Targeted Oncogene Mutation Detection in NSCLC by Multiplexed Array Mass Spectrometry Genotyping: Results of a Clinical Validation Study

By

Phillip Shepherd

A thesis submitted to Auckland University of Technology in fulfilment of the requirements for the degree of Master of Medical Laboratory Science.

Health & Environmental sciences, Applied Sciences, Master of Medical Laboratory Science

March 2017

Abstract

Cancer is the second leading cause of death in New Zealand and despite being the fifth most commonly diagnosed cancer, lung cancers are the leading cause of cancer related deaths and one of the major health issues affecting New Zealand (Ministry of Health, 2016).

The relatively recent discovery of cancer driver genes and somatic mutations in lung cancer patient sub-groups has seen the emergence of genetic abnormalities being used in the treatment of lung cancer as predictive bio-markers and molecular targets for targeted cancer therapy especially in Non-small Cell Lung Cancer (NSCLC) (Mitsudomi & Yatabe, 2010).

There are several different technologies available for the detection of Epidermal Growth Factor Receptor (EGFR) mutations in NSCLC. The Roche Cobas EGFR gene mutation test and Agena Mass Array OncoFocus gene mutation assay are two examples of these technologies. There is very little in the literature in relation to the cross validation of these technologies. The aim of this thesis was to provide this validation data while also ensuring the clinical utility of such testing.

The study consisted of 532 patients that were both tested by using the Roche Cobas EGFR gene mutation test and retested using the Agena Mass Array OncoFocus gene mutation assay.

To evaluate the diagnostic accuracy of this novel genetic testing strategy, data was analysed by agreement analysis. Overall survival and progression free survival after gefitinib or erlotinib treatment were analysed with the Kaplan–Meier method to assess the time to death or progression. Cox-regression was used for multivariate survival analysis. A log-rank test was employed to compare cumulative survival in different groups. All P-values were two-sided and a P-value <0.05 was considered statistically significant.

There was moderately high agreement between the Cobas EGFR Mutation Test and the Agena Mass Array OncoFocus assay for the detection of EGFR mutations in this study.

There was a very high level of agreement between the Cobas EGFR Mutation Test and the Agena Mass Array OncoFocus assay for the identification of specific EGFR mutations in patient samples that were mutation-positive in both assays.

The clinical validity of the assay was established by demonstrating high levels of overall agreement in the detection of EGFR mutations compared to a reference assay.

This study has demonstrated the clinical validity and utility of the Agena Mass Array OncoFocus assay for detecting EGFR mutations in tumour specimens from NSCLC patients.

In addition to ERFR mutations the Agena Mass Array OncoFocus assay identified a large number of patient samples with KRAS, NRAS and BRAF mutations that were not tested for by the Cobas EGFR Mutation Test. These mutations were also found by the study to have clinical relevance.

In conclusion the validity of the Agena Mass Array OncoFocus assay for detecting not only EGFR but also KRAS, NRAS & BRAF mutations in NSCLC tumour specimens, and the potential clinical usefulness for prediction of prognosis and clinical benefits from EGFR-TKI treatment has been demonstrated.

Aims

1. To use statistical tools to determine and evaluate the diagnostic accuracy of the Agena Mass Array OncoFocus assay in comparison to the Roche Cobas EGFR Gene Mutation assay.
2. To use survival data in comparison with the clinical testing results to assess predictive and prognostic value of the Agena Mass Array OncoFocus assay.
3. To establish a viable method for using the Agena Mass Array OncoFocus assay in a working molecular diagnostic laboratory.

Acknowledgements

Firstly I would like to thank Professor Mark McKeage and Dr Yan Li for their supervision, time and enthusiasm. Without their wisdom, guidance and support throughout the course of my studies it would have been near impossible to accomplish this thesis.

I would also like to thank Professor Don Love, Mrs Karen Sheath and the molecular genetics team at LabPlus, Auckland District Health Board, for the hard work done providing the samples for retesting and their support and advice in regards to the Roche Cobas molecular results.

In addition I would also like to thank Ms Prashannata Khwaounjoo for all her assistance with data sorting and for the positive encouragement to complete the technical analysis on time.

Finally I would like to thank Nick, my friends and family for the unconditional support and love they have provided during this time. I couldn't have asked for better and look forward to seeing more of everyone again.

Table of Contents

Attestation of Authorship

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgements), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.

Signature:  *Date:* ***15/04/17***

List of Figures

List of Tables

Abbreviations

°C – Degree Celsius

% - Percent

ALK – Anaplastic lymphoma kinase

BRAF – v-Raf murine sarcoma viral oncogene homolog B

Cobas z 480 analyser – Instrument used to run the Roche Cobas EGFR gene mutation assay

ddNTP – Dideoxynucleotide

dNTP – Deoxynucleotide

DNA – Deoxyribose Nucleic Acid

EGFR – Epidermal Growth Factor Receptor

FDA – Food and Drug Administration

FFPE – Formalin Fixed Paraffin Embedded

g – Gram

gDNA – Genomic DNA

HER2 – Human epidermal growth factor receptor 2

HPLC grade water – High Performance Liquid Chromatography grade water

KRAS – Kirsten rat sarcoma viral oncogene homolog

L – Litre

MALDI-TOF – Matrix-assisted Laser Desorption/ionisation – Time of Flight

MAP Kinase – MAPK pathway also known as the Ras-Raf-MEK-ERK pathway

MEK – Mitogen-activated protein kinase kinase

MET – Tyrosine-protein kinase Met

MTP – Micro titre plate (384 well PCR plate)

mL – Millilitre

n – Sample number

n/a or N/A – Not Applicable

NanoDrop – UV/visible light Spectrophotometer

nM – Nano Molar

NMD – No Mutation Detected

NRAS – Neuroblastoma RAS viral oncogene homolog

NSCLC – Non-Small Cell Lung Cancer

NTC – Non-Template Control

PFS – Progression Free Survival

RET – Rearranged during transfection

ROS-1 – Proto-oncogene tyrosine-protein kinase ROS

RT-PCR – Real Time - Polymerase Chain Reaction

SAP – Shrimp alkaline phosphatase

SD – Standard Deviation

SNP – Single Nucleotide Polymorphism

Spectro Chip – Mass Array chip with matrix pads, for use in the Agena Mass Array system

TKI – Tyrosine Kinase Inhibitor

µg – Microgram

ng – Nano gram

UV light – ultra Violet Light

Chapter 1

Introduction

1 Introduction

Cancer is the second leading cause of death in New Zealand and one of the major health issues affecting the population, new approaches to diagnosis and treatment are required. In the 2013 year there were 22166 new cancer registrations (Ministry of Health, 2016). For females the most common sites were breast followed by colorectal and melanoma. For males the most common sites were prostate followed by colorectal, trachea, bronchus and lungs. Of these new cases 2037 cases were classified as lung cancer, ICD-10 codes C33-C34. 1005 of these were female and 1032 male. There is also data for Māori vs non-Māori with Māori appearing over represented based on ethnicity as a percentage of population. Māori accounted for 421 of the 2037 cases with a wider disparity between male and female, where females were significantly more affected by the disease, accounting for 233 of the 421 cases (Ministry of Health, 2016). Despite being the fifth most commonly diagnosed cancer, lung cancers are the leading cause of cancer related deaths in New Zealand accounting for approximately 20% (Ministry of Health, 2015). In addition to the proportionately high mortality rate lung cancers have poor survival outcomes with one year survival sitting marginally above 30% and 5 year survival sitting near 10% in the 2010-2013 years (Ministry of Health, 2015).

A relatively recent emergence in the treatment of lung cancer is the discovery of cancer driver genes and somatic mutations in lung cancer patient sub-groups (Mitsudomi & Yatabe, 2010), and the clinical use of these genetic abnormalities as predictive bio-markers and molecular targets for targeted cancer therapy. The Epidermal Growth Factor Receptor (EGFR) gene was the first cancer driver gene to be clinically exploited in personalised lung cancer targeted therapy (Pao *et al*., 2004).

To date detection methods for driver mutations in the EGFR gene have been largely limited to Sanger sequencing, RT-PCR based methods or more recently, expensive Next Generation Sequencing based methods. The pace of testing and gene analysis has not kept pace with

drug discovery. This is highlighted where initial EGFR tyrosine kinase inhibitor drug treatment, with gefitinib or erlotinib, showed limited (Shepherd *et al*., 2005) or no therapeutic efficacy (Herbst *et a*l., 2004) (Giaccone *et al*., 2004) (Herbst *et al*., 2005) (Gatzemeier *et al*., 2007) in international randomised clinical trials undertaken in unselected lung cancer populations. However, occasional lung cancer patients were observed to have major therapeutic responses in these clinical trials and genetic analyses of their tumour DNA led to the discovery of lung cancer driver EGFR somatic mutations in 2004 (Lynch *et al*., 2004) *(Pao et al*., 2004) (Paez *et al*., 2004) (Huang *et al*., 2004) (Kosaka *et al*., 2004). Work elsewhere then showed the incidence of EGFR mutation positive lung cancer to vary widely between different ethnic groups and geographical regions from about 10 to up to 50% when expressed as a proportion of the total population diagnosed with lung cancer of any type (Sharma, Bell, Settleman, & Haber, 2007).

A subsequent series of randomised clinical trials, which were restricted to lung cancer patients demonstrated to have tumours harbouring EGFR gene mutations, then showed beneficial therapeutic efficacy for EGFR tyrosine kinase inhibitors relative to standard chemotherapy (Mok *et al*., 2009) (Maemondo *et al.*, 2010) (Zhou *et al*., 2011) (Rosell *et al*., 2012) (Mitsudomi *et al*., 2010). This prompted a move in international standard oncology practice towards undertaking EGFR gene mutation testing prior to systemic therapy in advanced lung cancer patients, and selecting an EGFR inhibitor treatment or standard chemotherapy for those with or without EGFR gene mutations, respectively (Keedy *et al*., 2011).

Currently, no international consensus exists about a preferred method for detecting EGFR gene mutations in lung cancer patients. A wide range of different genotyping assays have been reported and various opinions about their relative merits have been expressed. The Roche Cobas EGFR gene mutation test for detecting exon 21 L858R substitution and exon 19 deletion mutations was the first EGFR gene mutation test to gain FDA or equivalent regulatory approval (Laine, 2013)). The fast pace and high numbers of genetic markers available in other detection methods may be a barrier to other molecular companies seeking regulatory approval. The FDA process, for example, is laborious and costly adding significant barriers to its uptake in regard to medical testing.

Direct sequencing was originally the historical standard but has now been superseded by other techniques developed over the last decade for detecting oncogenic somatic mutations in clinical samples (Ellison *et al.*, 2013). In addition, direct sequencing has the disadvantage of low sensitivity for detecting mutant EGFR alleles in the presence of excess wild type DNA, as often occurs in clinical tumour biopsies due to the presence of normal tissue components (Angµlo *et al*., 2012).

1.1 Other lung cancer genes

Recent advances in the scientific understanding of lung cancer have led to the development of new models for lung cancer treatment using genotype-directed targeted therapies (Garraway, 2013). At a molecular level, mutated lung cancer genes are now known for being critical for initiating and driving the progression of the disease (Network, 2014). Lung cancer genes, such as ALK, BRAF, EGFR, HER2, KRAS, MET, NRAS, RET and ROS-1, acquire somatic mutations via nucleotide substitutions, insertions, deletions, amplification or by gene rearrangement (Network, 2014). These mutated oncogenes then encode activated mutant oncoproteins with constitutive and unregulated protein kinase activity, which drives aberrant downstream signalling and phenotypic changes in cells characteristic of cancer (Reungwetwattana, Weroha, & Molina, 2012). Blocking this pathogenic kinase activity with drug inhibitors, can suppress aberrant signalling, and induce tumour cell death and durable tumour responses in clinical trials (Reungwetwattana *et al*., 2012).

International standard oncology practice guidelines now recommend undertaking genetic testing prior to systemic treatment of patients with advanced lung cancer (Keedy *et al.*, 2011). Genetic testing enables the identification of key genetic drivers of lung cancer in individual patients and selection of the most appropriate targeted drug (Garraway, 2013). According to data published in 2014 (Kris *et al*., 2014), genotype-directed targeted therapy of advanced lung cancer can achieve survival outcomes that are unprecedented compared to those achieved by standard treatments not selected by genotyping. In the Lung Cancer Mutation Consortium study (Kris *et al*., 2014), multiplexed genotyping was used to test for lung cancer

driver mutations across ten different genes to enable clinicians to select targeted therapies or enrol patients in clinical trials. Fifty percent of metastatic lung cancer patients with EGFR and ALK gene mutations lived four or more years after enrolment in this study (Kris *et al.*, 2014), which is far superior to the survival outcomes achieved in New Zealand lung cancer patients (Ministry of Health, 2015).

Further technological developments have enabled the clinical implementation of these new models of lung cancer healthcare. An expanding formulary of clinically tested and approved targeted drugs is becoming available for treating specific genetically-defined subtypes of lung cancer. For example, targeted therapies are now available for treating lung cancers driven by mutated ALK (crizotinib, ceritinib) (Shaw, Kim, *et al*., 2014) (Shaw *et al.*, 2013), BRAF (dabrafenib) (Planchard *et al*., 2013), EGFR (erlotinib, gefitinib) (Maemondo *et al*., 2010) (Rosell *et al*., 2012), HER2 (trastuzumab) (Cappuzzo, Bemis, & Varella-Garcia, 2006), MET (crizotinib) (Ou *et al*., 2011), RET (cabozantinib) (Drilon *et al*., 2013) and ROS-1 (crizotinib) (Shaw, Ou, *et al*., 2014). Previously, all lung cancer patients were treated with the same chemotherapy regimen irrespective of the specific mutation driving their individual lung cancer. To screen for hundreds of mutations across numerous lung cancer genes simultaneously in small tumour samples, large-scale high-throughput multiplexed genotyping assays have been designed and developed (Thomas *et al*., 2007) (Dias-Santagata *et al.*, 2010). These assays have been evaluated for their technical accuracy, reliability, clinical validity and utility (J McKeage, Shepherd, Yozu, & R Love, 2013). With these developments it is now possible to envisage the introduction of a broad range of genotype-directed targeted therapies for treating lung cancer in New Zealand in the near future.

1.2 Genes targeted using the OncoFocus panel

A new laboratory test for detecting oncogenic mutations in clinical tumour specimens, the Agena Mass Array OncoFocus assay, was the subject of the research described in this thesis. There are four common oncogenes whose mutations are detected by the OncoFocus panel. Each of these oncogenes have been reported in associated with Non-small Cell Lung Cancer (NSCLC). These are BRAF, EGFR, KRAS & NRAS.

1.2.1 v-Raf murine sarcoma viral oncogene homolog B (BRAF)

Mutant BRAF has been implicated in the pathogenesis of several cancers, including NSCLC. BRAF is a member of the serine-threonine protein kinases family that includes ARAF, BRAF, and CRAF (RAF1). RAF kinases in the Mitogen-activated protein (MAP) kinase signalling cascade are fundamental mediators and exercise their effect predominantly through phosphorylation and activation of MEK. This happens after dimerization (hetero- or homo-) of the RAF molecules. Given the significance of the MAP kinase pathway, RAF is involved in multiple cellular processes, including cell proliferation, differentiation, and transcriptional regulation (C Lovly, L Horn, & W Pao, 2015a). In NSCLC somatic mutations in BRAF occur in approximately 1-4% of all presenting cases where the predominant presentation is adenocarcinoma (C Lovly, L Horn, & W Pao, 2015b).

1.2.2 Epidermal growth factor receptor (EGFR)

The epidermal growth factor receptor (EGFR; ErbB-1; HER1) is a cell surface receptor and is part of a complex cell signalling pathway that results in gene transcription and cell cycle progression (cell proliferation, prevention of apoptosis, angiogenesis, migration, adhesion & invasion), mutations affecting the expression or activity of the EGFR gene can result in cancer (Yarden & Schlessinger, 1987).

A specific ligand binding in the Ligand-binding domain in the extracellular space activates EGFR by transition from an inactive monomeric form to the active homodimer and starts the signalling cascade (Yarden & Schlessinger, 1987). The dimerization of EGFR activates the intracellular protein tyrosine kinase activity, this leads to autophosphorylation of several tyrosine residues. The stimulation of this autophosphorylation results in the downstream activation signalling proteins. These in turn initiate several signal transduction cascades which then lead to DNA synthesis and cell proliferation. Figure 1 below gives a schematic overview of this process (Oda, Matsuoka, Funahashi, & Kitano, 2005).

