole plasma sample >4 ml was used. The more plasma volume is available, the higher the cfDNA yield. Two types of cfDNA isolation kits were used for cfDNA isolation. The QIAamp Circulating Nucleic Acid Kit was used for the UKE samples, while ring trial samples were extracted with the NextPrep-Mag cfDNA Isolation Kit.

2.1.1 cfDNA isolation using QIAamp Mini column

The isolation of cfDNA is based on column technology (silica membrane). The procedure comprises four steps: lyse, bind, wash and elute. The column can bind cfDNA fragment up to 1000 pb, and the cfDNA binding capacity is up to 100 ng/ml.

Lysing samples: The plasma samples are usually sheathed in proteins, lipids, and vesicles requiring a lysis step to release the cfDNA. Performed in presence of proteinase K and Buffer ACL and under highly denaturing terms at increased temperatures, this ensures the inactivation of DNases and detachment of cfDNA from the matrix.

Membrane adsorption (column): The column is coated with a silica membrane where the adsorption of cfDNA takes place in the presence of a binding buffer. Primarily, a large sample volume was transferred onto a column, with cfDNAs binding onto the silica membrane. Proteins and other contaminants (lysate), which can affect the PCR, were drawn through the column by vacuum pressure. Salt and pH conditions ensured the denaturation of the proteins.

Cleaning step: To ensure that all contaminants were removed from the column, three wash steps were required.

Elution of cfDNA: The elution volume can be as low as 50 µl using the elution buffer. For higher cfDNA concentration, the elution volume can be reduced to as low as 20 µl.

The elution volume can be up to 150 µl for some applications that require a larger starting volume. If the elution volume increases, the DNA concentration will decrease (QIAGEN; 2019).

2.1.2 cfDNA isolation with NextPrep-Mag

In contrast to the QIAamp Mini column Kit, which is based on the silica membrane adsorption method, the NextPrep-Mag cfDNA Isolation Kit uses the magnetic bead binding method. Up to

5 ml of human serum or plasma can be extracted with the NextPrep-Mag Kit. The cfDNA isolation took place in three steps, namely protease digestion/cfDNA binding, wash, and elution.

Lyse and binding sample: The cfDNA was coated with proteins, lipids, and other contaminants. To remove these, the plasma sample was initially treated with proteinase K. At the same time, the cfDNAs bind on magnetic beads.

Cleaning step: The magnetic beads, which include the product, were attracted using a magnetic stand. The supernatant was gently discarded without disturbing the magnetic beads. To make sure that all matrix is removed from the product, four wash steps with two different buffers are required. The beads were resuspended with the wash buffer and the supernatant was discarded. This process step was repeated four times.

Elution of cfDNA from magnetic beads: After a couple of rounds of washing steps, the cfDNA was ready to be eluted. The elution volume is dependent on the plasma sample volume used. Large plasma sample volume requires more elution volume; for example, the elution volume of 5 ml plasma is 60 µl volume and 36 µl for 3 ml of a plasma sample. Decreasing the elution volume decreases the cfDNA yield, but the concentration increases. The elution process took 5 minutes of incubation at 55°C. In the end, the cfDNA eluate was transferred to a new tube by using a magnetic stand (PerkinElmer, 2019).

2.2 Detection of cfDNA concentration

Quantitation and sizing of cfDNA is a challenge, because of the low input concentration and short cfDNA fragments. During this bachelor thesis, methods like LabChip, Qubit, Nanodrop, and LiquID IQ with MassARRAY were used.

2.2.1 cfDNA concentration determination using LabChip GX

The LabChip GX Touch Nucleic Acid Analyzer instrument is an electrophorese in small, high performance automated format, an alternative to traditional gel electrophoresis. The LabChip DNA NGS 3K Assay is designed for a quantity of low input concentration cfDNA down to 0.5 pg/µl-50 pg/µl (fragment), 50 bp - 3000 bp and minimal sample consumption of 1 µl samples (Jun Yan, 2019).

There are three steps required to detect cfDNA concentration: chip and sample preparation and measurement in LabChip GX Touch.

Preparation of microfluidic chip: First, the Gel-Dye was prepared by adding 13 µl of DNA NGS 3K Dye Concentrate to the tube of DNA Hi Sens/NGS3K Gel Matrix. The Gel-Dye solution was spin filtered at 9200 rpm for 7.5 minutes to ensure a particle-free solution.

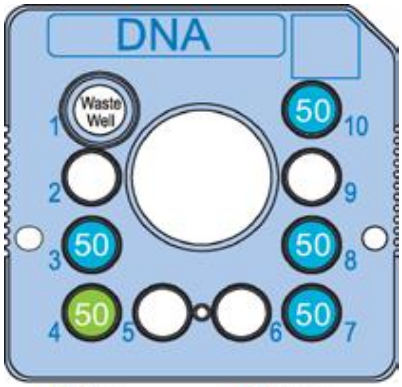

Figure 2. Microfluidic chip, layout for up to 48 samples throughput. Numbers from 1 to 10 are the number of wells. 1. Waste well. 3, 7, 8, and 10 are active well filled with 50 ul of Gel-Dye. Well 4 is filled with 50 ul of Marker. (DNA NGS 3K Assay Quick Guide, 2019)

Each active well (1, 3, 7, 8 and 10) is cleaned twice with water. The water was fully aspirated from each active well.

50 µl of Gel-Dye filled into wells 3, 7, 8, and 10 (Figure 2.) by using a reverse pipetting technique. Before placing the chip on the LabChip GX Touch, make sure that the Gel-Dye has no air-bubbles. That can disturb the measurement.

Preparation of cfDNA samples, Ledder, and Buffer: DNA NGS 3K Marker Booster was diluted 1:10 before use. In a 384-well plate, 1 µl of sample to 9 µl of a marker was added and was mixed ten times by pipetting up and down. The plate was centrifuged to ensure an air-bubble-free solution.

Sample Workflow: The 384-well sample plate was placed, along with other equipment such as LabChip, Ladder Tube (add 12 µl Ladder to 108 µl Marker), and Buffer Tube (750 µl of buffer or water) into the LabChip GX Touch (see Figure 3).

The prepared reagent quantities named above are sufficient for 48 samples throughput.

After the microfluidic LabChip, including Gel-Dye, was loaded in the LabChip GX Touch system, the chip's wells are linked with the platinum electrodes that enable current and voltage control. Approximately 150 nl of sample was used for each examination. Electrophoretically, each sample's analytes were separated into individual bands that were detected via

Figure 3. LabChip GX Touch, 1. 384-well plate (yellow), 2. microfluid LabChip (blue), 3. all six electrodes, 4. touch screen, 5. Ladder Tube, 6. Buffer Tube. (High Performance Electrophoresis for

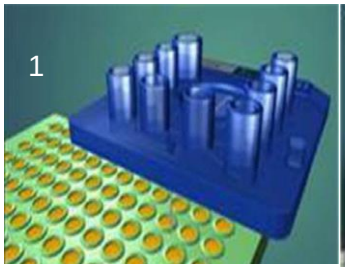

Figure 6.1. Systemic overview of the LabChip workflow, samples are aspired from microtiter plate (yellow) well onto the microfluidic LabChip (blue).

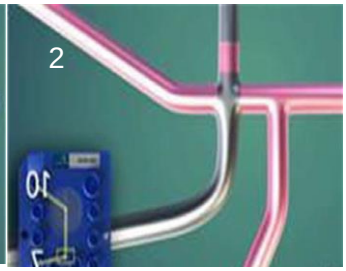

Figure 6.2. Systemic overview of the sample injection into a separation channel from well 10 to 7 in microfluidic LabChip.

Figure 6.3. Systemic overview of the laser induced fluorescence. Calculated the size and concentration of the sample analytes from the signal.

(**High Performance Electrophoresis for Genomics, 2019)**

laser-fluorescence. By using the ladder and internal markers, sizing and concentration for each band were determined (High Performance Electrophoresis for Genomics, 2019; DNA NGS 3K Assay Quick Guide, 2019).

2.2.2 cfDNA concentration determination using Qubit and NanoDrop

Qubit Fluorometer and NanoDrop 1000 are both instruments that measure the DNA concentration photometrically. Both instruments are designed to measure DNA in general, which means the assay cannot differentiate between gDNA and cfDNA. The length of the cfDNA fragment is approximately between 167 – 334 bp. LabChip and LiquID IQ Panel (used on MassARRAY System) are methods better suited to quantify cfDNA without DNA contamination.

Qubit 2.0 Fluorometer: Qubit is a benchtop fluorometer for the quantitation of RNA, DNA, and protein. A special dye is used that only fluoresces when bound to DNA, RNA, or Protein for ssDNA dsDNA to minimize the effects of contaminants in the sample that falsify the quantitation. The sample volume is between 1 µl and 20 µl with the quantitation range between 2 ng and 1000 ng.

Qubit 2.0 workflow: It is essential to use only thin-wall 0.5 ml tubes for standard and samples. DsDNA BR Assay requires two different standards that were used in the right order in the instrument. The dsDNA BR Reagent was diluted 1:200 with the dsDNA BR Buffer to create the working solution. The final volume of each tube was 200 µl. 10 µl of each standard was added to each 190 µl of the working solution. 1 µl of sample volume was added in 199 µl c working solution so that the final volume was 200 µl. Each prepared solution was vortexed for 2-3 seconds and incubated at room temperature for 2 minutes. The right setting profile was verified for the particular assay of interest; in this case, the right settings for the dsDNA measurement. The standard _1 and standard_2 were inserted in order to calibrate the instrument followed by the samples. The instrument showed the results in ng/µl (ThermoFischer, 2019).

Figure 7. Qubit 2.0 Fluorescence analytical instrument, demonstration of how to insert a 0.5 ml tube (including working solution) into the sample chamber. The display shows the calibration unit (The Chinese University of Hong Kong, 2019).

NanoDrop 1000 Spectrophotometer: The instrument measures 1 µl of samples directly at the device without using cuvettes. The NanoDrop spectrophotometer (full spectrum 220nm-750nm) uses a patented sample retention technology that applies surface tension. In contrast to the standard cuvette spectrophotometer, NanoDrop is capable of measuring 50 times higher concentration: up to 3700 ng/µl (dsDNA) without dilution.

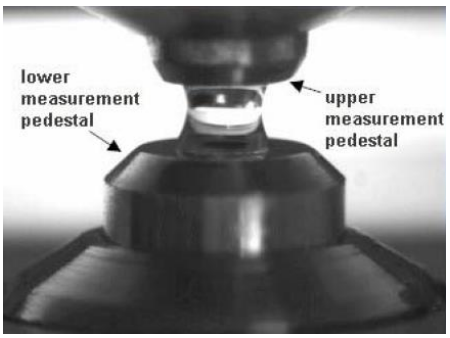

Figure 8. NanoDrop 1000, sample liquid between upper and lower measurement pedestal (Scientific, 2019).

The upper measurement pedestal was connected with a fiber optic cable, named the receiving fiber, and the lower measurement pedestal was connected with a second fiber optic cable named the source fiber. The sample liquid was in contact with both fiber optic cables by building a controlled gap of 1 mm and 0.2 mm between the fiber optic ends. The light source is a pulsed xenon flash lamp, and the spectrometer is a linear CCD array that analyzes the light after passing through the sample. The whole instrument is controlled by software that stores the results in a timestamped archive file on the Computer.

NanoDrop workflow: The sampling arm was opened and 2 µl of deionized water was pipetted to clean the upper and lower measurement pedestal. The sampling arm was closed and a spectral measurement started. After the measurement, the sample column between the upper and lower measurement pedestal was drawn automatically. The sampling arm was opened and the rest of the water wiped from both upper and lower measurement pedestals to clean the surface. The procedure was repeated until the result of the measurement was 0 ng/µl.

Figure 9. NanoDrop 1000 with open sampling arm with upper measurement pedestal (1), pipetting 1 µl sample onto the lower measurement pedestal (2). Cleaning the upper and lower measurement pedestal with soft laboratory wipe (3) (Scientific, 2019).

1 µl of sample was pipetted onto the lower measurement pedestal and the sampling arm closed to measure the sample. After processing each sample, it was required to clean the lower measurement pedestal with a water sample to avoid contamination (Scientific, 2019).

2.2.3 cfDNA concentration determination using the LiquID IQ Panel

The LiquID IQ Panel (Agena Bioscience, Inc, CA) not only allows the assessment of quality and quantity of cfDNA but also analyses the total amplifiable copies as well as any contamination with genomic DNA from white blood cells (gDNA). The assay also detects long DNA template from other sources such as necrotic tissue, infection, physical damage. Besides, the panel uses a sample ID method to indicate the gender of the sample.

The recommended cfDNA input into the LiquID IQ Panel is 1.5 µl from 50-100 µl (758-1515 copies) extraction. The assay will fail if the limit of sample quantitation is lower than 0.1 ng (30 copies equivalent input).

For the sample examination, five steps were required: PCR, SAP, extension, desalting, and measurement in the MassARRAY instrument. The PCR step was performed to increase the cfDNA amount. The SAP was a cleaning step which removes the dNTPs. The next step was the extension reaction, a single base reaction using dNTPs and gene-specific primers. Before spotting the sample of a SpectroCHIP, the sample was desalted with resin. After measurement in MassARRAY, the results were analyzed using MassARRAY TyperAnalyzer software (LiquID Panel Protocol, 2019).

2.3 UltraSEEK Lung Panel and MassARRAY

The MassARRAY System technology combined with the UltraSEEK Lung Panel can detect specific somatic mutations in liquid biopsy samples (Agena Bioscience Inc, CA). Mutations can be detected as low as 0.1% allele frequency from cfDNA. The panel identifies 67 mutations across *KRAS, ERBB2, BRAF, EGFR*, and *PIC3CA* genes (see Table 1).

Table 1. List of genes, their mutation number, and their codon position

Gene	Number of Mutations	Coverage
BRAF	4	Codon 469 of exon 11; codons 594, 600 of exon 15
EGFR	43	Exon 19 indels, exon 20 insertions, and substitution across exons 18, 19, 20, and 21
ERBB2	2	Exon 20 insertions
KRAS	14	Codon 12, 13, of exon 2; codon 61 of exon 3
PIC3CA	4	Codon 542, 545 of exon 9; codon 1047 of exon 20

The UltraSEEK somatic mutation analysis is based on the following six processes: PCR amplification, SAP treatment, single nucleotide targeted extension reaction, bead capture, clean-up, elution, and desalting. Subsequently, samples are transferred onto a SpectroCHIP

ARRAY and then loaded into the MassARRAY Analyzer 4, a Matrix-Assisted Laser Matrix-assisted Laser Desorption/Ionization - Time of Flight (MALDI-TOF) mass spectrometer. The data will finally be processed by the MassARRAY Typer and UltraSEEK software, which are generating reports of mutation calls (UltraSEEK Panels User Guide, 2018.

2.3.1 MassARRAY Analyzer 4

The MassARRAY Analyzer 4 (MA4) system consists of two components, the MALDI-TOF MS analyzer and the CPM (Chip Prep Module). The CPM dispenses resin into the sample wells and afterwards, transfers a few nanoliters of the analyte and 3-point-calibrant from the sample well onto the SpectroCHIP. The CPM can process up to two sample plates in one run. The prepared SpectroCHIPs are then transferred to the MALDI-TOF MS for measurement. Figure 10 shows in image A the microtiter plate 96-well, in image B two SpectroCHIP (96-format) on the chip holder, in image C the CPM deck with the appliance for the 3-point-calibrant above the chip holder (green circle) and the two analyte microtiter plates (red circle) and in image D the complete MassARRAY system. The three components showed in image C need to be placed in the MA4 before starting a run.

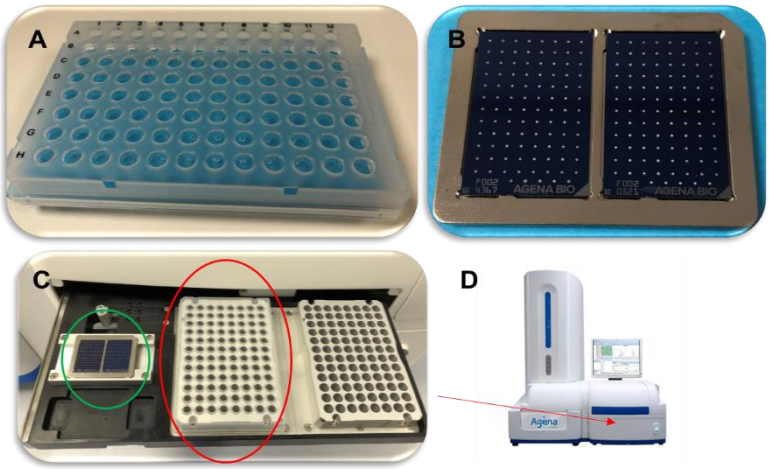

Figure 10. The image A shows a microtiter plate 96-well, image B shows two SpectroCHIP ARRAY (96-format, a silicon chip with pre-spotted matrix crystal) placed on a CPM chip holder. Image C shows the CPM deck with Chips (marked in green) and two microtiter plate (96 format) holders. The left holder is covered with a microtiter plate (marked in red). The image D shows the MassARRAY Analyzer 4.

The functionality of MALDI TOF

The MALDI TOF analyzer uses laser desorption assisted by a matrix to ionize the sample. The ionized sample is accelerated in an electric field; ions of different sizes will be separately detected by their time of flight. Particles with identical charge will carry identical kinetic energy,

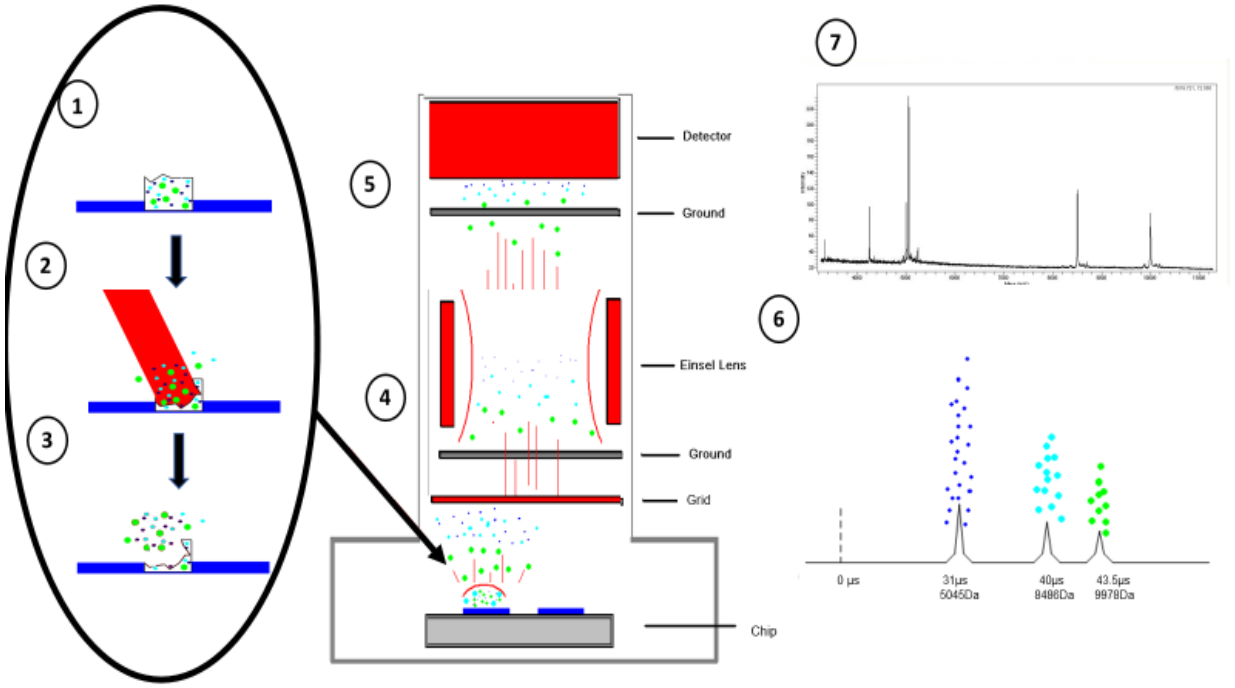

Figure 11. Schematic representation of MassARRAY Analyzer 4. Images 1-3 shows a laser beam (in red) fires on the patch. Image 4 shows how the Einzel lens is used to focus the analyte-molecules towards the detector and image 4. shows analyte-molecules arriving at the detector in order of their mass/charge. Images 6 and 7 shows a spectrum of the 3-point-calibrant. The peaks indicate the time of fight that translated into mass/charge.

therefore their velocities will depend exclusively on their masses. Figure 11 shows in 7 images the essential functions of MALDI-TOF.