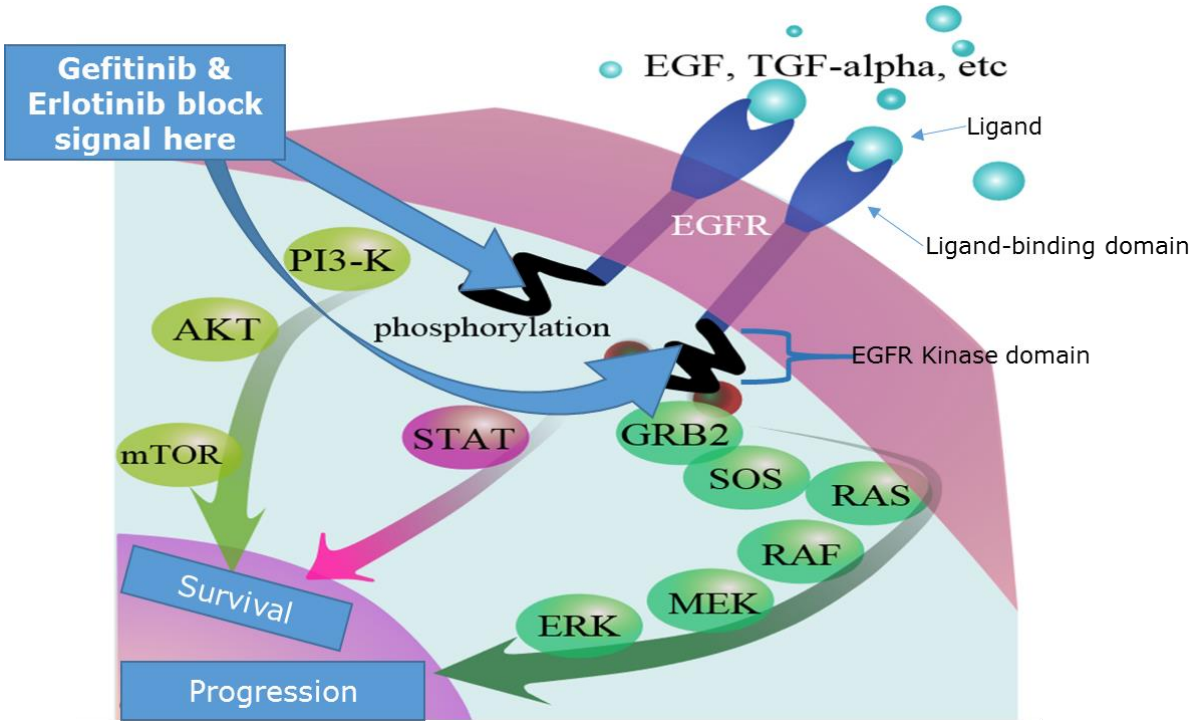


Figure 1 Schematic showing the EGFR transmembrane protein and the signal transduction cascades that are activated by it. The blue arrows depict the point at which gefitinib and erlotinib block the signal transduction, therefore preventing downstream cascade activation and ultimately cellular proliferation (Eikuch, 2007).

1.2.3 RAS - Kirsten rat sarcoma viral oncogene homolog (KRAS), Harvey rat sarcoma virus (HRAS), Neuroblastoma RAS viral oncogene homolog (NRAS)

Three different human RAS genes have been identified: KRAS (Kirsten rat sarcoma virus), HRAS (Harvey rat sarcoma virus), and NRAS (first isolated from a human neuroblastoma). The different RAS genes are highly homologous but functionally distinct (Goodsell, 2001). RAS proteins are small GTPases which cycle between inactive guanosine diphosphate (GDP)-bound and active guanosine triphosphate (GTP)-bound forms (C Lovly, L Horn, & W Pao, 2015d). RAS proteins are positioned downstream of growth factor receptor signalling and are central to this process. This makes them key in the process of cell proliferation, survival, and differentiation, Figure 1.

RAS is implicated in the pathogenesis of NSCLC. The activating mutations within the RAS gene result in constitutive activation of the RAS GTPase, where by this process is able to proceed even in the absence of growth factor signalling, resulting in a sustained cell proliferation signal (Lovly *et al*., 2015d).

1.3 Treatments in lung cancer

Surgical resection is a viable treatment option depending on the type and stage of the tumour and whether it has metastasised or not. If surgical resection can remove the entire tumour then this is the treatment option of preference. Where the entire tumour cannot be removed or if surgery is not an option then chemotherapy, radiotherapy or targeted drug therapies become the treatment option of preference (Mitsudomi & Yatabe, 2010). Non-surgical treatment options have progressed over time, in the late 1960's single agent chemotherapy was associated with a response in lung cancer patients (Mok *et al*., 2009). However it wasn't until the 2000's that targeted therapy was used, initially with sporadic benefit. It wasn't however until 2004 that the link between patients responsive to the tyrosine kinase inhibitor gefitinib and the EGFR pathway was discovered (Paez *et al*., 2004). In New Zealand it took until 2009 for gefitinib to be approved in the treatment of NSCLC harbouring an activating EGFR mutation.

1.3.1 Broad vs targeted therapy

Using targeted therapy in the treatment of lung cancer refers to using agents specifically designed to selectively target molecular pathways know to enable the proliferation of the lung cancer malignant phenotype. As a positive consequence of this targeted approach there is usually less negative affect to surrounding normal tissue.

Traditional chemotherapy drugs used for the treatment of cancer have activity that is particularly nonselective and can adversely affect surrounding tissue or bodily organs. The exact mechanisms of action for each chemotherapy drug are varied and complex and can be highly variable among patients for which they are administered, however they generally work by causing damage to cells undergoing mitosis, because increased cellular division is more prevalent in malignant cells than in normal tissue there is a generalised targeting of tumour cells (Corrie, 2008). Targeted therapies are drugs designed to be specific and selective in their effects by modulating the activity of proteins necessary and essential for oncogenesis and maintenance of cancer, in particular these target enzymes driving the unchecked growth, angiogenesis, invasiveness, and metastasis characteristic of malignant cancers. The increased specificity of their activity usually results in fewer negative side effects for patients receiving these drugs, particularly less of the common side effects of traditional anti-cancer drugs such as nausea, vomiting, and death of cells in the bone marrow and gastrointestinal tract. Having a more targeted therapy also leads to an increase in effectiveness against cancer cells (Sawyers, 2004).

1.4 Roche Cobas EGFR gene mutation test

LabPlus medical laboratory, at Auckland City Hospital, began offering publicly-funded EGFR gene mutation testing using a Roche Cobas EGFR gene mutation test platform from the 1st August 2012. In a ten-month period up to 31st May 2013, the laboratory had received a total of 318 referrals for EGFR gene mutation testing of samples from patients with lung cancer. The study described in this thesis used the Roche Cobas EGFR gene mutation test as a reference for the evaluation of the clinical performance of the Agena Mass Array OncoFocus Assay in regards to its detection of EGFR gene mutations in clinical samples from New Zealand lung cancer patients under local testing conditions. In addition to EGFR, the Agena Mass Array OncoFocus assay is capable of detecting mutations in the BRAF, KRAS, and NRAS genes. In this way, the study may verify whether the Agena Mass Array OncoFocus assay, currently in place at the University of Auckland, Faculty of Medical & Health Sciences, has suitable performance characteristics for use in a national coordinated testing programme, when applied in a local context. The OncoFocus panel, assayed on the Agena Mass Array, has been used extensively in research studies however this cross validation will be critical for confirming its potential for clinical use in the New Zealand medical laboratory space.

Chapter 2

Clinical Validation Studies

2.1 The Roche Cobas EGFR gene mutation assay

2.1 Introduction

DNA extraction and analysis using the Roche Cobas EGFR gene mutation assay was carried out at LabPlus, Auckland City Hospital, and results made available for this thesis. An overview of the extraction and analysis procedure carried out by LabPlus is provided below. DNA for the Roche Cobas EGFR gene mutation assay was extracted from unstained formalin fixed paraffin embedded (FFPE) slides, with a corresponding marked up H&E stained slide, which was then used in downstream analysis. The extracted DNA was used in PCR amplification and in the detection of target DNA using complementary primer pairs and oligonucleotide probes labelled with fluorescent dyes. The Roche Cobas EGFR gene mutation assay was designed to detect 41 mutations in exons 18, 19, 20 and 21, with the highly relevant deletions and complex mutations within exon 19 and L858R in exon 21 of the EGFR gene well covered.

Mutation detection was achieved through Real-time PCR analysis with the Cobas z 480 analyser. A mutant control and negative control were included in each run to confirm the validity of the run.

2.1.1 Specimen Preparation

A Roche Cobas DNA extraction kit was used to isolate tumour DNA from the supplied FFPE tissue. This was a manual sample preparation kit based on nucleic acid binding to glass fibres. Where possible a deparaffinised 5µm section of FFPE tissue was lysed by incubation at an elevated temperature with a protease and catotropic lysis/binding buffer that released the DNA while protecting the released DNA from DNases. Isopropanol was added to the lysis mixture that was then centrifuged through a column with a glass fibre filter insert. The DNA

was bound to the surface of the glass fibre filter allowing unbound substances, such as salts, proteins and other cellular impurities, to be washed through. The bound DNA was washed and eluted with an aqueous solution. The DNA was then spectrophotometrically determined and adjusted to the working concentration before being added to the amplification/detection mixture.

2.1.2 PCR Amplification

Target Selection

In order to detect relevant and specifically targeted mutations, the Roche Cobas EGFR gene mutation assay uses primers with specific base-pair sequences. For the exon 19 deletion mutations, considered to be particularly relevant, base pair sequences ranging from 125 to 141 are targeted while for the equally clinically relevant exon 21 L858R substitution mutation, a 138 base pair sequence is targeted. The Roche Cobas EGFR gene mutation assay employs an internal control, in exon 28, which targets an 87 base pair sequence.

The Roche Cobas EGFR gene mutation assay amplifies and detects mutations only in the regions of the gene between PCR primers, the entire EGFR gene is not amplified.

Target Amplification

For Targeted DNA amplification in the Roche Cobas EGFR gene mutation assay a DNA polymerase is utilized. Steps associated with conventional PCR are followed. Utilising a thermal cycler the PCR reaction mixture is heated to a temperature able to denature double stranded DNA exposing each single strand, forming the DNA template. When the reaction mixture is cooled again, the target specific forward and reverse primers anneal to the target DNA sequences within the single stranded DNA. The DNA polymerase is then, in the presence of magnesium and excess dNTP, able to extend each annealed primer, this extension replicates the template copy forming an identical DNA strand. This newly formed DNA strand is then available for use in the next cycle of PCR in which previous steps are repeated, with each full cycle doubling the amount of amplified DNA sequence. Only DNA targeted by the primers is amplified resulting in a large increase in the sequence of interest.

Automated Real-time Mutation Detection

The Roche Cobas EGFR gene mutation assay uses real-time PCR for detection of the targeted mutations within the amplified sequences. Each probe in the target specific reaction is labelled with a florescent dye that functions as a reporter in the reaction. This allows detection of each mutation target in the reaction. The reporter probe also contains a quencher molecule that retards any fluorescent emission from the reporter dye while it is an intact probe. During each amplification cycle, if a probe complementary to the single stranded DNA sequence, it binds and is subsequently cleaved by 5' to 3' nuclease activity of the DNA polymerase thus allowing the reporter molecule to exhibit fluorescence un-retarded. The fluorescence produced from this reaction is of a specific wavelength and can be measured when the reporter dye is excited by the required spectrum of light. There are three different reporter dyes used to label the mutations targeted by the Roche Cobas EGFR gene mutation test. Amplification of the targeted EGFR sequences is then achieved by independently detecting the three characteristic wavelengths across the three reaction wells for each sample. This is done using dedicated optical channels within the Cobas z 480 instrument.

Selective Amplification

Samples must be selectively amplified in the regain of the amplification target, this is achieved in the Cobas EGFR mutation test by the use of a specific enzyme. This is an AmpErase enzyme (uracil-N-glycosylase) and deoxyuridine triphosphate (dUTP). The AmpErase enzyme is able to recognise and catalyse the destruction of DNA strands containing deoxyuridine but not DNA containing thymidine. This method works on the principle that Deoxyuridine is not present in naturally occurring DNA but is always present in a synthesised amplicon owing to the use of dUTP in the Master Mix reagents in place of deoxythymidine triphosphate as one of the nucleotide triphosphates. This allows only for the synthesis of amplicons containing deoxyuridine. Consequently this incorporation of the Deoxyuridine renders the amplicon susceptible to destruction by the AmpErase enzyme prior to target amplification of the DNA. The AmpErase enzyme which is included in the Master Mix reagents catalyses the cleavage of deoxyuridine-containing DNA at the deoxyuridine residues by opening the deoxyribose chain at the C1-position. Subsequently when the amplicon is

heated in the first thermal cycling step at an alkaline pH, the amplicon DNA chain breaks at the position of the deoxyuridine, thereby rendering the DNA non-amplifiable. The AmpErase enzyme is inactive at temperatures above 55ºC preventing it from destroying the target amplicon in further raised temperature thermal cycling steps (Roche, 2013).

2.1.3 DNA Extraction from FFPE Samples using the Roche Cobas method.

Tissue Requirements

For optimal DNA extraction from FFPE tissue samples, the starting material was freshly cut sections of FFPE tissue (air exposed block surfaces were discarded where possible), each with a thickness of between 2-4 microns.

This was done using standard Histological sectioning techniques.

Total tumour area per slice was ideally 1 cm^2. At least 3 slides (or tubes) were made for each sectioning depth allowing the option of H&E staining to confirm tumour cells are present in the representative tissue.

Transport and Storage of Tissue Samples

Owing to the fact FFPE tissue is formalin fixed they can be transported and stored at room temperature. Sample tissue slices were however protected from contamination after sectioning by either being placed directly in a micro centrifuge tube or if slide mounted, the slide was placed into a protective case. Tubes and/or slides were clearly, accurately and uniquely identified.

Extraction Kit principle

The ability to extract DNA from these samples provides the potential for correlating disease state and tissue morphology with genotype. Extraction of DNA from FFPE tissues historically has been a challenge because the formalin fixation process results in cross-linking between proteins and DNA, as well as between different strands of DNA (Roche, 2013). The non-optimal preservation of genomic DNA in FFPE complicates its use in many standard downstream analysis applications. The Roche Cobas FFPE gDNA extraction kit uses incubation conditions optimized to partially reverse this cross-linking without the need for an overnight digestion (Roche, 2013).

The cross-linking introduced by the formalin fixation and paraffin embedding process results in nucleic acids that are partially degraded. The degree of DNA fragmentation will vary

depending on sample type, the age and storage conditions of the sample as well as the conditions used during formalin fixation. When designing downstream amplification assays, best results will be achieved when targeting regions of 200 nucleotides or less.

2.1.4 Quality Control

The eluate (suspended in DNase free water) produced by the extraction kit were quantitated by Nanodrop. Ideally the concentration was above 30 ng/µl and 260/280 ratio above 1.7 and 260/230 ratio above 1.3. The FFPE DNA Extraction Kit was function-tested by assaying for a PCR product from DNA extracted from a slide-mounted, FFPE tissue slice. Due to the unpredictable nature of FFPE tissue the above quality was not always be reached.

2.1.5 Contaminating Activity

The FFPE DNA Extraction kit reagents are known to be free of detectable RNase, exonuclease, and endonuclease activities as indicated on the package insert.

2.1.6 DNA Recovery

The yield of extracted DNA varied by tissue type, size, and preservation method. Approximately 25 ng/µl of DNA was obtained per square centimetre of tissue section. It is accepted that nucleic acids isolated from paraffin-embedded tissues are generally of poor quality.

2.2 The Agena Mass Array OncoFocus gene mutation assay

2.2 Introduction

The Agena Mass Array OncoFocus assay by Agena Bioscience was chosen for assessment in this study above other platforms on the market largely due to its ability to accommodate a high level of assay multiplexing, with the ability to easily interrogate multiple gene mutations simultaneously on low DNA input volumes, with decreased hands on time. The Agena Bioscience Mass Array system employs sensitive and robust chemistry coupled with advanced data analysis software to meet the diverse needs of a diagnostic molecular laboratory. Previously platforms detecting these clinically relevant gene mutations have largely been limited to single gene analysis. In an instance where a clinician requires a panel of genes in lung cancer for example EGFR, KRAS & ALK testing, then multiple assays would currently be run requiring increased amounts of limited DNA and vastly increasing the turnaround time. Most gene assays can be completed over a few hours however current testing is routinely batched to increase reagent efficiencies. This batching leads to lengthy increases in turnaround times or vast increases to the per sample testing cost. Agena Mass Array is a very specific method for detecting clinically significant mutations even at low allelic frequency. Mass Array is also comparable in sensitivity to other technologies such as NGS and is significantly more sensitive than Sanger sequencing, the currently accepted gold standard (Kriegsmann *et al*., 2015a).

The OncoFocus Panel employs two multiplexed PCR reactions, requiring 80 ng of input DNA per sample. Amplification is followed by a single base extension reaction. The extension products are then dispensed onto a Mass Array chip and detected via MALDI-TOF mass spectrometry on the Mass Array System. After the sample analysis on the Mass Array, an advanced software package interprets the acquired spectra and provides a list of any positive mutations identified in the samples. The entire workflow, from DNA to results, can be completed in a single 8-hour day, with less than 40 minutes of hands on time.

2.2.1 OncoFocus method

Genetic mutations resulting in the activation of oncogenes increase the probability that a normal cell will develop into a tumor cell. The Agena Mass Array OncoFocus Assay Panel provides a highly sensitive group of assays for the detection of over 200 oncogenic mutations in four clinically significant oncogenes, namely EGFR, KRAS, NRAS and BRAF.

Workflow Overview

- Purify sample and control DNA from FFPE, fresh tissue or cell line of choice. This is carried out in a dedicated DNA Extraction lab.
- Prepare DNA Working Dilutions.
- PCR Amplify DNA. PCR preparation is carried out in a Pre-PCR lab
- Clean up the PCR reaction with a SAP master mix. Post PCR processes are carried out in a post-PCR lab.
- Process the OncoFocus Extend reactions.
- Dispense the OncoFocus Extend reaction products to a SpectroCHIP array.
- Analyze samples on the Mass Array Analyzer instrument.
- Generate an OncoFocus assay report. Processed using Typer 4 software.