At the steady-state, the MALDI-TOF has a constant vacuum, and the electric potential difference is zero.

1. A close-up shows the SpectroCHIP with its silicon base (blue). To the pre-applied matrix pad, the analyte is dispensed, forming a combination-crystal (patch).
2. A 337nm nitrogen laser fires on the patch so that the matrix co-crystallizes with the analyte after the evaporation of the solvent. The analyte-molecules are ionized by the laser-desorption. The matrix consists of small organic molecules that are absorbed at the laser wavelength. A proton is transferred from the matrix to the analyte molecule so that all ions have the same charge of +1.

3. An ion-cloud of positively charged analyte-molecules (+AM) forms above the chip surface. The chip and the grid each have 20kV. To create a potential difference of -3kV that accelerates the +AM, the grid drops down from 20 kV to 17kV. The electric energy is converted to kinetic energy. The +AMs with low mass are accelerating faster than the +AMs with high mass.
4. The +AMs pass the ground of 0 kV and enter the field-free drift region. In this region, the +AMs are not accelerating anymore, so that now they travel at speed based on their mass/charge. An Einzel lens is used to focus the +AMs on the detector.
5. Finally, the +AM is arriving at the detector in order by their mass/charge. +AMs with low mass are striking the detector grid first, followed by +AMs with high mass. The detector is converting the electrons into digital signals. The SpectroACQUIRE software processes the spectra and displays them.
6. A schematic representation spectrum of 3-point-calibrant. The peaks indicate the time-of-flight that translates into mass/charge. The Spectra shows the molecular mass (x-axis): the first peak, at 31µs (5045Da), the second peak at 40µs (8486Da), and the third peak at 43.5µs (9978Da).
7. The same 3-point-calibrant spectrum is acquired in the MA4. The x-axis shows the molecular mass per charge in Da, and the y-axis shows the number of analyte-molecules which have been detected at the detector. The amount of analyte-molecules is displayed as intensity.

2.3.2 UltraSEEK biochemistry and workflow

From the cfDNA sample to the analyte requires many steps such as PCR, SAP, Extension, bead capture, clean-up, elution, and desalting.

Preparing Sample plate

A 96-well microtiter plate was used for one UltraSEEK Lung Panel run. The plate has eight well (vertical A to H) for samples and 12 well (horizontal) for 12 multiplex reactions (see Figure 12).

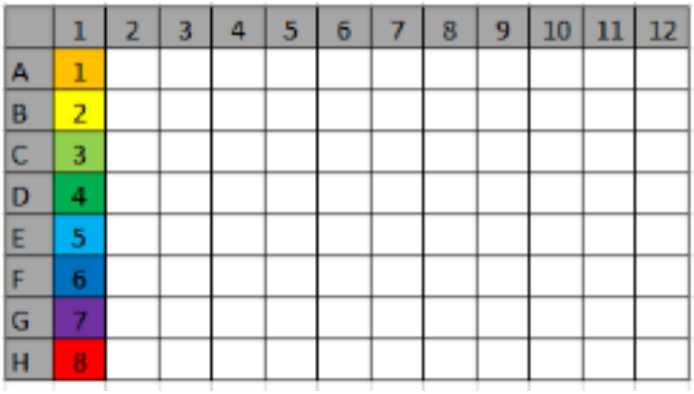

Figure 12. Sample plate layout for eight samples showed in different colors (A to H) (UltraSEEK Panels User Guide, 2018) .

Each UltraSEEK Lung Panel run included five samples, one positive control, one wildtype control, and one negative template control (NTC). The panel required ≥10 ng of cfDNA input in 35 µl volume.

Performing PCR Amplification

The panel is designed to run a global PCR to use less amounts of DNA input (10 ng, or at least 2000 amplifiable copies of cfDNA). In liquid biopsy samples, the yield of cfDNA is small; therefore, it is essential to run a PCR to increase the amount of the cfDNA. The PCR primers are designed to amplify the 67 mutations and their wildtype in the UltraSEEK Lung Panel. Besides, the panel includes quality controls that rate the DNA addition, PCR, and extension steps. Each channel, such as C termination, T termination, and G termination, includes one process control. A process control confirms the successful performance of the PCR reaction.

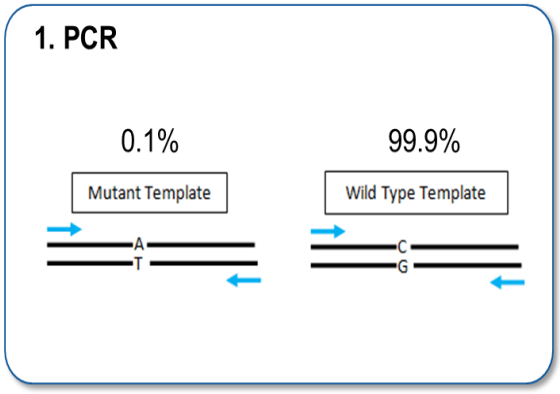

Figure 13. Schematic representation of PCR amplification. Primers are amplifying the mutation template and their wild type template. UltraSEEK panel capture 0.1% mutation of 99.9% wild type (UltraSEEK Panels User Guide, 2018).

The reagents for the PCR cocktail are listed in Table 2. 35 µl of this cocktail was added into 35 µl of the sample well, for a total PCR reaction volume of 70 µl. The sample plate was placed in a thermocycler for PCR reaction.

Table 2. Composition of PCR Cocktail for one reaction sample

Reagent	Per Well (µl)
HPLC-grade water	6.30
10x PCR Buffer	7.00
$MgCl_2$	2.80
dUTP/dNTP Mix	0.35
UNG Enzyme	1.75
PCR Enzyme	2.80
Global PCR Primer	14.0
PCR Cocktail Final Volume	**35.0**
Sample Volume	**35.0**
Total Volume	**70.0**

Performing SAP Amplification

The Shrimp Alkaline Phosphatase (SAP) treatment is a cleaning step. After the PCR reaction, the reaction mixture contains some free dNTPs, which would disturb the extension reaction in the next step. The enzyme SAP dephosphorylates free single dNTPs and converts them to dNDPs, making them inactive for the following extension reactions.

The SAP treatment follows the PCR reaction. The list of reagents is shown in Table 3. 28 µl of SAP cocktail was added to 70 µl of PCR reaction volume, for a final volume of 98 µl. The sample plate was placed once again in the thermocycler for SAP treatment.

Table 3. Composition of SAP Cocktail for one sample reaction

Reagent	Per Well (µl)
HPLC-grade water	21.42
SAP Buffer	2.38
SAP Enzyme	4.20
SAP Cocktail Final Volume	**28.0**

After the SAP treatment, the global PCR/SAP reaction volume of 98 µl was dispensed in a new 96-well microtiter plate. Each sample was dispensed (aliquots of 7 µl) 12 times (see Figure 14).

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	1	1	1	1	1	1	1	1	1	1	1
B	2	2	2	2	2	2	2	2	2	2	2	2
C	3	3	3	3	3	3	3	3	3	3	3	3
D	4	4	4	4	4	4	4	4	4	4	4	4
E	5	5	5	5	5	5	5	5	5	5	5	5
F	6	6	6	6	6	6	6	6	6	6	6	6
G	7	7	7	7	7	7	7	7	7	7	7	7
H	8	8	8	8	8	8	8	8	8	8	8	8

Figure 14. Layout of a new 96-well microtiter plate. The numbering corresponds to each sample and the colors represent eight different samples (UltraSEEK Panels User Guide, 2018).

Performing UltraSEEK Extension Reaction

After the global PCR performance, the extension reaction runs in 12 different multiplex reactions. The single base extension reaction targets residues within the amplified products. Each reaction includes process controls (T, C, or G) to confirm that the PCR performance was positive and five capture controls with different molecular weights to gauge the performance of the beads capture, clean-up, and elution steps. These capture controls are internal standards that make a statement about the quality of each sample well, classified as either

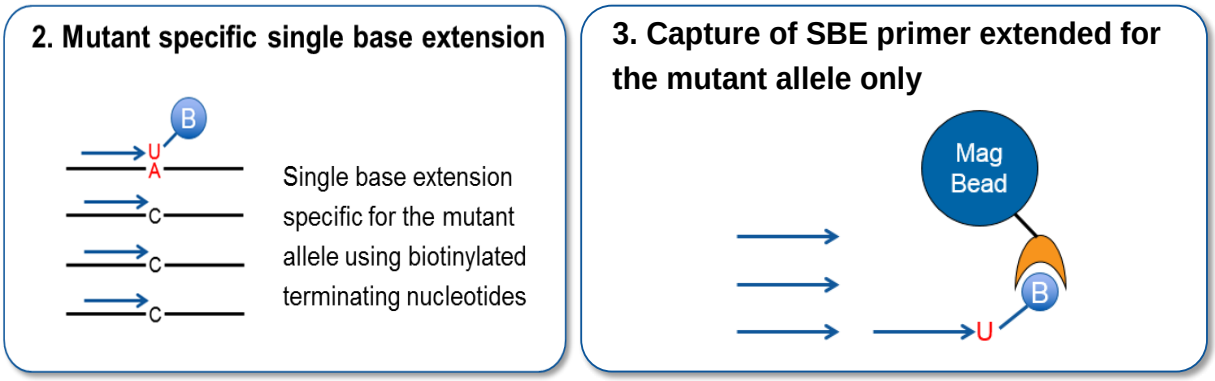

Figure 15. Image 2 shows mutation specific single base extension using biotinylated terminating nucleotide. Image 3 shows that only mutation allele is captured by a magnetic bead (UltraSEEK Panels User Guide, 2018).

good or poor/failed. If one or more of the assays fail, the sample well quality is considered poor/failed.