Reagent and Consumable Requirements

Assay Reagents

- PCR master mixes (2 vials).
- SAP master mixes (1 vial)
- Extend master mixes (12 vials)
- SpectroCHIP II 384 array chip
- CLEAN Resin

Consumables

- Un-skirted 96 well PCR plate
- Fµll - skirted 384-well Reaction Plate
- Clear adhesive sealing film-PCR CS/100 (for PCR cycling)
- Sealing film, non-sterile
- Sealing roller tool
- Single and multichannel pipettes 0.5, 10, 20, 200, 1000 µl (2 sets pre- and post-PCR)
- Repeater pipettes
- Combitips Plus 0.1 ml or 0.5 ml (2 sets pre- and post-PCR)
- Filtered pipette tips (10, 20, 300, 1000 µl)
- Micro tubes (0.5, 1.5, and 2 ml)
- Water, nuclease free high quality
- DNA AWAY
- 100% ethyl alcohol

Work areas

The laboratory space must include three separate (non-contiguous) work areas to prevent cross contamination from PCR products. These should be:

1. DNA extraction area, where nucleic acids are extracted from the supplied primary sample. Dilution of the stock elution to working concentration is also carried out within this area.
2. Pre-PCR area, where Master Mix is made up and template is added to the PCR master mix.
3. Post-PCR area, for all post PCR processing, including Extension reaction preparation.

Isolation of DNA

The OncoFocus Assay can be performed on genomic DNA isolated from fresh tissue, frozen tissue, formalin-fixed paraffin-embedded (FFPE) tissue, or cell lines. DNA isolated from FFPE samples is usually of lower molecular weight than DNA from fresh or frozen samples. The degree of fragmentation depends on the type and age of the sample and the conditions used for fixation. Quality checks using the Sample ID panel prior to analysis using the OncoFocus panel will assess the suitability of each sample irrespective of its origin.

DNA for the assay comparison validation study described in this thesis was provided by the Molecular Genetics department at LabPlus, as extracted DNA from FFPE tissue that had previously been in analysis by the Roche Cobas gene mutation assay. DNA for this section of the thesis was already extracted using the Roche Cobas extraction method described above.

2.2.1 OncoFocus Method

PCR Amplification

Genomic DNA was amplified from the sample using OncoFocus PCR primers that have specific base pair sequences targeting and amplifying mutations only in the regions between the PCR primers. This reaction is carried out in a 96-well plate in a final reaction volume of 30 µl. The sample DNA and PCR cocktail mixes were manually dispensed individually into the PCR plate.

Preparation

Prior to performing pre-PCR cycling work the bench areas, pipettes, and work areas were cleaned with a dilute solution of sodium hypochlorite, followed by a 70% ethanol wipe down. The Pre-PCR hood was irradiated with UV light for not less than 30 minutes prior to use. These steps set to ensure there is no environmental contaminates that could affect the steps that followed. Next all tubes and plates that contained reagents or samples were vortexed and centrifuged before proceeding to the next step in the protocol. When not in use, all plates were sealed with adhesive PCR sealer and stored at the 4 degrees if same day processing was to occur, plates were stored at -20 ^{o}C if any delay in processing was expected. All reagents were returned to their storage temperatures immediately after use to ensure assay integrity. Throughout all steps in the procedure gloves were worn and changed as required.

PCR Primer Master Mix and sample addition

The PCR master mix was made up based on sample number in the batch with a 20% over hang to allow for reagent loss in pipette tips and tubes. The following x1 recipe was used as the baseline x sample number + 20%. Two identical master mixes were made up, one for each primer set in the PCR assay.

Well 1	x1
H_2O	18.4 µl
Buffer	4 µl
Magnesium	3.2 µl
dNTP's	0.8 µl
Enzyme	1.6 µl
Primer **w1**	8 µl
Total Vol	36 µl

Aliquots of well 1 and well 2 PCR master mix (for OncoFocus) were used on the day they were made up. Prior to use, the master mix was mixed well by brief vortex and brief centrifuge. 26 µl of master mix was dispensed into each well (A1-A2) of a 96-well plate. This was repeated for each additional sample, sample 2 was in row B1-B2 and so on, including and additional NTC row. 4 µl of Sample 1 DNA was then pipetted into wells A1 – A2, and repeated in wells B1-B2 for the second sample and so on. A non-template control was set up as the last sample in each batch. The MTP plate was then sealed with film and vortexed briefly to mix, before a brief centrifuge.

PCR

Thermal cycling was performed using the following conditions:

94.0 °C	2 minutes	
94.0 °C	30 seconds	45 Cycles
56.0 °C	30 seconds	
72.0 °C	1 minute	
72.0 °C	5 minutes	
4.0 °C	Hold	

SAP Treatment

After amplification, any remaining free deoxynucleotides in the amplification reaction mixture needed to be dephosphorylated to prevent interference with the iPLEX Pro reaction that followed.

Shrimp alkaline phosphatase (SAP) is an enzyme that dephosphorylates the unincorporated dNTPs present from the PCR reaction and converts them into dNDPs, making them unavailable for further reactions. The SAP was then heat inactivated at 85°C leaving the PCR products ready for single base extension.

The SAP master mix was made up based on sample number in the batch with a 20% over-hang to allow for reagent loss in pipette tips and tubes. The following x1 recipe was used as the baseline x sample number x12 + 20%. One master mix was made up in a volume suitable for each sample in the assay batch.

All wells	x1
H_2O	1.53 µl
Buffer	0.17 µl
Primer **w1**	0.3 µl
Total Vol	2 µl

The aliquot of SAP master mix (for OncoFocus) was used on the day it was made up.
12 µl of SAP master mix was dispensed into each well of the 96-reaction plate containing the PCR products (W1 & W2) using a repeater pipette, before the plate was sealed with sealing film. The plate was then vortexed briefly to mix, and centrifuged.

Thermal cycling was performed using the following conditions:

37.0 °C	40 minutes	
85.0 °C	5 minutes	1 cycle
4.0 °C	Hold	

After completion of the SAP thermal cycling step the PCR products were ready to proceed in performing iPLEX Pro extend reaction.

Performing the iPLEX Pro Extend Reaction

The OncoFocus extension reaction reagent set is designed to detect insertions, deletions, substitutions, and other polymorphisms in amplified DNA PCR products. The iPLEX Pro Reagent Set reaction cocktail was added to the amplification products produced in the previous step. The amplification products and extension reaction cocktail were then thermal cycled, allowing the enzymatic addition of a dideoxynucleotide into the diagnostic site. Thus the amplicon/primer was extended by one nucleotide, terminating the primer extension. The iPLEX Pro Reagent Set reaction produces allele-specific extension products of different masses depending on the sequence analysed. These sizes are known and are predetermined in the assay design. This size information is part of an assay file that is uploaded to the mass array and used in MALDI-TOF analysis and subsequent analysis by the Typer 4 software.

Extend reaction

The Extend master mix was made up based on sample number in the batch with a 20% over-hang to allow for reagent loss in pipette tips and tubes. The following x1 recipe was used as the baseline x sample number + 20%. Twelve master mixes were made up, one for each primer set in the assay.

Well 1	x1
H_2O	0.62 µl
Buffer	0.2 µl
Term Mix	0.2 µl
Enzyme	0.04 µl
Primer **w1**	0.94 µl
Total Vol	2 µl

Aliquots of well 1 through to well 12 Extend master mix (for OncoFocus) were used on the day they were made up.

6.5 µl of PCR product from well 1 (W1) of the 96 well plate was dispensed into rows 1-6 of the labeled 384 well plate. 6.5µl of PCR product from well 2 (W2) of the 96 well plate was dispensed into rows 7-12 of the labeled 384 well plate. These steps were repeated for each additional sample, each sample required a track (in the MTP plate) containing master mix W1 & W2 PCR master mix this followed the sample order established on the 96 well plate, including and additional NTC row. The used 96 well plate was then discarded as it was no longer required. 2µl of W1 master mix was dispensed into each well (A1-E1) of a 384-well plate. These steps were repeated for each additional extend primer master mix (there were 12 in total), each sample required a track containing master mix A1 – E1, including the NTC row. Any remaining master mix was discarded, master mix cannot be re-frozen for storage once made up.

Care was taken to ensure the tips of the pipette did not come into contact with the PCR product already in the plate avoiding assay cross-contamination.

After all OncoFocus Extend master mixes had been added, the plate was sealed and vortexed briefly to mix. The plate was then centrifuged at ~2,000 x *g* for 5 sec.

Thermal cycling was performed using the following conditions:

94.0 °C	30 seconds		
94.0 °C	5 seconds		40 Cycles
52.0 °C	5 seconds	5 Cycles	
80.0 °C	5 seconds		
72.0 °C	3 minutes		
4.0 °C	Hold		

Desalting the Extend Reaction

Materials used:

CLEAN Resin

384/6 mg dimple plate for 384-plate set up

iPLEX Pro Reagent Set reaction plate from previous procedure

PCR sealing film

HPLC-grade water

Pipette and pipette tip (16 µl)

Plate rotator (at room temperature)

Resin Clean up

In order to remove or reduce unwanted interference by adducts in the mass spectrum, caused by salts and other ionic contaminants, the extension reaction mix was desalted using Clean Resin, supplied with the assay reagents. The Clean Resin was spread out on to the clean dry dimple plate, using approximately three scoops of the Clean Resin mix, making sure the resin settled evenly into all wells using the scraper plate. Once scraped level into all dimple wells the Clean resin plate was left to dry for 10 minutes at room temperature. While the resin plates were drying, 16 µl HPLC-grade water was added to each well of the MTP plate containing OncoFocus extension reaction products. This was done to dilute the extension reaction products to the working dilution for detection of mutations by the MALDI-TOF Mass Array. Once the resin had been allowed to dry for 10 minutes it was added to the micro titer plate containing the extension reaction products by gently inverting the sample micro titer plate on top of the dimple plate, making sure that the plate wells were aligned over the resin samples. Gentle taping of the dimple plate let the resin fall into the sample wells. Once the Clean resin was added to all wells of the extension reaction micro titer plate the plate was checked to make sure that the resin height was the same in all wells. Any wells that had more or less resin were noted and adjustments made as necessary. The sample plate was then sealed and the plate and rotated for 30 minutes at room temperature. The plate rotator rotated the microplate 360° around its long axis.

At the completion of the plate rotation the sample plate was centrifuged at 3,200 x *g* for 5 minutes to pellet the resin. This step was to ensure the pins of the Nanodispenser, used to add the Extension reaction mix to the Mass Array Spectro chip, are not contaminated with the resin which could block the pin interfering with the correct volume being added to the Spectro chip. At the completion of the centrifugation step the extension reaction mix was dispensed on to a Spectro Chip array.

Dispensing onto Spectro Chip Arrays

If the sample plates had been stored frozen, for example over the weekend, they were allowed to thaw to room temperature and then rotated and centrifuge, as above, before the extension reaction mix was transferred to a Spectro Chip array.

After the OncoFocus product de-salting was complete the extension reaction products were dispensed onto a Spectro Chip array ready for analysis by MALDI-TOF mass spectrometry. 15nL of the extension reaction mix was added to each pad corresponding to the appropriate well in the micro titer plate. The Samsung Nanodispenser, Agena Bioscience, San Diego USA, user manual was consulted for detailed instruction for this procedure.

Defining Assays and Plates

Once the dispensing onto a spectro array chip was complete, the assays were defined in the Mass Array Typer 4 database. For instructions, the “Defining Assays” and “Defining Plates” chapters in the Typer 4 User manual, Agena Bioscience, San Diego, USA, were consulted. These definitions allow the identification of sample details and assay parameters to be loaded into the Mass Array which then accompany raw data acquired by analysis of the extension reaction products.

Acquiring and Analysing Spectra

Analysis using the Bruker MALDI-TOF Mass Spectrometer was carried out using parameters optimised for iPLEX chemistry allowing allele specific single base extensions from the OncoFocus Mutation assay to be resolved. Instruction for use were followed from the Mass Array Analyser Compact User manual (Agena Bioscience).

Visual quality checks were made of the generated raw data peaks in addition to the non-template control prior to report generation using Typer 4 analysis software (Agena Bioscience). Genotyping reports were output in .xls format.

2.3 Results

There was moderately high agreement between the Roche Cobas EGFR Gene Mutation Test and the Agena Mass Array OncoFocus assay for the detection of EGFR mutations in this study. Among 470 patient samples with valid results from both assays, 367 patient samples (78%) were EGFR mutation-negative in both assays and 71 patient samples (15%) were EGFR mutation-positive in both assays (Figure 2). In the remaining 32 patient samples (7%), EGFR mutations detected by one assay but not by the other. From these data, the levels of positive percentage agreement, negative percentage agreement and overall percentage agreement, between the Roche Cobas EGFR Gene Mutation Test and the Agena Mass Array OncoFocus assay for the detection or not of EGFR mutations, were 79.8%, 96.9% or 93.2%, respectively (Table 1).

There was a very high level of agreement between the Roche Cobas EGFR Gene Mutation Test and the Agena Mass Array OncoFocus assay for the identification of specific EGFR mutations in patient samples that were mutation-positive in both assays. Identical EGFR mutations were detected by both assays in 65 of 71 patient samples (92%) that were mutation-positive in both assays. Both assays detected EGFR exon 19 deletion mutations in 32 patient samples, exon 21 L858R point mutations in 28 patient samples, exon 20 insertion mutations in 3 patient samples and exon 18 G719X point mutations in 2 patient samples (Table 2).

There was disagreement between the Roche Cobas EGFR Gene Mutation Test and the Agena Mass Array OncoFocus assay for the identification of specific EGFR mutations in 6 of 71 patient samples (8%) that were mutation-positive in both assays (Table 3). Four of the six discordant patient samples had double EGFR mutations detected by one assay but only one of those two mutations was detected by the other assay. One discordant patient sample had double mutations detected by both assays but only one of those two mutations was the same and the other one differed between the two assays. One discordant patient sample had a single EGFR mutation detected by both assays but that mutation differed between the two assays.

There was disagreement between the Roche Cobas EGFR Gene Mutation test and the Agena Mass Array OncoFocus assay in the detection of EGFR mutations in a total of 32 patient samples (7%). Firstly, the Roche Cobas EGFR Gene Mutation test detected EGFR mutations in 18 patient samples (4%) that were mutation-negative in the Agena Mass Array OncoFocus assay. In these 18 patient samples in which no mutation detected by the Agena Mass Array OncoFocus assay, the Roche Cobas EGFR Gene Mutation test detected EGFR exon 20 insertions in 8 patient samples, exon 19 deletion mutations in 6 patient samples, exon 21 L858R point mutations in 5 patient samples, and exon 20 point mutations in 2 patient samples (Table 4). Secondly, the Agena Mass Array OncoFocus test array detected EGFR mutations in 14 patient samples that were mutation-negative in the Roche Cobas EGFR Gene Mutation Test. In these 14 patient samples that had no mutation detected by the Roche Cobas EGFR Gene Mutation test, the Agena Mass Array OncoFocus assay detected exon 21 L858R point mutations in 4 patient samples, exon 20 insertion mutations in 3 patient samples, exon 19 deletion mutations in 3 patient samples, exon 20 point mutations in two patient samples, an exon 19 insertion in one patient and an exon 21 point mutation in one patient sample (Table 4).

The discordant patient samples were retested independently by a third assay and the Roche Cobas EGFR Gene Mutation Test and Agena Mass Array OncoFocus Assay results categorised accordingly as true positive, false positive, true negative and false negative (Table 5). Nine discordant patient samples were true positive Roche Cobas EGFR Gene Mutation Test results but false negative Agena Mass Array OncoFocus Assay results. Six discordant patient samples were true negative Roche Cobas EGFR Gene Mutation Test results but false positive Agena Mass Array OncoFocus Assay results. Four discordant patient samples were true positive Agena Mass Array OncoFocus Assay results but false negative Roche Cobas EGFR Gene Mutation Test results. Seven discordant patient samples were true negative Agena Mass Array OncoFocus Assay results but false positive Roche Cobas EGFR Gene Mutation Test results. Six patient samples could not be retested in the third assay.

The Agena Mass Array OncoFocus assay identified a large number of patient samples with KRAS, NRAS and BRAF mutations that were not tested for by the Roche Cobas EGFR Gene Mutation Test. Among 367 patients samples that were EGFR mutation-negative in both assays, a total of 127 patient samples (35%) had KRAS, NRAS and BRAF mutations identified by the Agena Mass Array OncoFocus assay (Table 6). One hundred and six patient samples had KRAS mutations that were most commonly KRAS codon 12 point mutations. NRAS mutations were detected in 7 patient samples and BRAF mutations in 3 patient samples. The spectrum and frequencies of specific KRAS, NRAS and BRAF mutations detected is shown in Table 6.

The Agena Mass Array OncoFocus assay predicted the clinical benefits of treatment with EGFR-TKIs in this study. Among 54 EGFR-TKI-treated patients, who had samples that were EGFR mutation positive in the Agena Mass Array OncoFocus assay, the median duration of treatment with an EGFR-TKI was 393 days, and 52% of the patients remained on treatment for at least one year (Figure 6 and Table 7). In contrast, among four EGFR-TKI-treated patients who had samples that were EGFR mutation-negative but KRAS, NRAS or BRAF mutation-positive in the Agena Mass Array OncoFocus assay, the median duration of treatment with an EGFR-TKI was only 99 days, and all of the patients had discontinued treatment before one year. Among 18 EGFR-TKI treated patients who had samples that were EGFR, KRAS, NRAS and BRAF mutation negative in the Agena Mass Array OncoFocus assay, the median duration of treatment with an EGFR-TKI was only 219 days, and only 36% of the patients remained on treatment for at least one year.

The Agena Mass Array OncoFocus assay predicted the overall survival of lung cancer patients in this study. Among 85 patients who had samples that were EGFR mutation-positive in the Agena Mass Array OncoFocus assay, the median overall survival was 991 days, and 68% of the patients were alive at one year (Figure 7 and Table 8). In contrast, among 131 patients who had samples that were EGFR mutation-negative and KRAS, NRAS or BRAF mutation-positive in the Agena Mass Array OncoFocus assay, median overall survival was

only 222 days, and only 36% of the patients were alive at one year. Among 270 patients who had samples that were EGFR, KRAS, NRAS and BRAF mutation-negative in the Agena Mass Array OncoFocus assay, the median overall survival was 353 days, and only 49% of patients were alive at one year.