The following mixes were prepared for the extension reaction (Tables 4 and Table 5).

Table 4. Composition of Extension Master Mix for one sample reaction

Reagent	Per Well (µl)
HPLC-grade water	0.519
iPLEX Buffer Plus	0.200
iPLEX Pro Enzyme	0.041
UltraSEEK Capture Controls	0.100
Extension Master Mix Volume	**0.860**

Table 5. List of Extension Reaction Cocktail

Tube number	Extension Master Mix	Extention Primer	Termination Mix	Final volume per Well
Amount, µl	0.86	0.94	0.20	2.00
1	same	EXT W1	CG	
2	same	EXT W2	CG	
3	same	EXT W3	CG	
4	same	EXT W4	CT	
5	same	EXT W5	CT	
6	same	EXT W6	CT	
7	same	EXT W7	CT	
8	same	EXT W8	CT	
9	same	EXT W9	GT	
10	same	EXT W10	GT	
11	same	EXT W11	GT	
12	same	EXT W12	T	

Table 5 shows the number of reaction tubes (12), which was prepared for extension reaction. Each tube includes 0.86 µl of Extension Master Mix, 0.94 µl of Extension Primer and 0.2 µl of Termination Mix. 2 µl of this Extension Cocktail was dispensed into each 7 µl of PCR/SAP reaction, for a total volume of 9 µl. The sample plate was placed a third time in the thermocycler for Extension reaction.

The mutant specific allele is labeled with biotinylated terminating nucleotides. These biotin-labeled single base extensions are captured by magnetic beads, which are coated with streptavidin. Only the mutant specific allele with biotin terminations binds to the streptavidin; the wildtype does not. The extension reaction is a selection method to select mutant alleles from the wildtype. A magnetic stand is used to bind the magnetic beads (the analyte) so that

the matrix is washed out. An elution buffer solution with a high streptavidin concentration (biotin competition solution) and thermal treatment was used to free the analyte of the magnetic beads.

The extension reaction was followed by the magnetic beads capturing process. Before this process, the magnetic beads need to be washed out of the storage buffer and transferred into a new buffer so that the magnetic beads were ready to bind the analyte.

Each reaction well was aliquoted with 4.25 µl of washed magnetic beads. The sample plate was placed on the plate rotator to incubate at least for 30 minutes at room temperature.

After incubation, the matrix (everything that did not bind to the magnetic beads) was discarded by using a magnetic stand. The analyte that bound to the magnetic beads was washed with water to remove remnants of the matrix.

The elution buffer, including analyte, contains some salt components, which by creating salt adducts to the molecules of interest, will disturb the measurement in MassARRAY. Resin treatment was performed to desalt the analyte so that the analyte was ready to be transferred to the SpectroCHIP and measured in the MassARRAY system.

To free the analyte from the magnetic beads, the samples were eluted with a 13 µL streptavidin competition solution. For the elution process, the sample plated was placed a last time in the thermocycler. The sample plate was placed in MA4 to desalt the samples with resin, dispense the analyte onto the SpectroCHIP, and measurement in MALDI-TOF MS.

3. Results

3.1 cfDNA quality analysis using LabChip and LiquID IQ Panel

Quality analysis of cfDNA is an essential step before mutation detection. LiquID IQ Panel and LabChip DNA NGS 3K assays are specifically designed to quantify cfDNA qualitatively.

The LiquID IQ Panel analyzes WBC (white blood cell) contamination, presence of long DNA template. It also detects total number of amplifiable cfDNA copies and indicates the gender of the sample, using a sample ID method to exclude sample mix-ups.

The LabChip DNA NGS 3K assay analyzes the length of cfDNA fragments in bp and gDNA contamination. In addition, the LabChip analysis shows the difference between human cfDNA and synthetically manufactured cfDNA (see Figure 16).

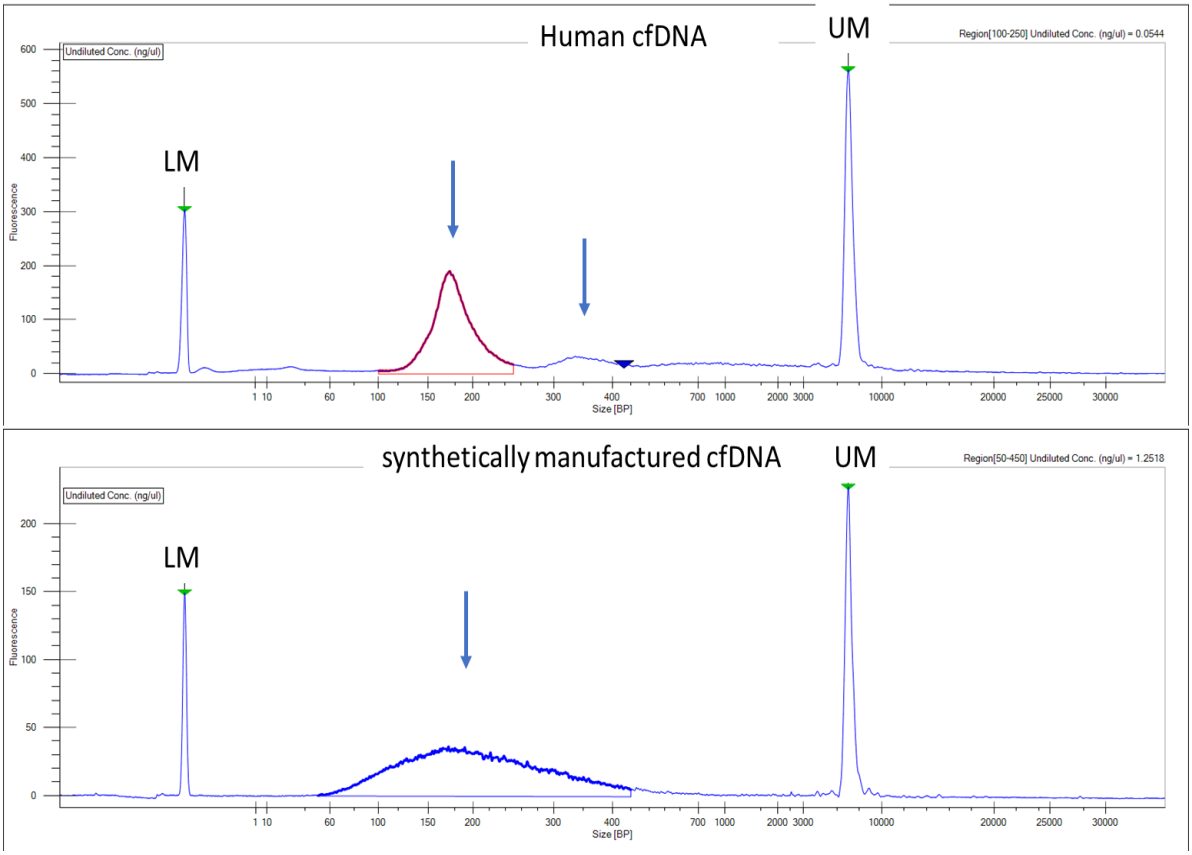

Figure 16. The Image show two diagrams of cfDNA, analyzed by LabChip. The diagram above shows a human cfDNA curve (marked in red) and the diagram below shows a synthetically manufactured cfDNA (marked in bold blue). Arrows indicate two individual peaks versus one broad peak. Lower marker (LM) and upper marker (UM) are internal standard. The LabChip software plots fluorescence intensity versus bp.

Lower marker (LM) and upper marker (UM) are two DNA fragments, which are run with each of these samples. LM and UM are internal standards used to align the samples with data from the ladder.

The human cfDNA peak has a spectrum of 100-250 bp fluorescence intensity of approximately 200 AU. The second peak, next to the cfDNA peak, is a double-sized cfDNA, with a peak spectrum of 290-410 bp and a fluorescence intensity of approximately 20 AU. The LabChip software calculates the amount of cfDNA without any contamination.

The synthetically manufactured cfDNA has a flat and broad peak with a spectrum of 60-410 bp and a fluorescence intensity of approximately 40 AU.

Eight samples of human cfDNA are analyzed by using the LiquID IQ Panel and LabChip DNA NGS 3K assay. The results are presented in Table 6.

Table 6. Quality analysis of cfDNA, liquid IQ panel (N=4) versus LabChip (N=1).