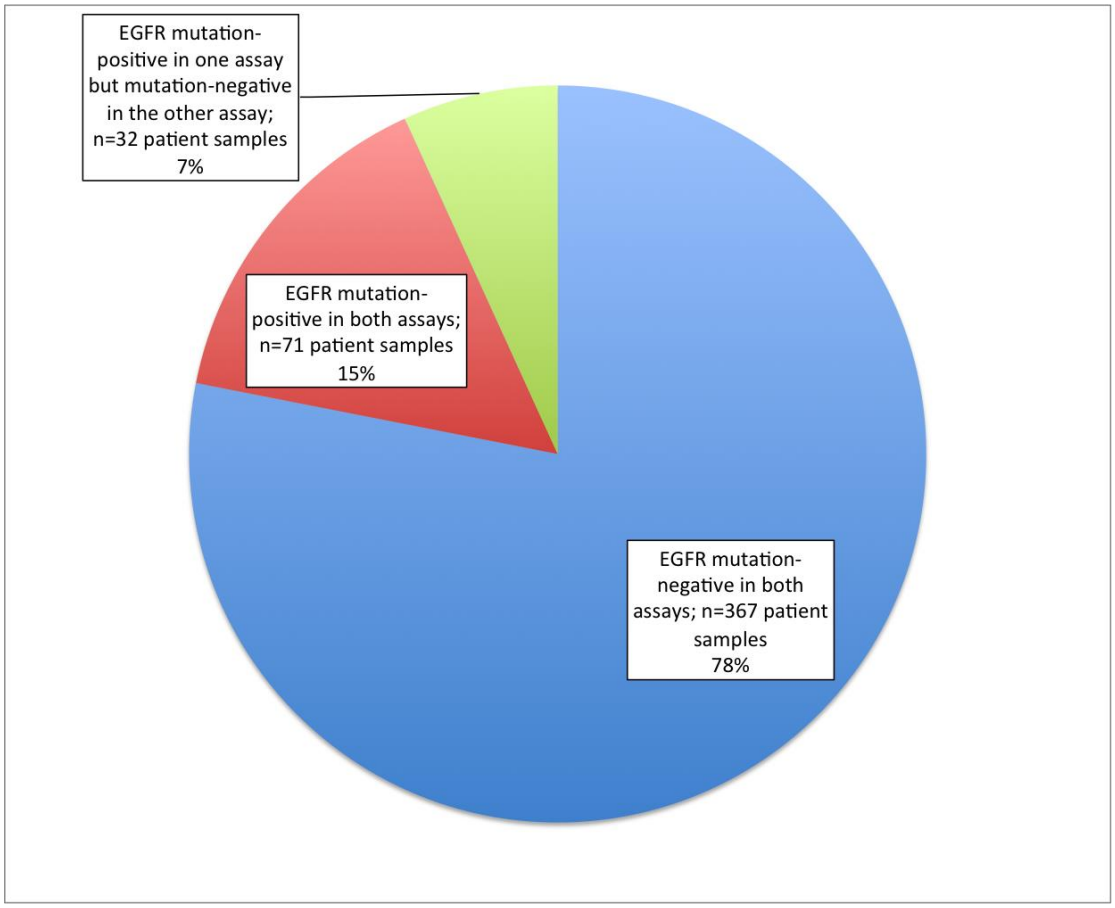


Figure 2 Agreement between Cobas EGFR Mutation test and Agena Mass Array OncoFocus assay for the detection of EGFR mutations in 470 patient samples.

	Agena Mass Array OncoFocus genotyping		
Cobas EGFR mutation test	Mutation detected	No mutation detected	Total
Mutation Detected	71	18	89
No Mutation detected	14	367	381
Total	85	385	470
Positive percentage agreement		71/89 = 79.8% (95%CI; 70.3 to 86.8%)	
Negative percentage agreement		367/381 = 96.9% (95%CI; 93.9 to 97.8%)	
Overall percentage agreement		438/470 = 93.2% (95%CI; 90.5 to 95.1%)	

Table 1 Agreement analysis between the Cobas EGFR mutation test and Agena Mass Array OncoFocus assay for the detection of EGFR mutations in lung cancer diagnostic specimens.

	Number of patient samples (%)
Exon 19 deletion	32 (49%)
L858R	28 (44%)
Exon 20 insertion	3 (5%)
G719X	2 (3%)
Total	65 (100%)

Table 2 Spectrum of EGFR mutations detected in 65 patient samples that were EGFR mutation-positive and identical mutations were detected by both assays.

Patient sample	Roche Cobas (Specific Detection)	Agena Mass Array (Specific Detection)
1	EGFR Exon 18 G719X and EGFR Exon 20 S768I	EGFR G719C
2	EGFR Exon 21 L858R	EGFR L858R and EGFR E709A
3	EGFR Exon 21 L858R	EGFR L858R and EGFR R108K
4	EGFR Exon 20 S768I and Exon 18 G719X	EGFR G719S and EGFR L861Q
5	EGFR Exon 18 G719X	EGFR G719S and EGFR L861Q
6	EGFR Exon 21 L858R	EGFR E709G

Table 3 EGFR mutation test results in six patient samples in which different EGFR mutations were detected by the Roche Cobas EGFR Gene mutation test and the Agena Mass Array OncoFocus assay.

Roche Cobas EGFR Gene Mutation test	Agena Mass Array OncoFocus assay	Sample Number
Exon 20 Insertion	NMD	6
Exon 19 Deletion & Exon 20 S768I	NMD	1
Exon 19 Deletion	NMD	4
Exon 21 L858R & Exon 20 Insertion	NMD	1
Exon 19 Deletion & Exon 20 T790M	KRAS G12C	1
Exon 21 L858R	NMD	3
Exon 20 Insertion	BRAF V600E	1
Exon 21 L858R	KRAS G12V	1
NMD	EGFR E746_A750del	2
NMD	EGFR H773_V774insNPH	1
NMD	EGFR D770_N771insSVD(5' Detection Only)	1
NMD	EGFR D770_N771insG/D770_N771insGD(5' Deletion Only)	1
NMD	EGFR L858R	4
NMD	EGFR Exon 19 Insertion; KRAS G12V	1
NMD x2	KRAS G12C; EGFR E746_A750del	1
NMD	EGFR T751_I759>N	1
NMD	EGFR L861Q	1
NMD x2	EGFR A750P (Rev Detection only),	1

Table 4 Specific EGFR mutations identified in 32 discordant patient samples that were EGFR mutation-positive in one assay but mutation-negative in the other assay.

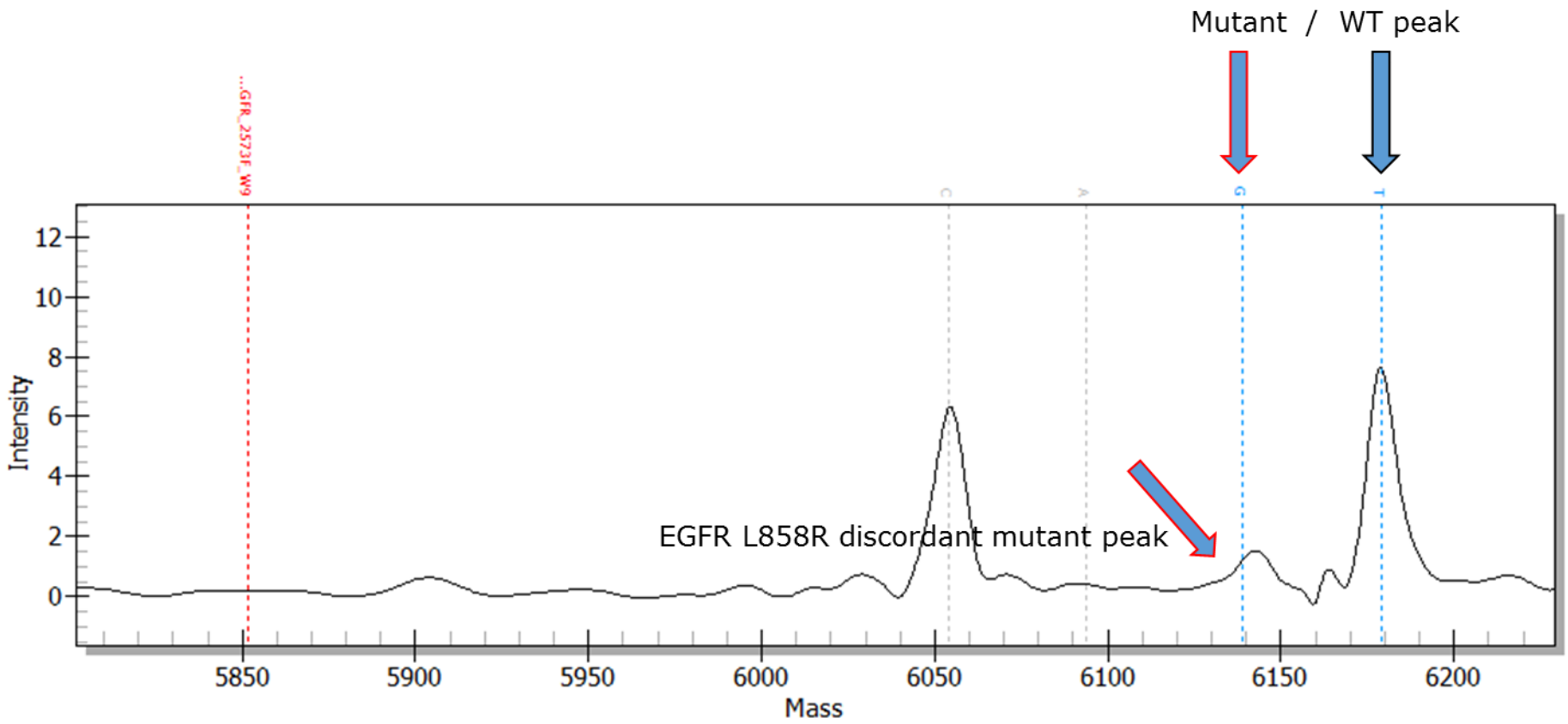


Figure 3 Mass spectra showing the presence of a discordant allele. This was resulted as EGFR L858R mutation negative, a possible a low abundance mutation peak, potentially being hidden by the poorly shaped off centred peak, is shown by the red & blue arrow.

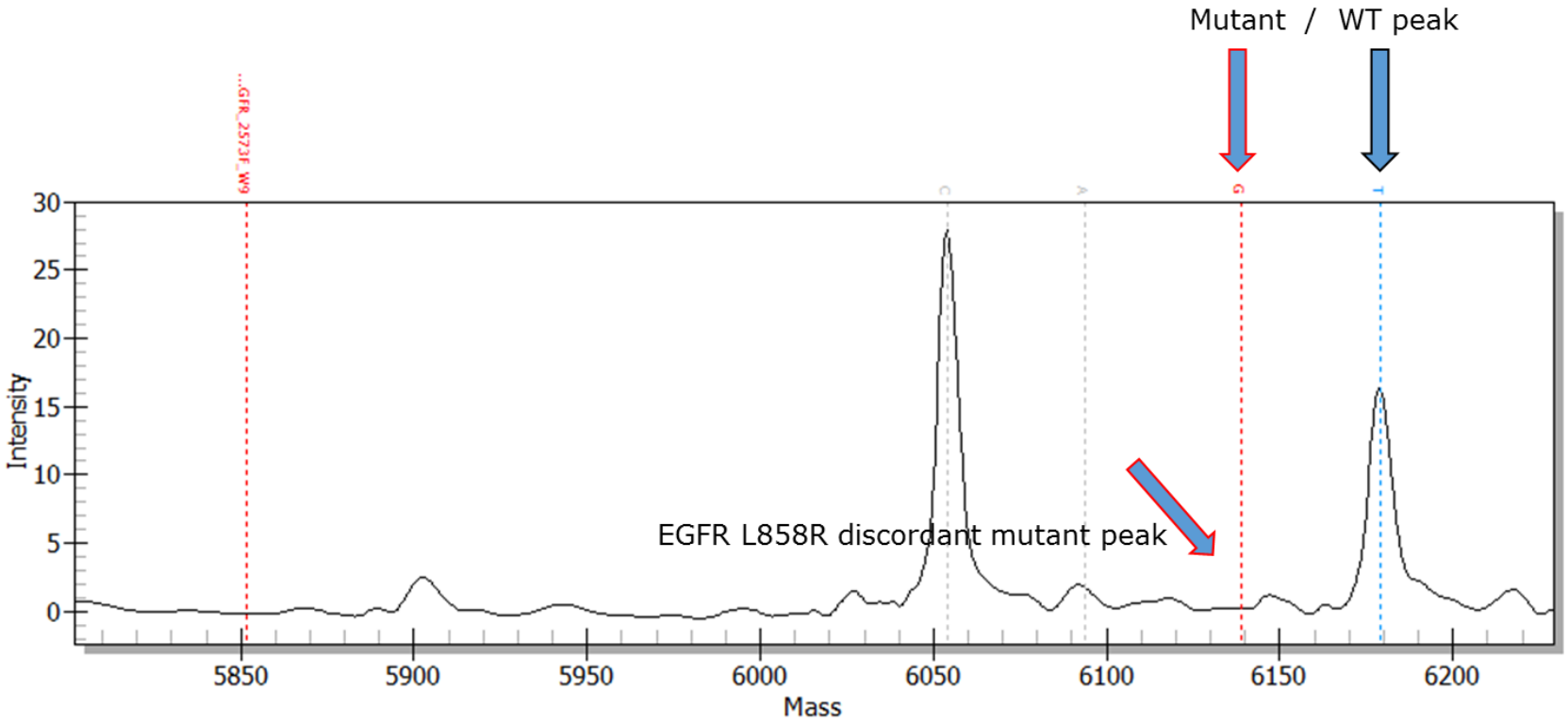


Figure 4 Mass spectra of a discordant result showing the presence of no mutated allele. This was resulted as EGFR L858R mutation negative, and it can be clearly seen that there is no mutant peak present in the spectra as indicated but the red & blue arrow.

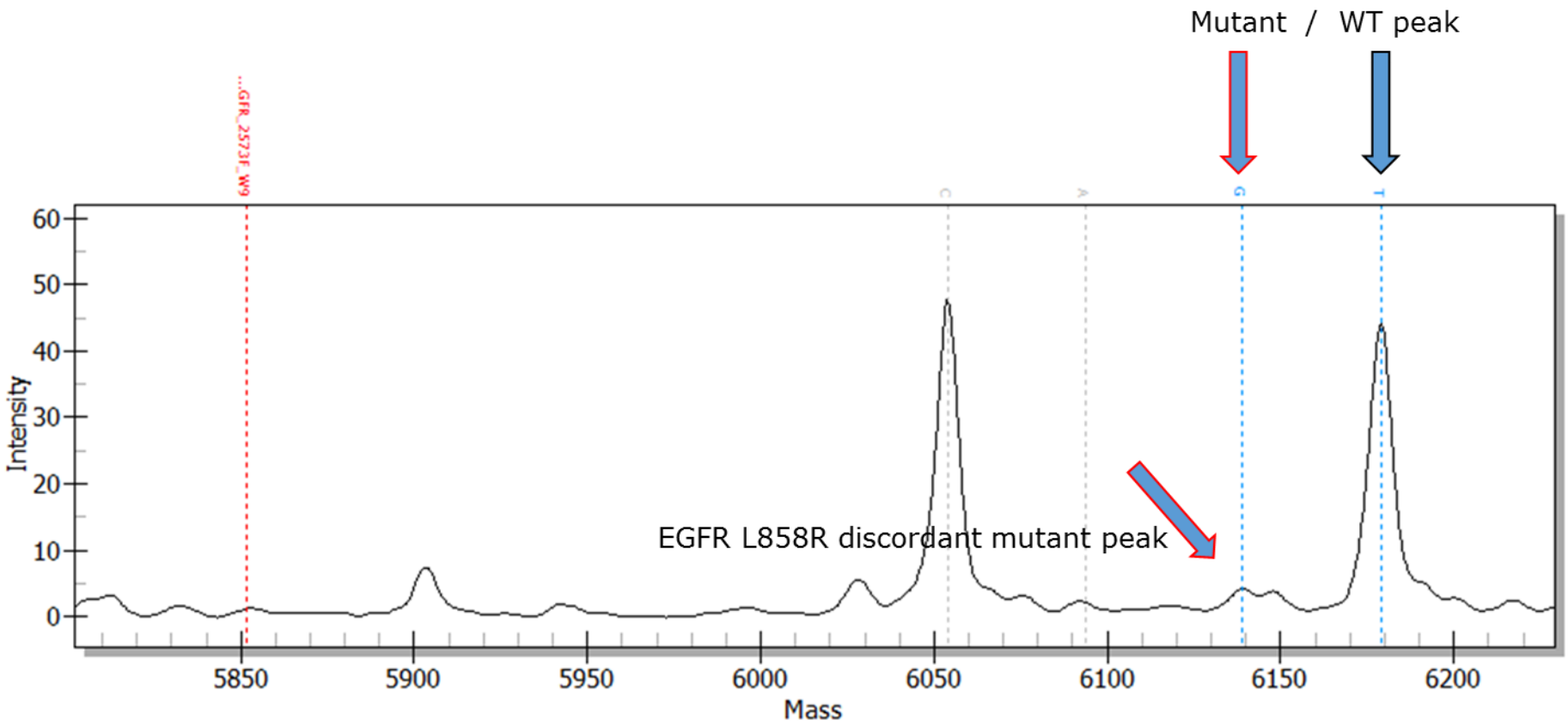


Figure 5 Mass spectra showing the presence of a second discordant allele. This was resulted as EGFR L858R mutation negative, however as can be seen, as indicated by the red & blue arrow there is a clear mutant peak present, indicating the presence of an L858R mutation in the sample.