	LiquID IQ Panel						LabChip	Deviation, %
Sample	WBC Contamination	Long DNA Template	Gender	Amplifiable Copies	Conc., ng/µl	AVG, ng/µl	ng/µl	
sample_1	0%	negative	M	93	0,19	**0,19**	**0,37**	**9%**
sample_1	0%	negative	M	95	0,19			
sample_1	0%	negative	M	90	0,18			
sample_1	0%	negative	M	92	0,18			
sample_2	0%	positive	F	61	0,12	**0,11**	**0,15**	**2%**
sample_2	0%	negative	F	50	0,10			
sample_2	0%	negative	F	50	0,10			
sample_2	0%	negative	F	54	0,11			
sample_3	0%	positive	M	124	0,25	**0,26**	**0,24**	**1%**
sample_3	0%	positive	M	127	0,25			
sample_3	0%	positive	M	140	0,28			
sample_3	0%	positive	M	131	0,26			
sample_4	0%	positive	F	195	0,39	**0,37**	**0,44**	**4%**
sample_4	0%	positive	F	201	0,40			
sample_4	0%	positive	F	178	0,36			
sample_4	0%	positive	F	165	0,33			
sample_5	0%	negative	F	171	0,34	**0,35**	**0,83**	**24%**
sample_5	0%	negative	F	192	0,38			
sample_5	0%	negative	F	173	0,35			
sample_5	0%	negative	F	163	0,33			
sample_6	0%	positive	M	173	0,35	**0,34**	**0,36**	**1%**
sample_6	0%	negative	M	165	0,33			
sample_6	0%	positive	M	178	0,36			
sample_6	0%	negative	M	156	0,31			

sample_7	0%	negative	F	618	1,24	**1,24**	**0,59**	**33%**
sample_7	0%	negative	F	656	1,31			
sample_7	0%	negative	F	603	1,21			
sample_7	0%	negative	F	603	1,21			
sample_8	0%	negative	F	772	1,55	**1,59**	**2,48**	**45%**
sample_8	2%	negative	F	785	1,57			
sample_8	4%	negative	F	833	1,67			
sample_8	0%	negative	F	780	1,56			

Each of these eight samples has been processed four times by using the LiquID IQ Panel and one time by using LabChip. Results of previous independent studies analyzing replicate samples (N=x) showed the reproducibility of LabChip. Hence the samples have been analyzed once.

WBC contamination was detected only in Sample 8 (2/4). A long DNA template was detected in Sample 2 (1/4), sample 3, sample 4, and sample 6 (2/4).

Using the two different methods, the results of cfDNA concentration are mostly similar. Five out of eight samples showed a deviation value of ≤ 9%, and the other three out of eight showed a deviation value between 24-45%.

With one exception, samples measured with the LiquID IQ Panel shows fewer cfDNA concentration values than the samples measured with the LabChip.

After cfDNA quality analysis, next task is the verification of mutation detection using the UltraSEEK Lung Panel.

3.2 Verification of UltraSEEK Lung Panel using reference materials

The performance of the UltraSEEK Lung Panel was verified using commercial-grade reference materials. For this purpose, the SeraCare$_{TM}$ cfDNA Reference Material v2 Panel has been used. The reference material is diluted in human genomic DNA as background wildtype material spiked in tumor DNA. This panel contains the following mutations shown in Table 7 and allele frequencies (AF) that are expected to be detected by the UltraSEEK Lung panel (SeraCare, 2019).

Table 7. List of genes, mutations and allele frequencies in SeraCare ctDNA reference material (N=2)

Gene	Nucleotide (mutation)	Amino Acid	Range of AF, %				
BRAF	c.1799T>A	p.V600E	2.0	1.0	0.5	0.25	0.13
EGFR	c.2236_2250del15	p.E746_A750delELREA	2.0	1.0	0.5	0.25	0.13
	c.2573T>G	p.L858R	2.0	1.0	0.5	0.25	0.13
	c.2369C>T	p.T790M	2.0	1.0	0.5	0.25	0.13
ERBB2	c.2324_2325ins12	p.A775_G776insYVMA	2.0	1.0	0.5	0.25	0.13
KRAS	c.35G>A	p.G12D	2.0	1.0	0.5	0.25	0.13
PIK3CA	c.1633G>A	p.E545K	2.0	1.0	0.5	0.25	0.13
	c.3140A>G	p.H1047R	2.0	1.0	0.5	0.25	0.13

Six tubes of SeraCare (SC) reference material were used, with AF of 2.0%, 1.0%, 0.5%, 0.25%, 0.125% and a SC WT with concentrations of 10 ng/µl, each. Additionally, a haplotype map (HapMap) sample of the human genome with a concentration of 10 ng/µl was used as a wildtype control sample, and water was used as the negative template control (NTC) for this test.

Table 8 below shows the samples that were used for the UltraSEEK Lung Panel (96 well format) run. All samples have been processed in duplicate with a cfDNA input of 33.3 ng. Each plate includes five SC samples as positive controls, one SC WT sample, one HapMap WT, and one NTC sample.

The results of the verification trial are presented in Table 9 and Figure 16.

Table 8. Sample designation and cfDNA input for one UltraSEEK Lung Panel run (N=2)

well	Sample	cfDNA input, ng/µl
1	SC_2.0	33.3
2	SC_1.0	33.3
3	SC_0.5	33.3
4	SC_0.25	33.3
5	SC 0.125	33.3
6	SC_WT	33.3
7	HapMap WT	33.3
8	NTC	-

Table 9. Results of verification (N=2)

Gene (amino acid)	Sample	AF (Setpoint), %	AF (detected), %	Mutation call rate	Mutation call rate, %
BRAF(p.V600E)	SC 2.0 1&2	2.0	1,3	positive	**100%**
	SC 1.0 1&2	1.0	0,8	positive	
	SC 0.5 1&2	0.5	0,6	positive	
	SC 0.25 1&2	0.25	0,4	positive	
	SC 0.125 1&2	0.125	0,3	positive	
EGFR (p.E746_A750delELREA)	SC_2.0_1&2	2.0	1,9	positive	**90%**
	SC_1.0_1&2	1.0	1,5	positive	
	SC_0.5_1&2	0.5	1,2	positive	
	SC_0.25_1&2	0.25	1	positive	
	SC_0.125_1	0.125	failed	negative	
	SC_0.125_2	0.125	0,8	positive	
EGFR (p.L858R)	SC 2.0 1&2	2.0	1,4	positive	**90%**
	SC 1.0 1&2	1.0	1,1	positive	
	SC 0.5 1&2	0.5	0,8	positive	
	SC 0.25 1&2	0.25	0,7	positive	
	SC 0.125 1	0.125	0,5	positive	
	SC 0.125 2	0.125	failed	negative	
EGFR (p.T790M)	SC_2.0_1&2	2.0	1,1	positive	**80%**
	SC_1.0_1&2	1.0	0,8	positive	
	SC_0.5_1&2	0.5	0,5	positive	
	SC_0.25_1&2	0.25	0,3	positive	
	SC_0.125_1	0.125	failed	negative	
	SC_0.125_2	0.125	failed	negative	
ERBB2 (p.A775_G776insYVMA)	SC 2.0 1&2	2.0	1,4	positive	**70%**
	SC 1.0 1&2	1.0	1,1	positive	
	SC 0.5 1&2	0.5	0,1	positive	
	SC 0.25 1	0.25	failed	negative	
	SC 0.25 2	0.25	0,4	positive	
	SC 0.125 1	0.125	failed	negative	
	SC 0.125 2	0.125	failed	negative	
KRAS (p.G12D)	SC_2.0_1&2	2.0	1,1	positive	**70%**
	SC_1.0_1&2	1.0	1,1	positive	
	SC_0.5_1&2	0.5	0,5	positive	
	SC_0.25_1	0.25	0,2	positive	
	SC_0.25_2	0.25	failed	negative	
	SC_0.125_1	0.125	failed	negative	
	SC_0.125_2	0.125	failed	negative	
PIK3CA (p.E545K)	SC 2.0 1&2	2.0	0,8	positive	**90%**
	SC 1.0 1&2	1.0	0,5	positive	
	SC_0.5_1&2	0.5	0,4	positive	

PIK3CA (p.H1047R)	SC 0.25 1&2	0.25	0,2	positive	**100%**
	SC 0.125 1	0.125	0,2	positive	
	SC 0.125 2	0.125	failed	negative	
	SC_2.0_1&2	2.0	1,2	positive	
	SC_1.0_1&2	1.0	1,1	positive	
	SC_0.5_1&2	0.5	0,8	positive	
	SC_0.25_1&2	0.25	0,7	positive	
	SC_0.125_1&2	0.125	0,5	positive	

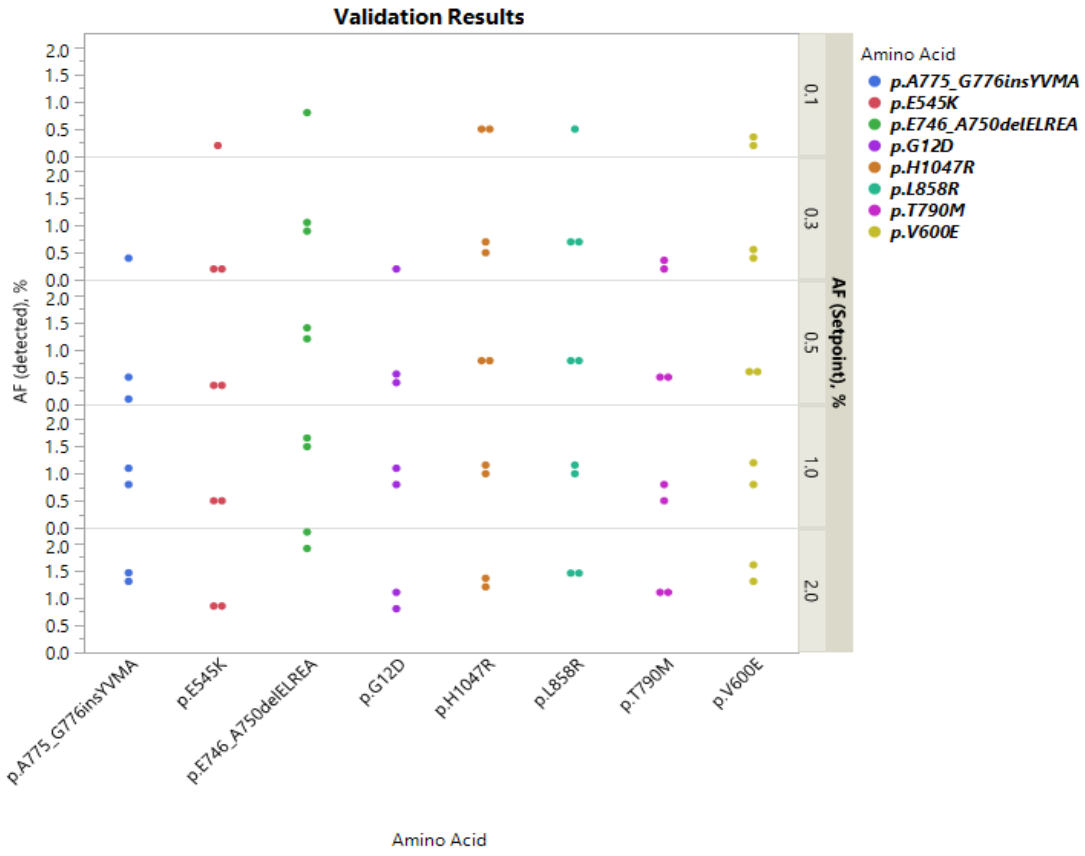

Figure 17. Graphic representation of verification results plotting AF detected value in percentage (left y-coordinate) per marker (x-coordinate). The coordinate on the left side shows the expected AF Setpoint in percentage.

Each sample was used twice. Table 9 shows the AF reference value (setpoint) in the SC samples and the AF, that was detected using the UltraSEEK Lung panel. The reference value deviation was different from sample to sample. The mutation call rate was also different from assay to assay, as outlined below.

- *BRAF*: The mutation call rate in this assay p.V600E was 100%, which means all mutations in the reference material panel have been detected.

- *EGFR*: This assay p.E746_A750delELREA has a call rate of 90%. The p.L858R assay also has a call rate of 90%. The p.T790M assay has a call rate of 80%.
- *ERBB2*: The p.A775_G776insYVMA assay has a call rate of 70%.
- *KRAS*: The p.G12D assay has a 70% call rate.
- *PIK3CA*: The p.E545K assay has a call rate of 90%. The pH1047R assay has a call rate of 100%.

3.3 Quality control of UltraSEEK Lung Panel with Ring Trial

Ring trial (RT) or comparison trial is a study that evaluates the performance, such as specificity, sensitivity, repeatability, and reproducibility of diagnostic methods. During an RT, identical sample materials are sent from a supervising laboratory to the participating laboratories. These laboratories are analyzing the samples using their routine methods and sending the results to a supervising laboratory.

Participation in a Ring Trial (RT) from the Reference Institute for Bioanalytics (RfB) along with the other 28 Laboratories was used to carry out a quality control of UltraSEEK Lung Panel. The RT includes nine blood plasma samples in three panels A, B, and C. Each panel includes three plasma samples with the following mutations:

- Panel A: *KRAS* codons 12 and 13 and wildtype
- Panel B*: BRAF* p.V600E and wildtype
- Panel C: *EGFR* p.T790M and wildtype

As *KRAS* mutations are not part of the UltraSEEK Lung Panel assay design, Panel A results were excluded for this bachelor thesis.

The Bioo Scientific Next-Prep-Mag cfDNA Kit was used to isolate cfDNA from plasma samples. For sample concentration determination, a NanoDrop 1000 Spectrometer was used. Each sample was eluted twice and each elution has been measured twice. Both elutions were collected in a 40 µl elution buffer. The results are presented in Table 10.

Table 10. cfDNA concentration determination of RfB samples (N=2)

	Elution I, ng/µl			Elution II, ng/µl			Collection Volume, µl
sample	1. Meas.	2. Meas.	Avg.	1. Meas.	2. Meas.	AVG.	
B1	20.2	19.1	19.7	11.3	11.8	11.6	40
B2	17.5	16.9	17.2	11.3	10.9	11.1	40
B3	20.6	19.4	20.0	12.0	11.5	11.8	40
C1	18.4	17.7	18.1	13.1	13.0	13.1	40
C2	22.7	20.2	21.5	12.7	11.9	12.3	40
C3	26.1	25.4	25.8	13.2	14.7	14.0	40

The cfDNA concentration for Elution I is between 17.0 - 25.8 ng/µl, and for Elution II, it is between 11.0 - 14.0 ng/µl.

Table 11 below shows the sample names and their respective cfDNA concentration that was used to run one UltraSEEK Lung Panel quality control and the results are presented in Table 12.

Table 11. Sample name and concentration cfDNA input for UltraSEEK biochemistry

Sample	cfDNA concentration, ng/µl
B1_E1	19.7
B1_E2	23.1
B2_E1	17.2
B2_E2	22.2
B3_E1	20.0
B3_E2	22.2
C1_E1	18.1
C1_E2	26.1
C2_E1	21.5
C2_E2	24.6
C3_E1	25.8
C3_E2	27.7
PC	20.0
HapMap WT	20.0
NTC	0

Table 12. Comparison of RfB Mutation Setpoint and recovery with UltraSEEK method (N=2)

Sample	RfB		UltraSEEK Lung Panel		Recovery detection	Setpoint deviation
	Mutation Setpoint	AF Setpoint, %	Mutation Detected	AF Detected, %		
B1	BRAF	1.0	BRAF	0.5	detected	50%
B2	BRAF	1.5	BRAF	1.0	detected	33%
B3	BRAF wild type	0.0	BRAF wild type	0.0	detected	
C1	EGFR wild type	0.0	EGFR wild type	0.0	detected	
C2	EGFR	0.5	EGFR	1.0	detected	100%
C3	EGFR	1.0	EGFR	0.7	detected	30%

All mutations and wildtype samples from the RfB were detected with the UltraSEEK Lung Panel.

BRAF (p.V600E): This mutation has been detected in sample B1 and B2, with AFs of 0.5% and 1.0%, which deviate from the reference values of the RfB which were expected to be 1.0% (deviation of 50%) and 1.5% (deviation of 33%). The *BRAF* wildtype has been detected in Sample B3.

EGFR (p.T790M): The presence of this mutation has been detected in sample C2 with an AF of 1.0% and in C3 with an AF of 0.7%. The RfB reference AF in sample C2 was expected to be 0.5% and in C3 1.0%; thus, the samples had deviations from the reference AF of 100% and 30%. The *EGFR* wildtype has been detected in sample C1.

Ring Trial Troubleshooting

During the RfB ring trial, unanticipated phenomena such as unexpected peaks were found in the spectral area, which initiated a troubleshooting session for further clarification. Closer observation revealed, that the unexpected peaks appear approximately 40 Da next to a mutation peak, but exclusively in some assays (see Figure 18).

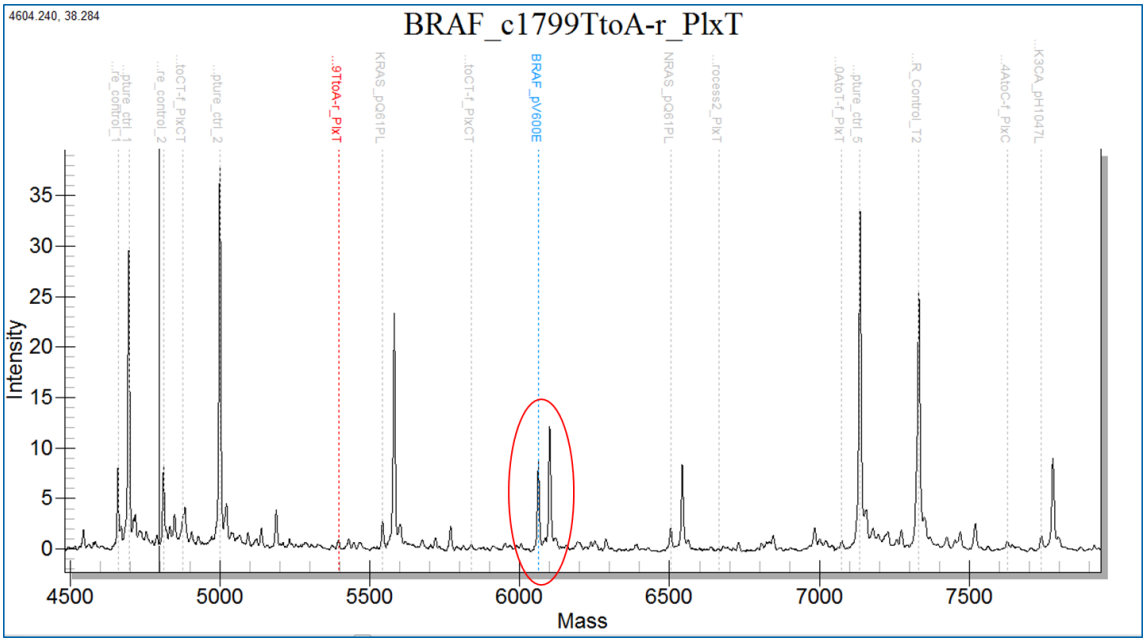

Figure 18. Mass spectrogram of *BRAF* (p.V600E) shows the expected mutation peak (blue), unexpected peak on the left side of mutation peak (circled in red) The Typer software plots intensity versus Mass in Da.

To investigate whether the UltraSEEK Lung Panel termination mixes could be the root causes of the unexpected peaks, a troubleshooting was performed by testing all available lots of termination mixes in comparison to each other. The terminators, C, G, and T have the following mass relations:

- **C to G = +40 Da**
- **C to T = +1 Da**
- **T to G = +39 Da**

To investigate whether it could be a G termination contamination in the C or T mixes that terminated the wildtype (unexpected peak), a detailed troubleshooting experiment was performed by using the following troubleshooting design (see Table 13).