Roche Cobas EGFR Gene Mutation test	Agena Mass Array OncoFocus assay	Sample Number
True positive	False negative	9
True negative	False positive	6
False positive	True negative	7
False negative	True positive	4
Unknown	Unknown	6

Table 5 Discordant EGFR mutation detection assay results categorised as true-positives, false-positives, true-negatives and false-negative according to the results of a third assay (Idylla).

Roche Cobas EGFR Gene Mutation Test	Agena Mass Array OncoFocus Assay	Sample Number
NMD	BRAF G469R	1
NMD	BRAF V600E	3
NMD	KRAS A146T	1
NMD	KRAS G12A	9
NMD	KRAS G12C	40
NMD	*KRAS G12C, KRAS G12V*	*1*
NMD	KRAS G12D	21
NMD	KRAS G12R	2
NMD	KRAS G12S	1
NMD	KRAS G12V	28
NMD	KRAS G13C	5
NMD	KRAS G13D/N	1
NMD	KRAS Q61H	4
NMD	KRAS Q61L	2
NMD	KRAS Q61R	1
NMD	NRAS G12D/E, KRAS G12D	1
NMD	NRAS G13R	1
NMD	NRAS Q61H	1
NMD	NRAS Q61L	1
NMD	NRAS Q61Q/K	1
NMD	NRAS Q61R	2

Table 6 Spectrum and frequency of specific KRAS, NRAS and BRAF mutations identified in EGFR mutation-negative patient samples by the Agena Mass Array OncoFocus assay.

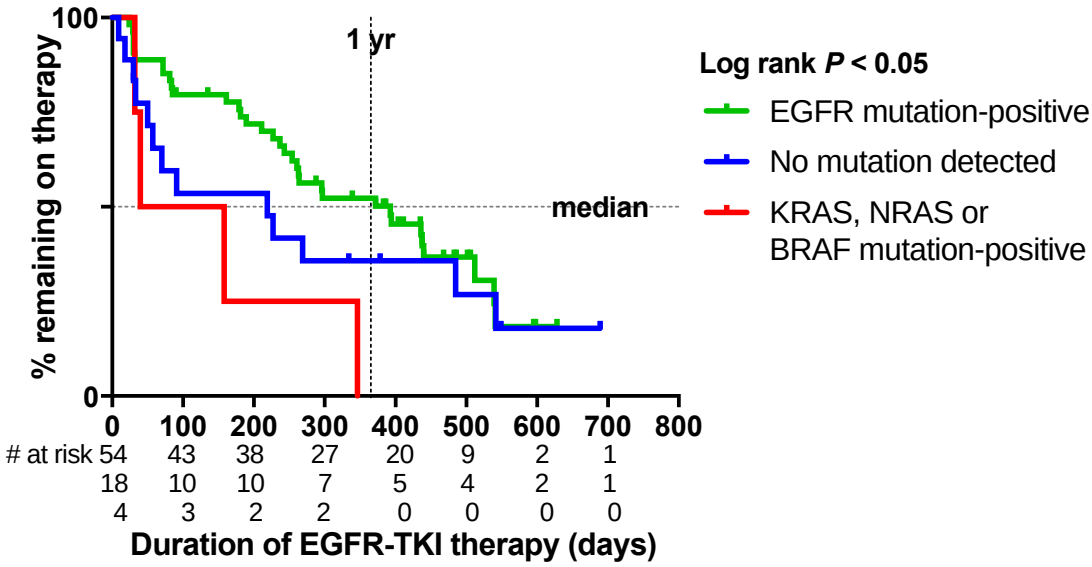


Figure 6 Prediction of duration of EGFR-TKI therapy by Agena Mass Array OncoFocus assay results.

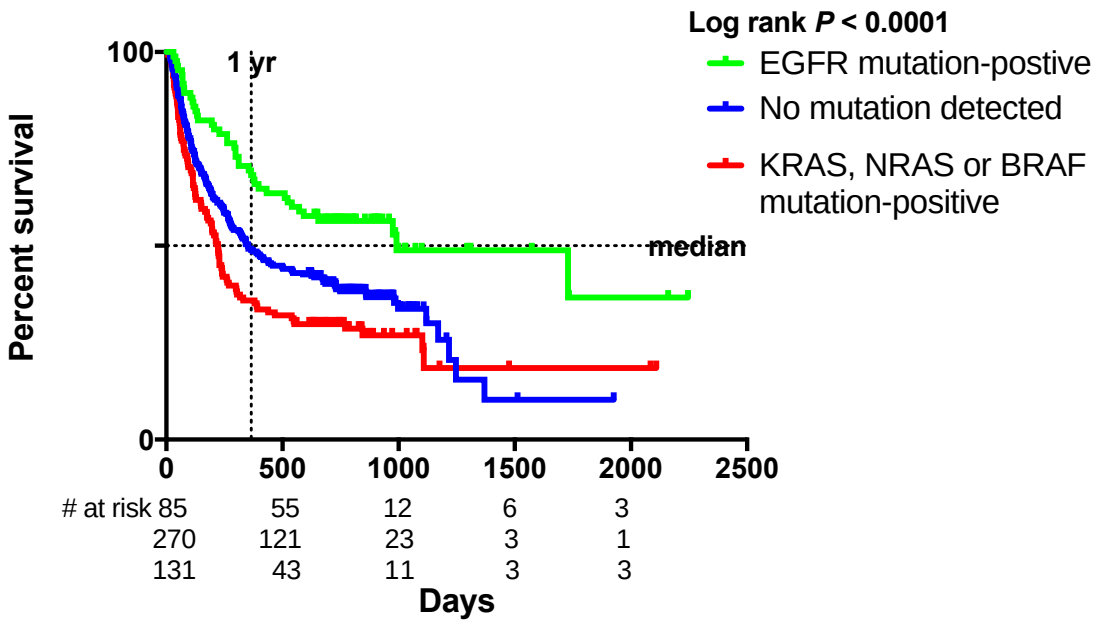


Figure 7 Prediction of duration of overall survival by Agena Mass Array OncoFocus assay results.

Agena Mass Array OncoFocus assay result	Duration of EGFR-TKI therapy	
	Median (95% CI)	Percent remaining on treatment at one year (95% CI)
EGFR mutation-positive	393 days (243-512)	52% (38-65)
No mutation detected	219 days (50-485)	36% (15-57)
KRAS, NRAS or BRAF mutation positive	99 days (0-346)	0% (n/a)

Table 7 Duration of EGFR-TKI therapy by Agena Mass Array OncoFocus assay result.

Agena Mass Array OncoFocus Assay result	Duration of overall survival	
	Median (95% CI)	Percent alive at one year (95% CI)
EGFR mutation-positive	991 days (520-not reached)	68% (58-78)
No mutation detected	353 days (270-498)	49% (43-55)
KRAS, NRAS or BRAF mutation-positive	222 days (168-244)	36% (28-44)

Table 8 Duration of overall survival by Agena Mass Array OncoFocus assay result.

2.4 Discussion

2.4.1 Agena Mass Array and OncoFocus genotyping

The Agena Mass Array OncoFocus Panel was designed using the iPLEX Pro single base extension chemistry. Single base extension has its limitations however it is also a very powerful method, enabling a high degree of specificity facilitating differentiation between deletions. Figure 8 demonstrates how single base extension allows specific deletions to be readily discerned within EGFR Exon 19. Next generation sequencing currently does not easily achieve this level of resolution, owing mainly to challenges around deletion size within this region (Treangen & Salzberg, 2012).

Tumour DNA extracts from about 750 New Zealand lung cancer patients referred to the LabPlus medical laboratory for EGFR gene mutation testing during a 2-year period (1 August 2012 to 31 July 2014), were re-tested using the Agena Mass Array OncoFocus panel assay, which is capable of detecting 128 different activating mutations in the EGFR gene and an additional 63 different mutations in the BRAF, KRAS and NRAS genes. Previous studies had found the Agena Mass Array and genotyping panels to be superior to direct sequencing for detecting oncogenic somatic mutations from tumour DNA extracts from cancer patients (Arcila, Lau, Nafa, & Ladanyi, 2011). Critically, the Agena Mass Array platform enables the simultaneous interrogation of multiple positions within multiple genes at a clinically relevant level of analytical sensitivity while also offering a rapid turnaround time for diagnostic purposes. These combined features compare favourably against other technologies such as digital PCR (Wang *et al*., 2010) and next generation sequencing strategies (Marchetti *et al.*, 2012), and allows for the rapid implementation of other diagnostic oncogene testing should this need arise in future. The Roche Cobas gene mutation test was regarded as the notional gold standard reference assay for the study and was used to identify both true and false positive and negative results generated by the Agena Mass Array OncoFocus assay. The Agena Mass Array OncoFocus assay had potential to demonstrate specific EGFR mutation genotypes present in the large and representative sample of New Zealand lung cancer patients, thereby indicating which of these need to be included in a national testing strategy to achieve target coverage of 90% or more of all cases of EGFR mutation positive lung cancer.

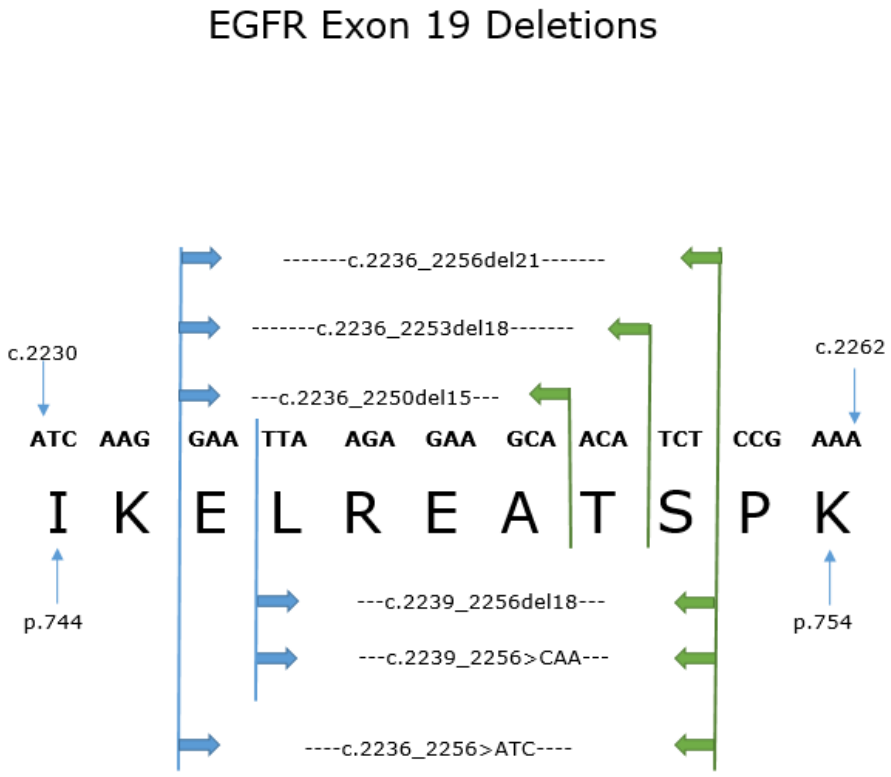


Figure 8 EGFR Coverage within the OncoFocus Panel

2.4.2 Agreement analysis

In evaluating the diagnostic accuracy of this novel genetic testing strategy, data was analysed by agreement analysis. Overall survival and progression free survival (PFS) after gefitinib or erlotinib treatment was analysed with the Kaplan–Meier method to assess the time to death or progression. Cox-regression was used for multivariate survival analysis. A log-rank test was employed to compare cumulative survival in different groups. All P-values were two-sided and a P-value <0.05 was considered statistically significant. All analyses were performed using SPSS software (version 13.0; SPSS Inc. Chicago, IL, USA). For example, the level of agreement between the Roche Cobas EGFR gene mutation test being used at the LabPlus medical laboratory and the Agena Mass Array and OncoFocus assay was measured in regard to their detection, or not, of an EGFR gene mutation, initially without specification of the particular type of mutation detected. Descriptive statistics were then used to calculate the proportion of overall agreement, positive agreement, negative agreement and disagreement, and their respective 95% confidence intervals. A sample size of more than 400 patient tests was sufficient for detecting proportions of agreement with 95% confidence intervals of less than 5%.

The Agena Mass Array OncoFocus assay is a set of revalidated assays covering 202 somatic mutations in four key oncogenes observed not only in lung tumours but also colorectal, and metastatic melanoma. The panel was designed to detect significant clinically relevant driver mutations in EGFR, KRAS, NRAS, and BRAF with extensive coverage of EGFR exon 19 and 20 insertions and deletions. The Roche Cobas EGFR Mutation Test uses primers that define specific base-pair sequences for each of the targeted 34 mutations. For the exon 19 deletion mutations, base pair sequences that range from 125 to 141 were targeted and; for the L858R substitution mutation in exon 21, a 138 base pair sequence was targeted; for the internal control in exon 28, an 87 base pair sequence was targeted. For both technologies amplification occurred only in the regions of the EGFR gene between the primers; the entire EGFR gene was not amplified. Table 9 below summarises the mutations covered by these two panels.

EGFR Missense
R108K, T263P, A289V, A289D, GS98V, E709K, E7090/H, E709A, E709G, E709V, E709fs 1111, G719S, G719C, G719A, G719D, E746K, E746V, L747P, L747S, T7511, S752P, S752Y, P753Q, P753S, 1759N, D761N, D761Y, S768I, S768N, D770N, R776C, R776H, T790M, T854A, L858M/K/R, L858R, L861Q, L861R
EGFR Exon 19
K745_E749del, E746_E749del, E746_A750del, E746_T751del, E746_T751>1, E746_A750>1P, E746_T751>1P, E746_S752>1, E746_T751>A, E746deV1744_K7451nsKIPVA1I , E746_S752del, E746_T751>1, E746_P753>1S, E746_T751>0, E746_A750>QP, E746_T751>L, E746_P753>LS, E746_T751>S, E746_S752>A, E746_T751>V, E746_T751>VAorvP, E746_P753>VS, E746_S752>V, E746_A750>VP, E746_T751>VA, E746_T751>VP, E746_P753>VQ, E746V/K74S_E746insVPVAIK1, E746_S752>D, L747_A750>P, L747_T751>Q, E746_A750>DP, L747_T751>P, L747_S752>Q, L747_S752>QH, L747_E749del, L747_S752del /L747_S752>Q, L747_P753>Q, L747_T751>A, L747_K754del, L747_K754>N 1, L747_T751>S, L747_T751del, L747_P753>S, L747S/l747_1<754>ST1, T751_1759>N, T751_1759>REA, T751_1759>St, T751_1759del1, S752_1759del, P753_1759del,1751_1759>5 1
EGFR Exon20
M766_A767insAI, A767_S768insll.A1, S768_V769>1L1, V769_0770insMASVD, 0770_P772>ASVDNR, V769_D770inscvt, V769_D770insASV, D770_N771>AGG, V769_D7701nsASV1, V769_D7701nsGSV/ V769_D7701nsGVV/D770>GY 1, D770_N7711nsG, D770_N7711nsAPW, D770_N7711nsGL, N771>GF, N771>GY, D770_N771insG, D770_N771insGD1, D770_N771insSVD, N771_P772>SVQNR, N771>TH, N771>SH, D770_N771insMATPl, H773_V774insNPH, H773_V774insH, H773_V774insPH, H773_V774insQ 1, V774_C775insHV,N771_P772insN1, D770fs•611, N771_P772insRH/ P772_H773insTHP1, P772_H773insV1, P772_H773insHvt, H773>NPY 1, V774_C77SinsHvt
BRAF
D594G, D594V, G469S, G469E, G469A, G469V, G469R, G469R/S, L5970, L597V, L597R, L597S, V600E, V600K, V600M, V600L
KRAS
G12S, G12R, G12T, G12V, G12F, G12P, G12A, G12C, G12W, G12D, Gl2N, G121, G12L, G12Y, G12E, G12D/V, G13C, G13S, G13A, G13V/I, G13D/N, G13R, AS9T, Q61K, Q61E, Q61L, Q61R, Q61P, Q61H
NRAS
G12S/N, G12R/P, G12C/Y, G12D/E, G12A, G12V, G13S/NG, G13R, G13C/Y, G13D, G13A, G13V, Q61H, Q61L, Q61R, Q61PQ, Q61K, Q61E

Table 9 Agena Mass Array OncoFocus Assay vs Roche Cobas EGFR gene mutation test list. Gene mutations detected with the OncoFocus panel and those detected with the Roche Cobas EGFR gene mutation test shown underlined.

The study consisted of 532 patients that were both tested by using the Roche Cobas EGFR gene mutation test at LabPlus and retested using the Agena Mass Array OncoFocus assay at the University of Auckland, Faculty of Medical and Health Sciences. Results were accepted from LabPlus for the study on the basis that there was remaining DNA suitable for retesting. Those patients with insufficient remaining DNA were excluded from the study. Samples that failed analysis at LabPlus were included in the study and retested at the Liggins Institute.

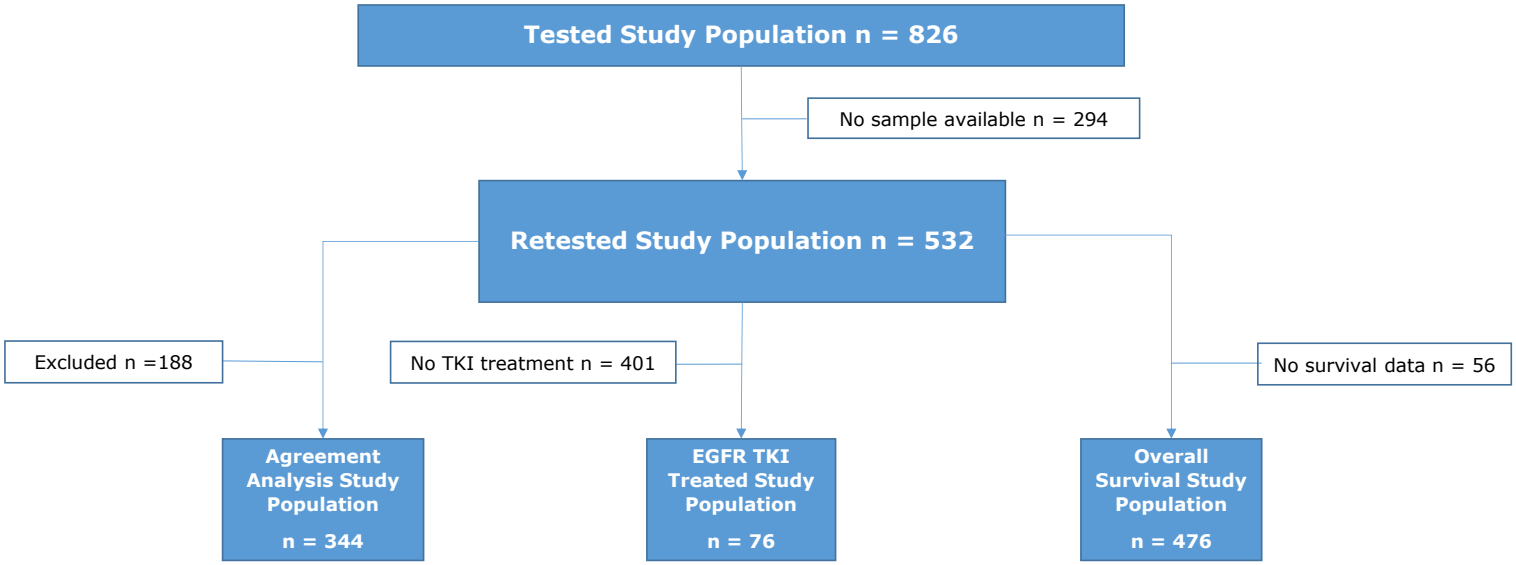


Figure 9 Consort diagram of the study

The consort diagram, figure 9, gives an overview of the study populations, which consisted of 826 eligible patients, eligible patients are those who have had sample sent to LabPlus for EGFR mutation analysis. Of the eligible 826 patients 294 had no remaining sample available for retesting, and were excluded from the study. This brought the retested study population to 532 and formed the retested study population that was available to 3 areas of the study – agreement analysis, EGFR TKI treated and overall survival analysis, figure 9. Of the 532 available for agreement analysis 188 were excluded due to invalid or unavailable results from one or both assays leaving 344 in this area. Of the 532 patients 401 did not receive TKI therapy leaving 76 patients that did receive TKI therapy, forming the EGFR TKI treated study population. Of the

532 patients survival data was not available on only 56 patients. This left 476 patients available for overall survival analysis.

Survival curves showing overall survival represented by the number of days each patient remained on TKI therapy. There were 3 major groups consisting of patients with a detectable EGFR mutation, patients where no mutation was detected and those who had a non-EGFR (BRAF, KRAS or NRAS) mutation detected. Analysis of the survival curves using both Logrank test for trend and Gehan-Breslow-Wilcoxon test showed statistical significance in the difference between each curve with P-values of 0.036 and 0.046 respectively. A P-value <0.05 was considered statically significant. The median survival for patients with a detectable EGFR mutation was 393 days with those having no mutation detected sitting at 219 days. Patients that had a non-EGFR mutation detected had a median survival of only 99 days. This is substantially reduced in relation to those patients for whom no mutation was detected.

These findings show the Agena Mass Array OncoFocus mutation test has the ability to predict prognostic outcome in a clinically relevant way.

While the agreement between both testing methods showed good agreement, with overall agreement 93.4%, negative agreement of 95.3% and positive agreement of 83.5%. Within the positive agreement fraction there are 6 of 71 patients that both testing platforms showed an EGFR mutation but the specific EGFR mutation differed between testing platforms, Table 3.

4 of the 6 discordant results showed a completely different EGFR mutation being detected by each testing platform. In the remaining 2 samples an additional EGFR mutation was deleted. Two of these 6 discordant results appear easy to explain in that there is data published elsewhere suggesting that it's not uncommon for these complex mutations to appear in small numbers in a large data cohort (Wu & Shih, 2016). This would suggest that where Roche Cobas detected a L858R and Agena Mass Array detected an E709G mutation, the L858R is likely to have been present in a low abundance leading to it being missed by the Mass Array. The Roche Cobas has a limited number of targeted mutations in comparison to the Mass Array and this explains why there are additional mutations detected by Mass Array and not by Roche Cobas in 3 of the 6 cases. On further detailed analysis of the spectra produced by the Agena Mass Array OncoFocus assay for the reported discordant L858R mutations there was clear evidence that in one case of the discrepant L858R results the OncoFocus assay was clearly a

negative result, in contrast to the positive reported by the Roche Cobas EGFR Gene Mutation assay. Figure 4 clearly shows no mutant allele present in the assay spectra, in addition the wild type peak and other peaks in the assay look of good quality. However figure 5 demonstrates a very clear low abundance peak present in the spectra indicating that there is an EGFR L858R mutation present. This result has been incorrectly resulted as negative in the raw study data. This low abundance peak highlights the critical need to carefully inspect the spectra where the analysis software flags a potential positive mutation call.

For full understanding of these discordant mutation results further detailed analysis using another sensitive method was required to confirm the actual mutation present. This testing was carried out by the molecular genetics department at LabPlus, Auckland City Hospital, using a third assay. The Idylla (Biocartis NV, Mechelen, Belgium) is a fully automated real-time PCR based molecular diagnostic system with a reported sensitivity of between 1-5% (Biocartis) and was used to analyse the discordant results. The outcome of this conformational testing is summarised in table 5. It can be seen that of the 32 discordant results 26 could be confirmed as either true positive, true negative or false positive, false positive for each if the compared assays (Agena Mass Array & Roche Cobas EGFR gene mutation tests). There were however 6 samples that were invalid for the assay. Of the remaining 26 samples 11 results were in favour of the Agena mass array system with 15 in favour of the Roche Cobas system. The results from this retesting helps clarify that there is a higher degree of false result reporting that is desirable. Means to minimise this risk should be taken and include processing only FFPE slides that have been examined by a histopathologist and had the tumour area estimated and macroscopically marked up on an accompanying H&E slide. In addition the accurate quantification of the amplifiable DNA input into each assay is recommended to avoid false negatives in cases where the DNA from an FFPE samples is insufficient.

This study has demonstrated the clinical validity and utility of the Agena Mass Array OncoFocus assay for detecting EGFR, KRAS, NRAS and BRAF mutations in tumour specimens from NSCLC patients. The clinical validity of the assay was established by demonstrating high levels of overall agreement in the detection of EGFR mutations compared to a reference assay. Clinical utility was established by demonstrating associations between patient outcomes and the detection of EGFR, KRAS, NRAS and BRAF mutations by the Agena Mass Array OncoFocus assay. Patients whose tumours were identified is being EGFR mutation-positive by the assay

had longer overall survival and duration of EGFR-TKI treatment as compared to those with no mutation detected. Patients whose tumours were identified as being KRAS, NRAS or BRAF mutation-positive had relatively shorter overall survival and duration of EGFR-TKI treatment as compared to those with no mutation detected. In this way, the Agena Mass Array OncoFocus assay for testing tumour specimens may aid the clinical assessment of NSCLC patients and decisions about treatment and prognosis.

The study has demonstrated the potential for EGFR mutation tests to generate false-positive and/or false-negative test results. The EGFR mutation status of 7% of the total of 470 retested patient samples were discordant between the two assays, i.e. EGFR mutation-positive in one assay but EGFR mutation-negative in the other assay. The profile of specific EGFR mutations detected in these discordant samples generally reflected those detected in the EGFR mutation-positive patient population as a whole, except for increased prevalence of exon 20 insertion mutations among the discordant patients samples. Retesting of the discordant samples by a third EGFR mutation assay showed that both the Agena Mass Array OncoFocus assay and Cobas EGFR Mutation Test had similar potential for generating false-positive and false-negative test results. Such false-positive and false-negative EGFR rotation test results may have important clinical consequences. False-positive results could lead to the use of EGFR-TKIs in patients with little chance of benefit but with risk of adverse events. False-negative results could deny patients the benefits of EGFR-TKIs therapy and increase the risk of early disease progression.

This study had both strengths and weaknesses worthy of comment. It evaluated a large and unselected group of NSCLC patients and remnant tumour DNA extracts that were likely to be representative of those encountered in reaching testing. The findings of this study may therefore be transferable to the general population of lung cancer patients in routine practice. However, the numbers of EGFR-TKI treated patients was small and limited the statistical power of correlations between test results in the duration of benefit of treatment. With its nonrandomised observational design, bias may have been introduced from uneven distributions of prognostic factors and confounding variables between groups, or from the nonrandomised allocation of treatment with EGFR-TKIs. For example, demographic factors such as gender and ethnicity, were unevenly distributed between the EGFR mutation-positive, KRAS, NRAS or BRAF mutation-positive and no mutation detected groups, although hazard ratios for differences in

overall survival between these groups remained unchanged in there magnitude and significance after statistical adjustment for these and other factors. Differences in the duration of EGFR-TKI therapy between the EGFR mutation-positive, KRAS, NRAS or BRAF mutation-positive and no mutation detected groups may have been influenced by whether that treatment was given first- or second-line all by the results of the original Roche Cobas EGFR Gene Mutation Test results.

2.5 Conclusion

In conclusion, this clinical validation study has demonstrated the validity of the Agena Mass Array OncoFocus assay for detecting EGFR, KRAS, NRAS and BRAF mutations in NSCLC tumour specimens, and its potential clinical usefulness for prediction of prognosis and clinical benefits from EGFR-TKI treatment. Each instrument had a number of results that were discordant with a third reference assay leading the study to highlighted, with the agreement analysis, that there is no one perfect test available in this area and consideration to testing platform should be given. Sample quality should always be assessed to avoid false negatives being reported and only samples provided with H&E slides indicating tumour area within the FFPE section should be considered for analysis.

Chapter 3

Analytical Validation Studies

3.1 Introduction

In New Zealand it is permissible to develop in house laboratory testing methods and validate them for clinical use. However, before the results produced by such methods can be considered reliable, test methods must be shown to be fit for purpose. Method validation and verification provides objective evidence that a method is in fact creditable, and is sensitive and specific for the assay targets.

In order for the Agena Mass Array OncoFocus assay method to be a practical streamlined diagnostic test, further verification steps for assessing the assay needed to be carried out. Hands on time needed to be reduced to an absolute minimum, and any area where mistakes could be made were removed, reduced or mitigated.

From a current clinical viewpoint the validation of a molecular method for specific onco-mutation targets would only be justifiable if the method can reliably detect mutations in FFPE tissue. It was therefore logical to apply the method used in chapter 2 to retest the EGFR study cohort, in order to validate this method. Method sensitivity and specificity was also established to ensure that the assay reaches the same performance level as other methodologies currently available, which have a reported mutation frequency down to 5-10%.

The aim of this chapter was to investigate and establish the suitability of the Agena Mass Array OncoFocus assay for application as a clinically validated diagnostic assay in our laboratory. In order to establish the suitability of this panel as a clinical test we investigated establishing a low abundance control. We also investigated the establishment of the OncoFocus master mix stability and a viable worksheet for creating these master mixes in batches suitable for clinical use. Following the establishment of the above mentioned we established the ability of the OncoFocus panel to detect mutations across the 4 key oncogenes covered by the panel. Quality control samples and samples with known mutation profiles were sourced for use in this validation study.

Chapter 2 of this thesis covers the comparison of clinically detected EGFR mutations using the Agena Mass Array OncoFocus assay against those found by the Roche Cobas EGFR Gene Mutation test. This chapter will focus on further validating the remaining gene mutations (BRAF, KRAS & NRAS) detected by the multigene OncoFocus assay not validated in the EGFR section above.

3.2 Materials and Methods

Specific extraction kits and samples, not previously available or used in chapter 2, were required to complete this section of the study and are described below.

3.2.1 Reagents & Consumables

The Promega FFPE gDNA extraction kit was purchased from InVitro Technologies, Auckland NZ. Sample extraction wet laboratory steps were carried out as per the method explained below. The OncoFocus PCR and extension reaction primers were purchased from Agena Bioscience, San Diego USA. PCR and extension reaction iPlex Pro reagent kits were purchased from Agena Bioscience, San Diego USA. PCR plates and plate seals were purchased from Thermoscientific, Auckland NZ. Water was Milli Q grade (Millipore, Bedford, MA, USA). PCR, shrimp alkaline phosphatase (SAP), extension and Mass Array wet laboratory steps were carried out as per the method explained in Chapter 2, with a brief explanation below.

3.2.2 Human samples

FFPE samples were received de-identified with fictitious patient details and simplified clinical detail. Cell lines were received as coded samples. All experimental procedures involving the use of human tissue were approved by Health and Disability Ethics Committees (HDECs) under the following approvals – 13/NTA/2 & 13/NTB/165.

Each FFPE sample was extracted using the Promega ReliaPrep FFPE gDNA Miniprep System. This extraction method was chosen as it provides a complete, all-inclusive method for purifying quality genomic DNA from FFPE tissue without using hazardous solvents or overnight digestion. Genomic DNA can be isolated from FFPE tissue using this method in less than three hours with approximately half an hour hands-on time.

3.2.3 Major Equipment/materials

The following were required and not supplied with the kit, micro centrifuge, 100% ethanol, vortex mixer, micro centrifuge tubes, heating block set at 56 ^{o}C, heating block set at 80 ^{o}C, electronic timer.

3.2.4 Extraction Method

Using a new pair of gloves one extraction tube, one spin column and one elution tube were set up and labelled for each sample to be extracted. For the 10-reaction size extraction kit 12ml of 100% ethanol was added to the bottle containing 3 ml of concentrated wash solution. The lid of the wash bottle was then marked to indicate the ethanol had been added. The reagent was then stable at room temperature when capped tightly.

For tissue that was slide mounted the following steps were then followed. Using a fresh pair of gloves and a new sterile scalpel blade the marked up tissue was scraped into a 1.5 ml micro centrifuge tube that had been pre-labelled with identifying information. Care was taken avoiding excess wax or areas of tissue that did not contain tumour, except in the instance the normal tissue was the desired material. Gloves were changed after each sample to avoid potential cross contamination of extracted samples.

3.2.5 De-paraffinisation Using Mineral Oil

Mineral oil was added to the sample in the 1.5ml micro centrifuge tube, 300 µl of mineral oil was added, for up to 5 x 6 micron sections (1 cm^2). This mixture was then incubated at 80 °C for 1 minute. The micro centrifuge tube as then vortexed to mix.

3.2.6 Sample Lysis

In order to lyse the tissue 200 µl of lysis buffer was added to the sample. The lysed sample was then centrifuged at 10,000 × *g* for 15 seconds at room temperature. Two phases formed within the micro centrifuge tube, a lower, aqueous, phase and an upper, oil, phase. 20 µl of Proteinase K was then added directly to the lower phase, and this lower phase was mixed by pipetting up and down. The mixed sample was then incubated at 56 °C in a preheated heating block for 1 hour before being moved to a second preheated heating block for incubation at 80 °C for a further 1 hour. After this two hour incubation period the sample was allowed to cool to room temperature. The sample was then centrifuged briefly at room temperature to collect any drops that had formed on the inside of the micro centrifuge tube lid.

3.2.7 RNase Treatment

To remove any unwanted RNA from the sample 10 µl of RNase A was added directly to the lysed sample in the lower phase. The lower phase of the sample was then mixed again by pipetting. The sample was then incubated at room temperature for 5 minutes.

3.2.8 Nucleic Acid Binding

In order to bind the remaining nucleic acids 220 µl of BL buffer was added to the lysed sample before 240 µl of 100% ethanol was also added to the sample. This mixture was then vortexed briefly to mix. Following the vortex the sample was then spun at 10,000 × *g* for 15 seconds at room temperature. As previously two phases formed, the lower aqueous phase and the upper oil phase. The entire lower aqueous phase was then transferred from the primary micro centrifuge tube into the prepared binding column/collection tube assembly. Care was taken to include any precipitate that had formed. The primary micro centrifuge tube containing the remaining mineral oil was then discarded. The binding column/collection tube assembly was then spun at 10,000 × *g* for 30 seconds at room temperature. Following this the flow through was discarded before the binding column was reinserted into the collection tube.

3.2.9 Column Washing and Elution

Immediately following the previous step 500 µl of 1x wash solution, with ethanol, was added to the binding column. The column was then caped before the sample was spun at 10,000 × *g* for 30 seconds at room temperature. The flow through was again discarded before the binding column was reinserted into the same collection tube used for the nucleic acid binding. 500 µl of 1x wash solution was again added to the binding column before it was caped. The sample was then spun at 10,000 × *g* for 30 seconds at room temperature. As previously the flow through was discarded prior to the reinsertion of the binding column into the collection tube used for the nucleic acid binding. The cap was then opened on the binding column, the binding column/collection tube assembly was then spun at 16,000 × *g* for 3 minutes at room temperature to dry the column. Centrifuging with the cap open was to ensure thorough drying of the column which is important to prevent carryover of ethanol to the eluate. The binding column was then transferred to a clean 1.5 ml micro centrifuge tube prior to discarding the original

collection Tube. 50 µl of ultrapure water was then added directly to the membrane in the column before it was capped. The capped column was then allowed to sit at room temperature for 2 minutes. Following the 2 minute room temperature incubation the binding column/collection tube assembly was spun at 16,000 × *g* for 1 minute, at room temperature. The binding column was then removed and discarded prior to the micro centrifuge tube being caped. The eluted DNA contained within the capped micro centrifuge tube was then able to be used or stored at an appropriate storage temperature.