Table 13. Troubleshooting with Termination mixes

Sample	Stop Mix	Stop mixes used in troubleshooting	W1-W2	W3	W4-W8	W19-11	W12
B1 B2	Mix_1	Existing top mixes which have been used for the ring trial	**CG**	**C**	**CT**	**GT**	**T**
B1 B2	Mix 2	New stop mixes prepared with **C1**, G, and T	**CG**	**C**	**CT**	**GT**	**T**
B1 B2	Mix_3	New stop mixes prepared with **C2**, G, and T	**CG**	**C**	**CT**	**GT**	**T**
B1 B2	Mix 4	New stop mixes prepared with **C3**, G, and T	**CG**	**C**	**CT**	**GT**	**T**
B1 B2	Mix_5	Existing stop mixes from another project	**CG**	**C**	**CT**	**GT**	**T**

Samples B1 and B2 have been used to perform the troubleshooting with different stop mixes. Mix_1 was used for the RfB samples in the first run and is suspected to be contaminated. Mix_2, Mix_3 and Mix_4 are prepared with the same G and T tubes but different C tubes (**C1, C2 and, C3**). Mix_5 is an existing mix from another project. The UltraSEEK Lung Panel extension reaction has 12 plexes (W1-W12). The stop mix CG is present in W1-W2, C in W3, CT in W4-W8, GT in W9-W11 and T in W12.

Table 14. Results of troubleshooting (N=5)

Sample		W1	W2	W3	W4	W5	W6	W7	W8	W9	W10	W11	W12
B1	Mix_1			x	x	x	x	x	x				
B2				x	x	x	x	x	x				
B1	Mix_2												
B2													
B1	Mix_3			x	x	x	x	x	x				
B2				x	x	x	x	x	x				
B1	Mix_4												
B2													
B1	Mix_5												
B2													
Stop mixes:		**CG**	**CG**	**C**	**CT**	**CT**	**CT**	**CT**	**CT**	**GT**	**GT**	**GT**	**T**

Table 15 shows the results of the troubleshooting experiment. The affected samples and assays are marked with an “x”. Samples B1 and B2 show unexpected peaks of +40 Da exclusively in Mix_1 and Mix_2 and only for Multiplex reactions W3-W8. As a conclusion, only C termination mixes which are not expected to include G were affected, suggesting that the C termination mix tube has been contaminated with the G termination mix.

3.4 UltraSEEK Lung Panel in a clinical feasibility study

Agena Bioscience GmbH has collaborated with a cancer research group in the UKE for the detection of mutations in blood plasma from seventeen patients (UKE_1 to UKE_17). The cfDNA concentration was determined with Qubit. The results of this study are presented in table 15.

Table 15. List of patient's plasma samples, cfDNA concentration, mutation AF and, mutation call rate (N=1)

Sample	cfDNA, ng	Mutation and AF, %										Mutation call rate
		KRAS_pG12RC	*KRAS_pG12AV*	*EGFR_pE746_A750del*	*KRAS_pG12D*	*EGFR_pL747_A750toP*	*EGFR_pE746_S752toV*	*PIK3CA_pE545K*	*BRAF_pV600E*	*EGFR_pT790M*	*EGFR_pL858R*	
UKE_1	10,4	**1.0**										positive
UKE_2	10,4		**0.3**									positive
UKE_3	10,4											negative
UKE_4	10,4			**3.0**								positive
UKE_5	10,4	**0.2**										positive
UKE_6	10,4											negative
UKE_7	10,4											negative
UKE_8	10,4				**0.1**							positive
UKE_9	**06,3**											negative
UKE_10	**06,9**					**0.4**						positive
UKE_11	**06,9**											negative
UKE_12	10,4	**0.3**					**0.3**					positive
UKE_13	**06,9**							**0.2**				positive
UKE_14	10,4											negative
UKE_15	10,4											negative
UKE_16	10,4											negative
UKE_17	**09,7**											negative
PK_SC_0.5%	17,0	**0.4**		**0.7**	**0.3**				**0.2**	**0.2**	**0.7**	positive
WT_SC	17,0											negative
NTC	00,0											negative

10 ng of cfDNA is the minimum requirement for the UltraSEEK Lung Panel to perform analysis.

- 5/17 UKE samples have less than 10 ng of cfDNA.
- 8/17 UKE samples were showing mutations in genes such as *KRAS, EGFR* and *PIK3CA*.

- 9/17 UKE samples were showing no mutation
- The positive control (PC_SC) sample was showing the expected mutations
- The wildtype (WT_SC) sample and negative control NTC were showing no mutation as expected.

4. Discussion

4.1 Quality analysis of cfDNA

Quality analysis of cfDNA is an important step before the detection of mutation. For this test, two different cfDNA have been used, human cfDNA samples, and synthetically manufactured cfDNA samples. These samples have been processed by using the LabChip DNA NGS 3K assay kit and measured by the LabChip GX Touch.

Results of the quality analysis are showing a clear difference between human cfDNA and synthetically manufactured cfDNA. The graph of the human cfDNA sample is showing two peaks. The spectrum of the first peak is between 100 bp and 250 bp (with a peak height of approximately 169 bp), and the spectrum of the second peak is 290-410 bp (with a peak height of approximately 338). This observation corresponds well with previous studies showing that the cfDNA length of the cancer patient is between 90–150 bp and 250–320 bp (Mouliere *et al*, 2018). Generally, the length of DNA that wrapped once around a nucleosome is approximately 167 bp, composed of 147 bp core DNA and 20 bp linker DNA (Hongde *et al*, 2008). During apoptosis, cfDNA fragments that appear in bloodstream are in range of 167-334 bp. Both peaks of human cfDNA confirm the length of a cfDNA that descended from the nucleosome.

In contrast to the human cfDNA peak, the peak of synthetically manufactured cfDNA is broad and flat, with a cfDNA fragment spectrum from 40 bp to 410 bp. The peak height is approximately 170 bp. Additionally, in comparison to humans cfDNA, the fluorescence intensity is five-time lower.

As mentioned in results, the reference material (synthetically manufactured cfDNA) is a tumor DNA fragmented by sonification. The sonification method is used to create DNA fragments in standard length using longer-wavelength acoustic energy. During the sonification procedure, a broad range of DNA fragments is manufactured to produce small amount of cfDNA (on average a length of 167 bp). The broader peak of cfDNA fragments and the smaller amount of cfDNA concentration are the reasons for the lower quality of synthetic cfDNA. (DW *et al*, 2006; McGinty *et al,* 2015). Despite this knowledge, the synthetically manufactured cfDNA is applicable for the verification of the UltraSEEK Lung Panel.

NanoDrop and Qubit are quantifying the whole amount of DNA, also the short cfDNAs fragment, which are not amplifiable. The length of the UltraSEEK PCR amplicons starts at 80 bp; therefore, DNA fragments shorter than 80 bp cannot be amplified by the PCR primers. This issue impacts the UltraSEEK Lung Panel ability to detect mutations. As mentioned, 10 ng of cfDNA is the minimum requirement for the UltraSEEK Lung Panel to perform analysis. A poor

quality of cfDNA (short cfDNA fragments) reduces the amount of cfDNA input and decreases the sensitivity of the mutation detection. In order to solve this issue, the amount of cfDNA input needs to be increased by 50-100%.

LiquID IQ Panel and LabChip DNA NGS 3K are two different tools for cfDNA quantification. Both methods are not just quantifying the cfDNA concentration but also displaying the range of the amplifiable DNA fragments.

Eight samples of human cfDNA have been analyzed by using the LiquID IQ Panel and LabChip DNA NGS 3K. The results of the cfDNA concentration of these two different methods are fairly similar. Five out of eight samples showed a deviation value of ≤ 9%, and the other three out of eight showed a deviation value of 24-45%.

Summarized, the results of quality analysis of the cfDNA study showed that the human cfDNA and synthetically manufactured cfDNA are having different characteristics. The peak spectrum of synthetically manufactured cfDNA is much broader than the peak spectrum of human cfDNA. Also, the peak intensity of synthetically manufactured cfDNA is five-time lower than peak intensity of human cfDNA. LiquID IQ Panel and LabChip are better suited to quantify cfDNA than NanoDrop and Qubit. However, the synthetically manufactured cfDNA samples are better suited for the next step, the UltraSEEK Lung Panel verification.

4.2 UltraSEEK Lung Panel verification

For the UltraSEEK Lung Panel verification, the SeraCare$_{TM}$ ctDNA Reference Material v2 panel has been used. This reference material is artificially produced cfDNA (spiked tumor DNA diluted in human genomic DNA). The SeraCare panel contains mutations of different allele frequencies.

Eight amino acids from five different genes have been used for this test. Each amino acid is presented in five samples (different AF), and each sample has been detected twice, which means that one amino acid has been detected ten times. The mutation call rate shows the detection rate of each amino acid.

The mutation call rate of gene *BRAF* (p.V600E) and *PIK3CA* (p.H1047R) is 100%, which means that all targeted mutations have been captured. *EGFR* (p.E746_A750delELREA) and (p.L858R) and PIK3CA (p.E545K) are showing a mutation call rate of 90%. Samples with low AF value (0.1-0.3%) are failing for those assays, resulting in a 10% error rate.

The recovery-rate at *ERBB2, KRAS,* and *EGFR* (p.T790M) is 70-80%, the samples with low AF value are the reason for the error rate. More data is needed to determine the individual limits of detection per marker of the UltraSEEK Lung Panel and to confirm its reliability as N=2 is not enough. Fortunately, the same experiment has been performed in five laboratories (independent from each other), with eight instruments, across the world. Thus, the data collection grows from N=2 to N=20 independent measurements to investigate the reliability of the UltraSEEK Lung Panel for mutation call rate. The results of this study show that the mutation call rate is dependent on assays and their AF levels. The mutation call rate of AF 2%, 1%, and 0.5% are in the range of 91-100%. The mutation call rate decreases (44-70%) with the decreasing of AF (0.1-0.3%). Assays like *BRAF* (p.V600E) and *EGFR* (p.E746_A750delELREA and p.T790M) are performing better even on lower AF (0.1-0.3%%) (Douglas *et al*, 2019).