3.2.10 Post extraction clean up

All unused kits were then packed away including equipment and consumables for safe storage and reuse at a later date. The hood was then wiped out with 70% ethanol before being shut down.

3.3 Instrumentation – PCR Thermal Cycler & Agena Mass Array System

The GeneAmp PCR System 9700 from Thermofisher Scientific, Auckland NZ, was used for PCR, SAP & extension cycles, and a summary of these steps is presented here. With 12 wells per sample, the Agena Mass Array OncoFocus assay was able to interrogate all required diagnostic sites. In each well, 2 µl of sample DNA was added to 3 µl of PCR master-mix containing the required PCR primers. The PCR plate was cycled using the iPlex Pro standard cycling conditions, thereby increasing the number of amplicon sequences. At the completion of the PCR cycle, 2 µl of SAP master mix was added to all 12 wells for each sample, in order to dephosphorylate the unincorporated dNTPs, eliminating them from the single base extension reaction that follows. In preparation for the final cycling step, 2 µl of the corresponding extension master-mix was added to each of the 12 primer pools. The ddNTPs included in this master-mix permit only a single base extension to occur under standard iPlex Pro cycling conditions, allowing the specific interrogation of each diagnostic site of interest. The final extension cycle of the PCR plate was carried out to complete the amplification process and target the relevant diagnostic sites. The extension products were then desalted with the addition of a resin to the plate. This mixture was diluted with 16 µl of milliQ water before being rotated for 30 minutes, exposing all of the reaction mix to the resin beads. Centrifugation was then used to pack the

resin at the bottom of the plate, allowing the supernatant to be aspirated by a Nano dispensing robot. 15 nL of this desalted reaction mixture was then spotted onto a Spectro Array chip, in preparation for analysis in the MALDI-TOF mass spectrometer. The Agena Mass Array MALDI-TOF system from Agena Bioscience, San Dieago USA, was used for analytical processing of the assay PCR products. Assay design files and sample parameters were entered into the MALDI-TOF-MS, allowing identification of the corresponding wells in relation to their original sample. Data from the MALDI-TOF-MS was available in the database server and was accessed and interpreted using Typer 4 software.

3.4 Analysis Software

The Typer 4 software, Agena Bioscience, San Dieago, USA, was designed to interpret the MALDI-TOF-MS output spectra and call any detected mutant peaks using the loaded assay design parameters with each assay chemistry, in this case the OncoFocus assay. Mutation calls detected by the Typer 4 software were output in a human readable excel file, as shown in table 13. This mutation file gives information on the gene & mutation detected and a mutation frequency for the detected mutation.

3.5 Establishment of a low abundance control

The Agena Mass Array method uses the relationship between the wild type allele and the mutant allele peak height in the mass spectra for each target site to calculate mutation frequency. The availability of this information allows for sample manipulation to be assessed.

3.5.1 Method

After identifying a sample containing the EGFR L858R mutation and determining its mutation frequency, the corresponding FFPE slides were analysed microscopically to identify normal tissue, outside the macro-dissected tumour region previously removed for mutation analysis. This remaining tissue was selected marked up and carefully scraped off the slide into a sample tube before DNA was extracted using the Promega FFPE DNA extraction kit and supplied protocol, described above. The sample extracted from the remaining non-tumour tissue area was presumed to be predominantly normal tissue harbouring the patient's wildtype genetic profile. The extracted normal (non-tumour) tissue was then run on the Agena Mass Array OncoFocus assay using the method described in chapter 2 above. The confirmed normal DNA was then used to dilute the known L858R mutation positive sample, from the same FFPE slides, to 10% mutation frequency. Normal DNA was mixed with the mutation positive DNA and retested before being adjusted with more normal DNA and retested until the sample had a detectable mutation frequency of ~10%, Figure 10 & Figure 11.

3.6 Establishment of Master Mix Stability

Reagents for the Agena Mass Array OncoFocus assay come frozen and sufficient to run 320 samples per assay kit. These reagents are in their component tubes and must be made up fresh as a master mix for each assay run. There is no information provided with the assay kit related to master mix stability once made up. The stability of pre-made master mix working stocks is investigated below.

3.6.1 Method

The provided standard Agena Mass Array OncoFocus method was followed, and the x1 recipe was used to create a batch of master-mix sufficient for 50 samples, including a reduced overhang of 5% (Table 10). The batch was then split evenly into ten iPlex Pro reagent sets, each with the required 12 PCR and corresponding 12 Extension master-mixes. These sets were immediately frozen to -20 ˚C. One reagent set was then removed from storage every seven days and thawed for use. The standard Agena Mass Array OncoFocus method described in chapter 2 was used to analyse the low abundance control. This process was repeated, over a course of eight 7-day cycles, to establish master-mix stability over a two month period.

3.7 Establishing OncoFocus Master Mix Work Sheet

PCR, SAP & extension master mixes can be made up as working stocks and frozen in convenient aliquots to be used with batch analysis. For this a worksheet is required to track reagent lot numbers and expiry dates and to provide a visual reference to be used in the process of making up the master mixes.

3.7.1 Method

Using the x1 recipe supplied with the OncoFocus assay a work sheet was devised that was able to be used as a visual reference during master mix batch creation. It was decided to set the batch size at 5 samples and 4 vails of each master mix, the calculations for each reagent were recorded on to the worksheet. Boxes were added to allow information written on each vial of master mix to be recorded on the sheet as a cross reference. On the reverse side of the sheet sections were added to record the reagents lot numbers and expiry dates. There was also a section added to record the master mix aliquot assigned lot numbers and expiry date and a position for the operator to sign for traceability. Figure 12 shows a working version of the designed sheet.

3.8 Establishing Multigene Panel Validity

3.8 Cell line & RCPA Quality Assurance Program Comparisons

The OncoFocus panel detects mutations across 4 key oncogenes. To evaluate the OncoFocus Panel for mutation detection across these 4 genes, samples from two sources were used: one sample set being of a known mutation status prior to analysis, while the second sample set consisted of RCPA-QAP samples for which the results were revealed only after analysis. In addition to the retesting covered in chapter 2, further panel cross validation studies were carried out to confirm the panel's ability to detect onco-mutations in clinical samples. The OncoCarta v.1 panel was used to detect and confirm somatic mutations in the New Zealand Melanoma cell lines, which were sourced from the Auckland Cancer Society Research Centre.

The mutations in the database with their corresponding Agena Mass Array result have been listed on the cross panel validation study BRAF/KRAS sheet (Table 11).

The EGFR, KRAS and BRAF testing was carried out in the Liggins Institute, as part of the Royal College of Pathologists Australasia – Quality Assurance Programme (RCPA-QAP) over a per of four years with 100% concordance. This programme carried out analysis of 69 clinical FFPE tissue samples with blinded results, as well as ten cell lines with known mutation signatures. The entire set of samples were previously shown to carry 41 mutations detectable by the OncoFocus panel, of which 100% of these were confirmed with the OncoFocus Panel (N=79). In the samples evaluated, 16 unique mutations were identified. The overall mutation status for the sample set was analysed (Figure 13 & Table 12) in which the number of observations for each unique mutation ranged from zero to eight.

3.9 Resµlts

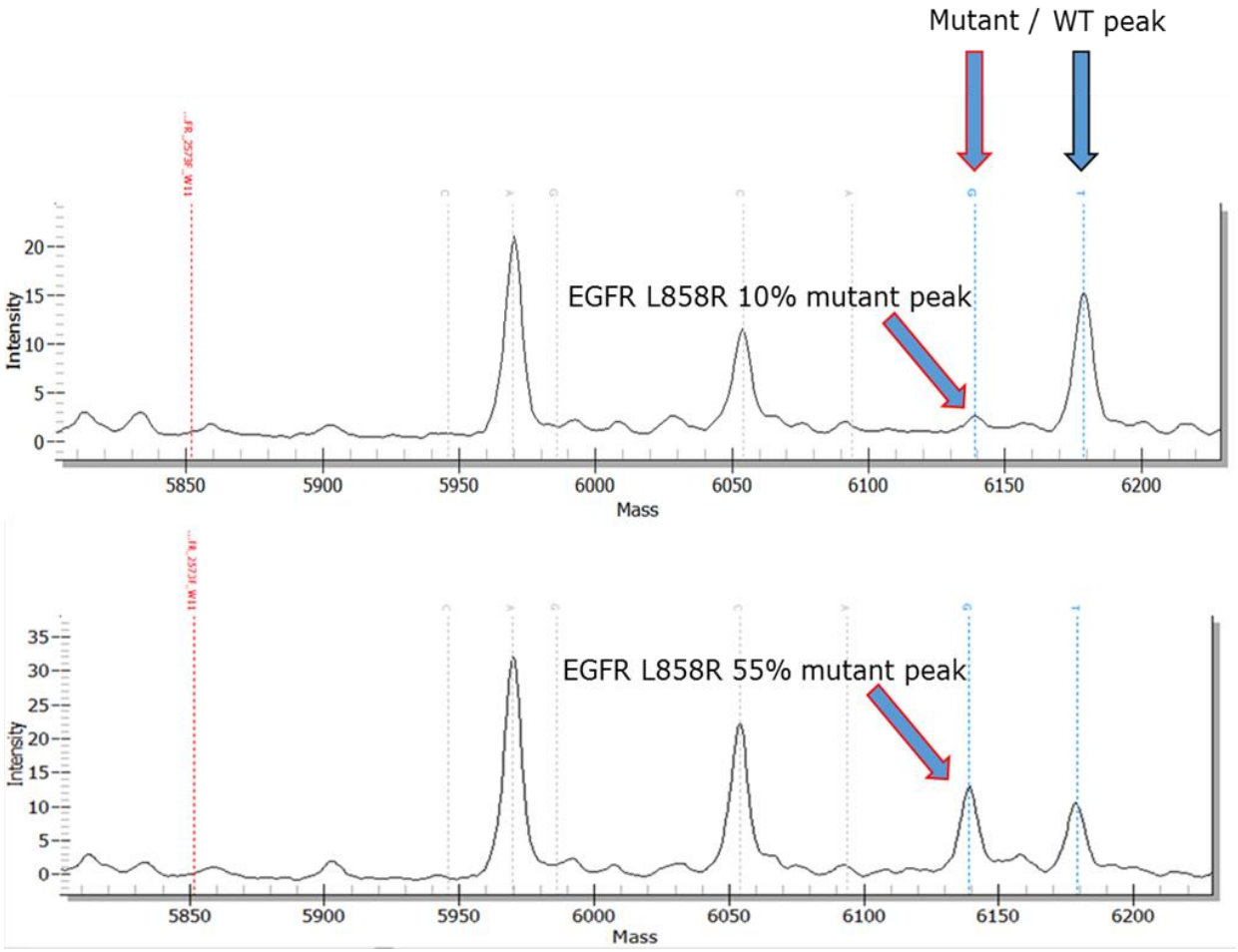


Figure 10 Low Abundance Control. Lower spectra showing the original EGFR L858R mutant peak (arrow – red outline) in relation to its wild type peak (arrow – blue). Upper spectra showing the L858R mutation peak diluted to 10% (arrow – red out line) in relation to its wild type peak (arrow – blue).

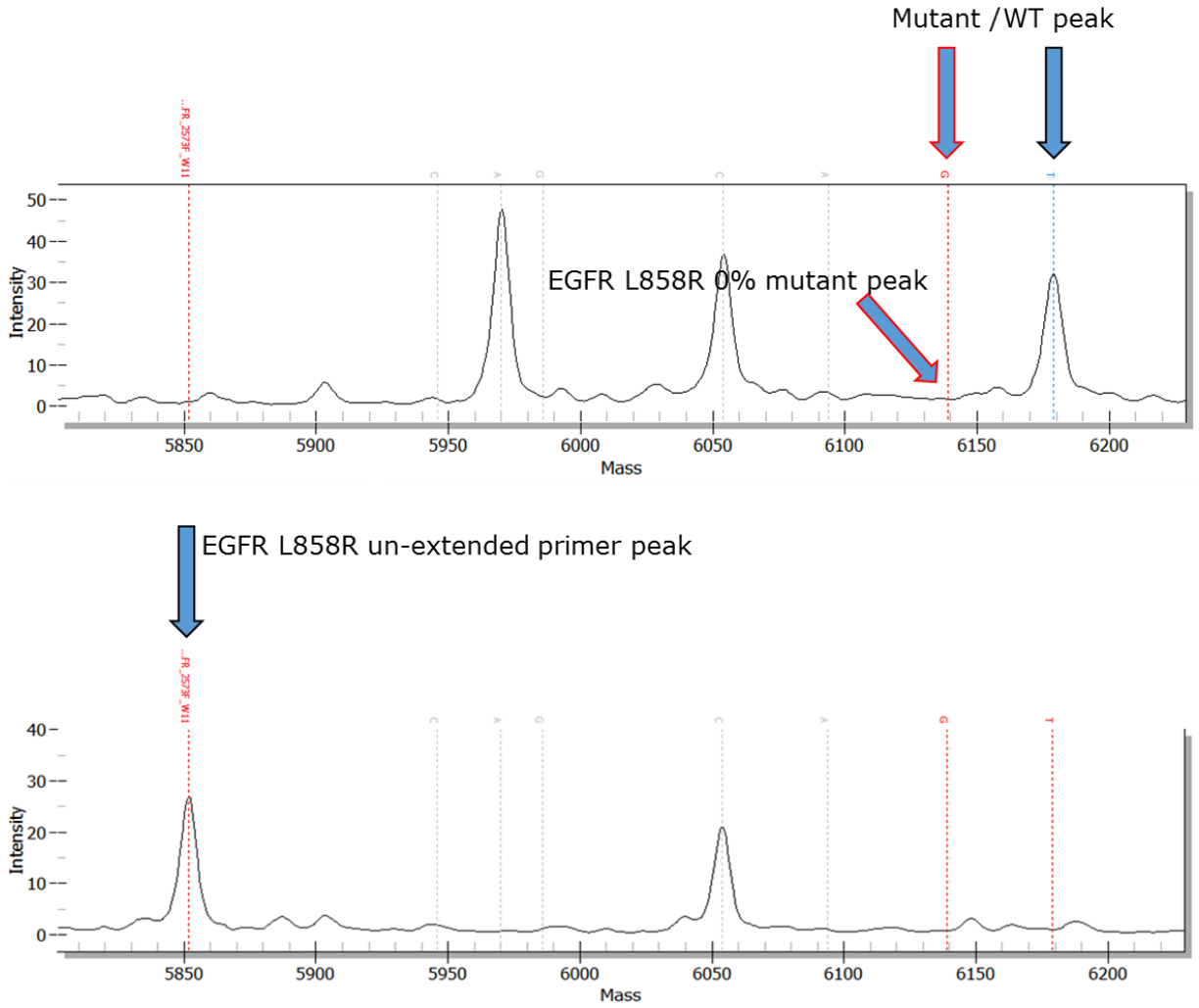
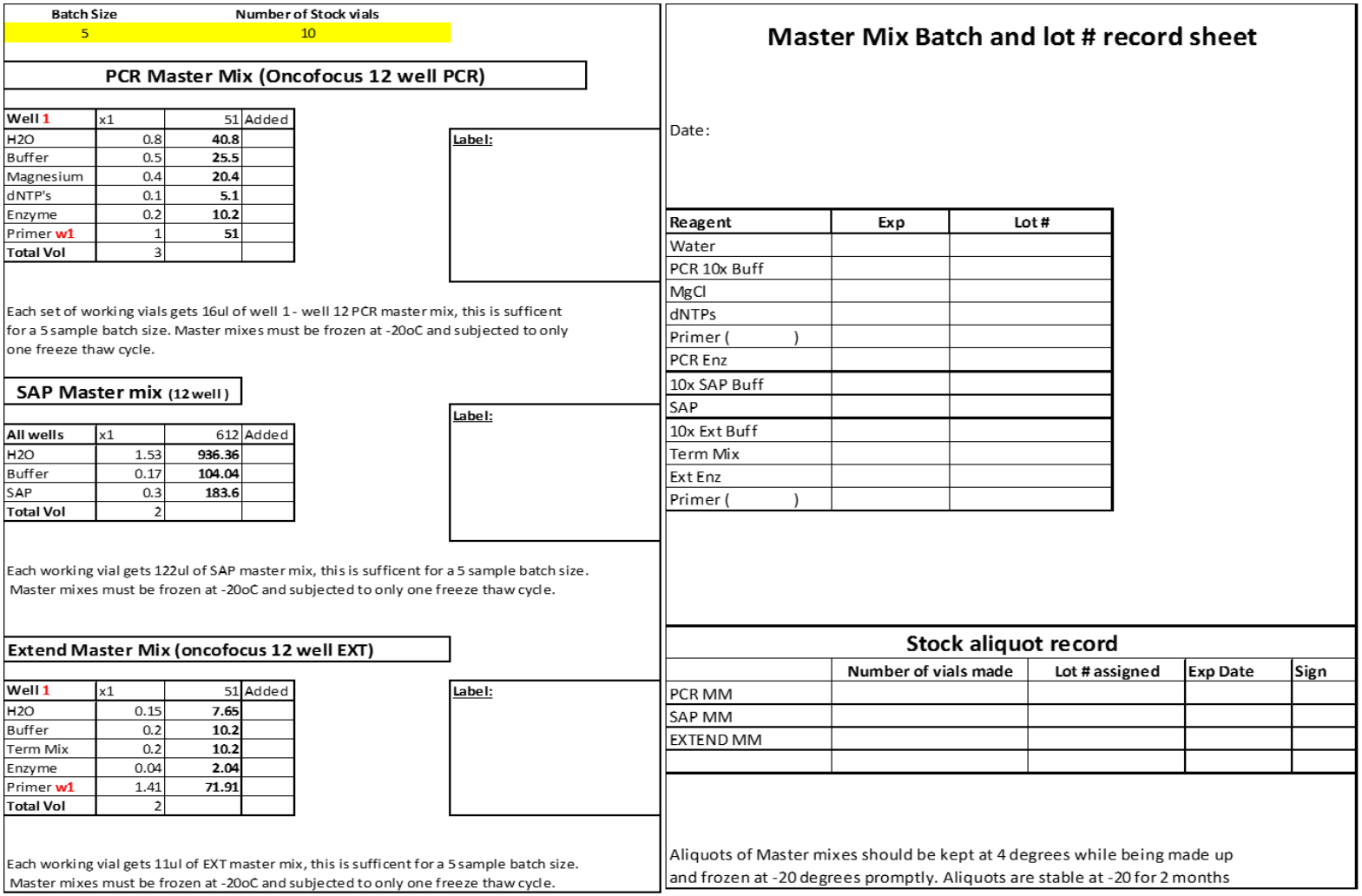


Figure 11 Lower spectra showing the EGFR L858R wild type and mutant peaks absent and the presence of the un-extended primer peak (arrow – blue) in an assay with very poor DNA quality. Upper spectra showing the L858R mutation peak absent indication no mutation detected (arrow – red out line) in relation to its wild type peak (arrow – blue).