The AF reference value deviation varied from sample to sample and from assay to assay. However, approximately 90% of all samples that had been run as “replicate” were recovered, which confirmed the general assay performance. The error rate of AF recovery is linear, which confirmed once again the reliability of the assay design.

To summarize, reference materials with low-level mutation (0.1 – 0.3%) have a high rate (44-70%) of false-negative results. However, it should be noticed that the reference materials are based on synthetic cfDNA. Even though SeraCare samples have been validated as reference materials by using NGS (Next Generation Sequencing) technique, they differ from true human cfDNA samples. Confirming “the good reason” for the fact that there is a clear difference between technical verification using artificial reference samples and the true validation of an analysis panel using real clinical samples.

4.3 UltraSEEK Lung Panel Ring Trial

The quality control of the UltraSEEK Lung Panel, for this study, has been performed by participating in a ring trial. The main goal of this participation was to evaluate the performance (specificity, sensitivity, repeatability, reproducibility) of the UltraSEEK Lung Panel. For this study, a Ring Trial (RT) from the Reference Institute for Bioanalytics (RfB), has been chosen.

The EQA (External Quality Assessment) scheme for ctDNA includes nine samples in three panels A, B and C, with three samples per panel. Each sample included 100 ng/ml of DNA, which was isolated from tumor cell lines with *KRAS, BRAF* and *EGFR* mutations. The tumor DNA was fragmented by sonification to match the nucleic acids to a similar size of DNA

fragments (cfDNA/ctDNA) that is present in the blood of tumor patients. This DNA was spiked in 3 ml of DNA-free human K3 EDTA plasma. The RfB samples material was set to different AFs of 1.5%, 1.0% and 0.5% and the goal was to detect mutations and determine the AF (Haselmann, 2019).

For cfDNA extraction, the Bioo Scientific Next-Prep-Mag Kit was used and the cfDNA concentration was determined using the NanoDrop 1000 Spectrometer. To increase the cfDNA yield, the samples have been eluted twice. The cfDNA concentration of the first elution (Elution I) varied between 17.0 ng/µl and 25.8 ng/µl and for the second elution (Elution II) between 11.0 ng/µl and 14.0 ng/µl. Due to double elution, the cfDNA yield increased from approximately 20.0 ng to 30.0 ng.

The UltraSEEK Lung Panel was carried out with approximately 20 ng of Elution I and Elution II samples (N=2).

The *BRAF* (p.V600E) mutation was detected in samples B1 and B2 and the corresponding wildtype in sample B3. Samples C2 and C3 showed *EFGR* (p.T790M) mutations and C1 showed the wildtype allele. The RfB report confirmed the ring trial test results to be correct. Hence, the UltraSEEK Lung Panel performance is verified and the panel is suitable for the clinical analysis.

During the UltraSEEK procedure, issues were identified that have led to troubleshooting.

The first run of UltraSEEK led to a troubleshooting investigation, resulting in a clear understanding and clarification of the underlying root cause. During data analysis of the RfB ring trial, unexpected peaks were observed in the spectrum. The peaks always appeared approximately 40 Da next to a mutation peak in assays, which have a cytosine (C) termination for mutation (wildtype G) or Thymine (T) termination for the mutation (wildtype G). To avoid wildtype calls the assays are designed covering twelve multiplexes with different termination mixes that only terminate the specific mutation allele. If an assay calls both mutation and wildtype, it means the termination tube includes a wrong terminator which calls the wildtype of the mutation. In this case, the tubes with the CG instead of C termination mix would lead to the wildtype call that has a G termination (C to G → +40 Da), and tubes with TG instead of pure T termination mix would call the wildtype with G termination (T to G → +39 Da). To investigate whether a whole termination lot was contaminated or whether it was just a handling issue such as pipetting error, a troubleshooting drafted with five different termination mixes was designed.

The results of troubleshooting clearly showed that there was a termination G (C to G) contamination. This issue has occurred in Mix_1, which was used for the ring trial samples and

Mix_3 which was compounded from a single C termination stock tube called C1. Thus, this particular C1 stock tube was contaminated with G terminator that cause unexpected peaks in the spectrum.

After a thorough worldwide complaint investigation, it has come to the conclusion that the observed error has only occurred when using this individual C1 stock tube in the Agena Lab in Hamburg, most probably due to a handling issue.

After the troubleshooting, the results of the ring trial were sent to the RfB, which confirmed the results to be correct by handing out a certificate. Thereby the UltraSEEK Lung Panel was verified for this study.

4.4 UltraSEEK Lung Panel clinical study

Agena Bioscience GmbH and UKE are members of the Cancer ID Consortium and are collaborating on studies to find new ways to track cancer (Cancer ID, 2015). This Bachelor thesis is contributing to The Brain Metastases project performed in collaboration with Agena Bioscience GmbH.

In contrast to the previous investigations described above, where synthetically manufactured cfDNA was used, real human cfDNA from 17 patients has been used for this clinical feasibility study.

A sample plate with eight sample wells, including five samples, one positive control, one wildtype control, and one NTC “blank”, has been designed to create reliable data.

Out of 17 samples, mutations have been detected in 9 samples. For the rest of the samples, no definitive conclusion could be drawn due to different potential scenarios, such as a healthy patient, or the level of AF being lower than 0.1% so that the UltraSEEK Lung Panel was not expected to capture anything or quality of cfDNA samples was below limit of detection.

For more reliable data, a sample analysis should optimally be carried out in duplicate or triplicate. This requires a large amount of cfDNA, which is critical. The amount of cfDNA occurring in blood samples is very low, which reduces the possibility of performing a statistically significant data analysis. For this study, a small amount of cfDNA was made available, which limited the number of tests.

The UltraSEEK Lung Panel requires a cfDNA input of 10 ng per replicate. Five of the UKE samples could not even fulfill this requirement. The cfDNA quantity of UKE samples was only

enough for a single test run. UltraSEEK Lung Panel detects mutation in patient samples even though the cfDNA concentration in some samples was lower than the panel recommendation. The detected mutations are likely to be positive, as confirmed by the control samples which were run with each examination plate.

To summarize, mutations in more than half of the samples have been detected with the UltraSEEK Lung Panel, even though the required amount of 10 ng cfDNA concentration was not always fulfilled. The mutations detected are likely to be positive, but it cannot be confirmed due to the limited amount of cfDNA available. All control samples confirmed that the UltraSEEK Lung Panel procedure was successful.

5. Conclusion and Outlook

Lung cancer is the most commonly diagnosed cancer type and responsible for 1.8 million deaths worldwide in 2018. An early diagnosis of lung cancer is difficult because the symptoms are only apparent when lung cancer is at an advanced stage. Low-dose computed tomography is the only recommended screening tool to diagnose lung cancer, and it also has certain limitations such as false positive results, overdiagnosis, and radiation exposure. Besides, to identify the type of tumor for further treatment, a risky surgical procedure for tissue biopsy is required.

In contrast to tissue biopsy, liquid biopsy is a minimally invasive method requiring only a blood sample to perform early diagnosis of cancer. Circulating tumor DNA (ctDNA) is one of the biomarkers that appear in small amounts, 0.01 percent in the bloodstream. Ultra-sensitive detection techniques are required to capture, identify, and detect the ctDNA.

This study aimed at detecting mutation in the blood of lung cancer patients. An experimental approach was designed on how to process the patient's samples. The design included a cfDNA quality investigation, validation of the detection method (UltraSEEK Lung Panel) using reference material, comparison of the results with other independent laboratories by participating in a ring trail, and finally, the processing of clinical samples.

The results of the cfDNA quality analysis study show that human cfDNA and synthetically manufactured cfDNA are different in quality. The synthetically manufactured cfDNA is heavy fragmented which indicates a lower product quality. cfDNA quantification methods such as LiquID IQ Panel and LabChip are able to show these quality differences. Therefore, these methods have been favored instead of NanoDrop and Qubit. Despite this knowledge, however, the synthetically manufactured cfDNA remains suitable for the verification of the UltraSEEK Lung Panel.

The sensitivity of the UltraSEEK Lung Panel using reference material (synthetic cfDNA) is higher with allele frequencies (AF) ≥0.3%. The detection rate of mutations is optimal in these conditions. Consequently, the number of false negative results is increasing with decreasing AF levels. It should be noticed that manufactured cfDNA is of lower quality than real human cfDNA. However, the reference materials are applicable for the UltraSEEK Lung Panel verification test.

Participating in a ring trial was an appropriate opportunity to evaluate the performance of the UltraSEEK Lung Panel with other detection methods such as Real-time PCR, sequencing,

BEAMing, Digital PCR, and other kits. The results of this trial verified once again the UltraSEEK Lung Panel performance for this study. With the UltraSEEK Lung Panel passing all preliminary tests, real patients' samples were ready to be analyzed.

Seventeen samples of lung cancer patients were analyzed with the UltraSEEK Lung Panel. Seven samples showed no mutation. Nine samples showed mutations in genes such as KRAS, EGFR, and PIK3CA. The detected mutations were expected to be positive as confirmed by the control samples. But due to the small amount of cfDNA that was made available, it was not possible to apply a second or triple analysis of the samples in order to confirm the mutation detection results. Furthermore, comparisons with pathological results of those tissue samples are necessary to make a statement on the accuracy of the mutation detection.

The UltraSEEK Lung Panel has shown to be suitable to detect mutations (ctDNA) of lung cancer patients with blood samples with an AF as small as 0.1%. Larger clinical studies are needed to strengthen the current UltraSEEK Lung Panel results, so in future, liquid biopsy could be considered as an officially recommended screening tool to diagnose lung cancer and to monitor its treatment.

The results of this study are handed out to colleagues in UKE to continue the Brain Metastases Study, which will be released in a research paper.

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