Stability Data for OncoFocus Master Mixes								
	7 days	14 days	21 days	28 days	35 days	42 days	49 days	56 days
L858R mutation peak (%)	10	10	10	10	9	10	10	9
WT peak (%)	90	90	90	90	99	90	90	99

Table 10 Master Mix stability. Stability data for the made-up OncoFocus master mixes. L858R mutation detected at ~10% when tested at 7 day intervals over a 56 day stability testing period.



Batch Size	Number of Stock vials
5	10

PCR Master Mix (Oncofocus 12 well PCR)

Well 1	x1	51	Added
H2O	0.8	**40.8**	
Buffer	0.5	**25.5**	
Magnesium	0.4	**20.4**	
dNTP's	0.1	**5.1**	
Enzyme	0.2	**10.2**	
Primer w1	1	**51**	
Total Vol	3		

Label:

Each set of working vials gets 16ul of well 1 - well 12 PCR master mix, this is sufficent for a 5 sample batch size. Master mixes must be frozen at -20oC and subjected to only one freeze thaw cycle.

SAP Master mix (12 well)

All wells	x1	612	Added
H2O	1.53	**936.36**	
Buffer	0.17	**104.04**	
SAP	0.3	**183.6**	
Total Vol	2		

Label:

Each working vial gets 122ul of SAP master mix, this is sufficent for a 5 sample batch size. Master mixes must be frozen at -20oC and subjected to only one freeze thaw cycle.

Extend Master Mix (oncofocus 12 well EXT)

Well 1	x1	51	Added
H2O	0.15	**7.65**	
Buffer	0.2	**10.2**	
Term Mix	0.2	**10.2**	
Enzyme	0.04	**2.04**	
Primer w1	1.41	**71.91**	
Total Vol	2		

Label:

Each working vial gets 11ul of EXT master mix, this is sufficent for a 5 sample batch size. Master mixes must be frozen at -20oC and subjected to only one freeze thaw cycle.

Master Mix Batch and lot # record sheet

Date:

Reagent	Exp	Lot #
Water		
PCR 10x Buff		
MgCl		
dNTPs		
Primer ( )		
PCR Enz		
10x SAP Buff		
SAP		
10x Ext Buff		
Term Mix		
Ext Enz		
Primer ( )		

Stock aliquot record

	Number of vials made	Lot # assigned	Exp Date	Sign
PCR MM				
SAP MM				
EXTEND MM				

Aliquots of Master mixes should be kept at 4 degrees while being made up and frozen at -20 degrees promptly. Aliquots are stable at -20 for 2 months

Figure 12 Master Mix Working Stocks. To ensure reliable results are produced a master mix worksheet is required to record details of each master mix batch. Volumes for each tube are shown and details entered on each tube can be recorded. Reagent lot numbers were recorded on the reverse side of the worksheet.

Cross Panel Validation

Number	Gene	Sample	Database result	OncoCarta V1 Result	OncoFocus Result	MelaCarta
1	BRAF	S4301	V600K	V600K	V600K	V600K
2	BRAF	S1402	V600K	V600K	V600K	V600K
3	BRAF	S5202	V600E	V600E	V600E	V600E
4	BRAF	S404	V600E	V600E	V600E	V600E
5	BRAF	S2805	G469A	G469A	G469A	G469A
6	KRAS	S2406	G12D	G12D	G12D	Not assayed
7	KRAS	S4807	Q61K	Q61K	Q61K	Q61K
8	KRAS	S6308	G13L	G13R	Not assayed	G13R
9	WT	S109	WT	WT	WT	WT
10	WT	S210	WT	WT	WT	WT

Table 11 Cross panel validation showing mutations identified by each Agena Mass Array panel compared against the New Zealand melanoma database result.

Samples not tested using a specific panel are resulted as “Not assayed”. WT = Wild Type allele detected.

Gene	Mutation	Expected Result
EGFR	L858R	4
EGFR	L861Q	1
EGFR	E746_A750Del	1
EGFR	L747_T751Del	1
EGFR	E746_T751>VAorVP	1
EGFR	T790M	1
EGFR	NMD	13
KRAS	G12D	5
KRAS	G12V	2
KRAS	G13D	2
KRAS	G12S	2
KRAS	G12C	1
KRAS	NMD	11
BRAF	V600E	8
BRAF	V600K	4
BRAF	NMD	10

Table 12 RCPA-QAP vs Mass Array. Summary of RCPA QAP results over a 4 year period, 2012 – 2015 inclusive.

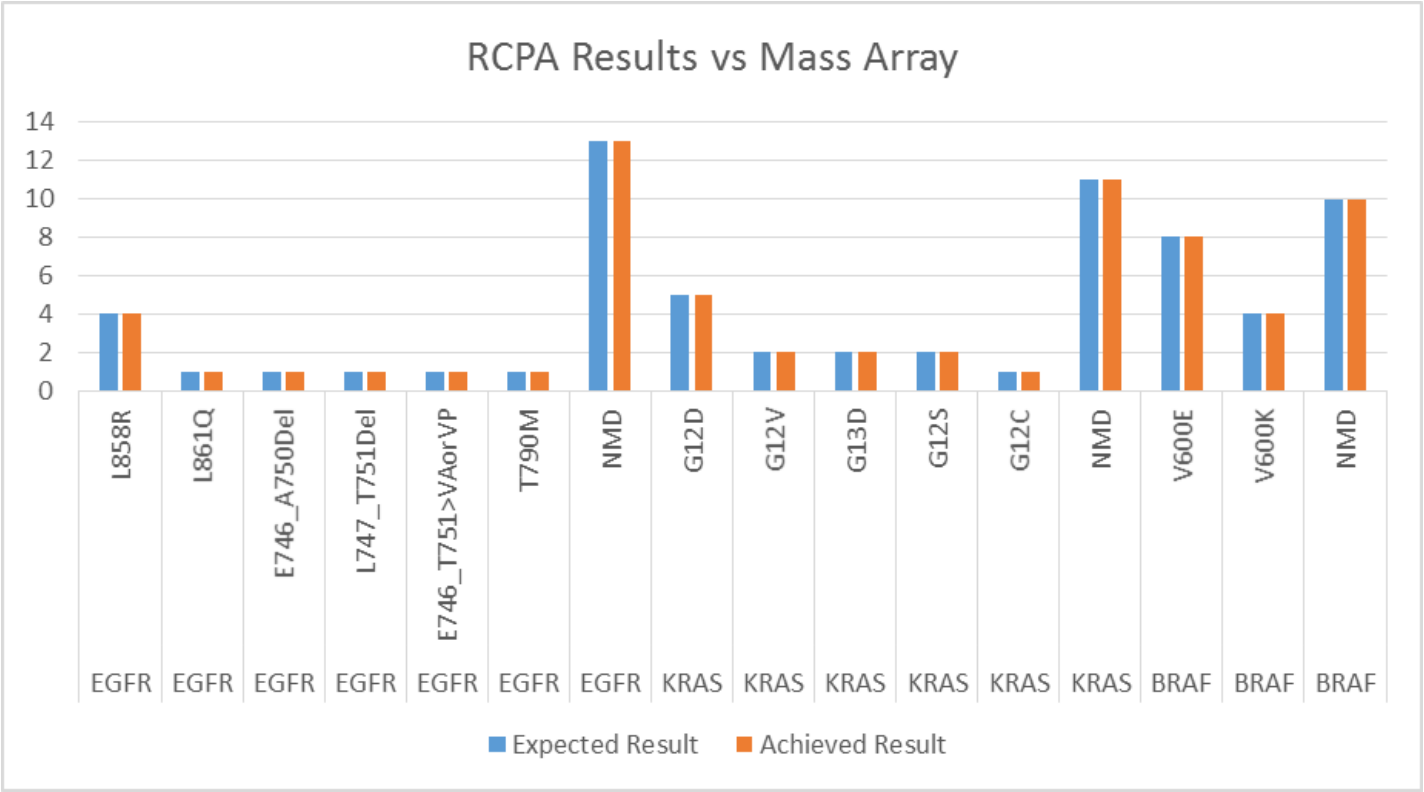


Figure 13 RCPA-QAP vs Mass Array. Confirmation of Mutational Status in RCPA-QAP samples using the OncoFocus Panel

Sample	Gene	Assay	Mutation	Allele	WT Freq	Mut Freq	Z-score	Confidence
1	KRAS	OF_KRAS_34-36 (CA)	G12C	T(KRAS_34F_W2) & G(KRAS_35R_W1) & T(KRAS_36R_W12)	0.858	0.142	10	1.High
2	KRAS	OF_KRAS_34-36 (CA)	G12D	G(KRAS_34F_W2) & A(KRAS_35R_W1)	0.492	0.508	10	1.High
3	EGFR	OF_EGFRExon19 (CA)	L747_T751del	DEL(EGFR_2240F_W4) & DEL(EGFR_2252R_W1)	0.442	0.502	10	1.High
4	KRAS	OF_KRAS_34-36 (CA)	G12C	T(KRAS_34F_W2) & G(KRAS_35R_W1) & T(KRAS_36R_W12)	0.613	0.387	10	1.High
5	EGFR	OF_EGFR_2572-2573 (CA)	L858R	C(EGFR_2572F_W7) & G(EGFR_2573F_W9)	0.427	0.573	10	1.High

Table 13 Typer 4 Mutation List. The Agena Mass Array Typer 4 analysis software produces an excel file displaying all detected or suspected mutations. Typer 4 uses the relationship between the wild type allele and the mutant allele peak height in the mass spectra at each target site to calculate mutation frequency for the detected mutation. The Wild Type Frequency & Mutation Frequency rows show the percentage that each peak contributes to the total. The total number can be greater or less than 100% in samples where multiple extension primers are used to deduce the mutation, this is shown in sample 3 were an exon 19 deletion has been detected.

3.10 Discussion

3.10.1 Establishment of a Low Abundance Control

In order to assess the sensitivity of the OncoFocus assay locally, a low abundance control was required. An aliquot of DNA from a sample containing a known EGFR L858R mutation was selected. This particular mutation was chosen as it is recognised as the single most common mutation found in non-small cell lung carcinoma (NSCLC), with a frequency of detection in this condition sitting at 43%, although as a group Exon 19 Deletions make up the most commonly occurring mutations detected in NSCLC at 48% (C Lovly, L Horn, & W Pao, 2015c). When the control sample was selected, specific checks were made to ensure that sufficient remaining normal tissue was available on slides previously extracted for the original assay. It was crucial that this sample possess a clinically relevant oncogenic mutation, while also containing an excess of remaining normal FFPE tissue, as the sample could then be used as wild type diluent to bring the mutation frequency in the chosen extraction to 10% while keeping the wild type mutation high. Commercial controls are available, however it was decided that an in-house control would be easier to acquire, as well as being more cost effective. The Typer 4 software gives a reliable comparison of wild type versus mutation percentage (Figure 10), allowing the in-house designed control to be crafted to suit the unique requirements of this work. The low abundance control was then able to be used to assess the sensitivity of the assay down to the detection limit of the control (a 10% mutation frequency) and also to carry out several other experiments in this chapter. The ability to detect mutations at this clinically significant level are important for any assay whose purpose is to detect oncogenic somatic mutations from FFPE samples, owing to the degraded quality of most samples and the heterogeneity of any tumour sample.

3.10.2 Establishment of Master Mix Stability

The main aim for this section of the thesis is to understand if there can be improvements and optimisations to the supplied OncoFocus assay. For a test method to be viable in the routine diagnostic laboratory environment the process must be as streamlined as possible with the chances of introducing error minimised or removed. The method provided with the OncoFocus assay indicates

that a master mix should be made up fresh on the day of use and in a quantity suitable for the number of samples being processed. The package insert indicates that freeze thaw cycles of the reagents should be minimised, with the main concern being that freeze thaw cycles of the PCR dNTP's are kept to 10 or less cycles. Given diagnostic samples come into a laboratory daily and require fast turnaround times multiple small batches of 5-10 sample at a time are required in a week these recommendations and requirements make the assay kit unsuitable for routine use in its current format. A reasonable solution was to pre-make and freeze a larger quantity of the master-mix containing the necessary enzyme and primers, however the stability of such a mix after thawing needed to be investigated before implementing this in the lab. Each iPlex Pro kit containing the dNTP vial is sufficient for approximately 300 samples, in the 384 well format. This would require in the vicinity of 30 freeze thaw cycles if master mixes were made up on an as needed basis. It was therefore necessary to devise and validate a suitable master mix format that both meet the manufacturer's recommendations and meets the needs of routine use. It is possible to aliquot the reagents into smaller volumes to limit the number of freeze thaw cycles however it was believed, after consulting laboratories on their expected weekly through-put, that making complete master mixes for a batch size of 5 would give the optimal use of reagents and convenience to staff. Having the master mix pre-made allows sample processing hands on time to be drastically reduced. Making up batches of master mixes allows the full concentration of the staff member to be given to this critical job. It is the aim of this chapter to identify steps for streamlining a method that enhances the laboratory workflow. The master mix batch size was set at 5 samples per batch and these aliquots were immediately frozen to -20 ^{o}C to protect the integrity of the enzyme. An aliquot was then removed from storage every 7 days and thawed for use, this was carried out for 8 7-day cycles to establish master mix stability over a 2 month period. The thawed master mix was used to analyse the low abundance control to ensure not only did the assay work but that it was able to detect a clinically relevant oncomutation at low mutation frequency, results are displayed in Table 10. These clearly demonstrate no loss of activity over a 2 month period and the ability of the assay to continue detecting the low abundance mutation.

3.10.3 OncoFocus Master Mix Working Stocks

After the validation study above confirmed that there was no loss of activity from a single freeze thaw cycle or from the combination of reagents into a pre-made master mix, PCR, SAP & Extend master mixes can be made up as working stocks and frozen in convenient aliquots to be used with batch analysis.

The x1 recipe was used to make up a set of 10 master mixes each sufficient to run 5 samples. The rationale behind this number was, if a batch of 10 master mixes was made up owing to the fact a laboratory in New Zealand is likely to receive in the region of 50-60 samples per month the batch will last approximately one month allowing 2-3 batches per week to be run. Given the assay turnaround time should be less than 5 days from sample receipt in the laboratory (recommendation from the faster cancer treatment initiative), but preferably closer to 3 days this configuration was identified as an ideal scalable format. Further changes to this format could be made by any laboratory using this test methodology citing the validation work done on the master mix stability. It should however be noted that these working stocks in any volume must be immediately frozen to -20 ^{o}C and be subjected to only one freeze thaw cycle, any remaining master mix must be discarded after use. The master mix is to be kept for a maximum of 2 months and discarded if not used. This is to ensure enzyme activity is not compromised by pushing beyond the validation parameters established. If a laboratory was experiencing occasions where reagents were not used before their 2 month expiry it would be advisable to reduce the batch number made up.

3.10.4 Cross Panel Validation

In addition to the retesting covered in chapter 2 further panel cross validation studies were carried out to validate the panel's ability to detect onco-mutations in clinical samples across other clinically relevant onco-genes. To evaluate the OncoFocus Panel for mutation detection, samples from 2 sources were used, the mutation status of the first sample set was known and is discussed below prior to analysis while the second sample set consisted of RCPA-QAP samples, and results for these samples were revealed only after analysis The OncoCarta v1 panel was used in a previous research study to detect and confirm somatic mutations in the New Zealand Melanoma cell lines held by the

Auckland Cancer Society Research Centre. This data comparison is shown in Table 11 with the corresponding Agena Mass Array result listed on the cross panel cross validation study BRAF/KRAS sheet. These results show 90% concordance across panels and with that of each categorised cell line. There were 10 samples for which 3 Agena Mass Array panels were cross validated. There was one result, number 8, which had a database result showing KRAS G13L, however both the OncoCarta v1 and MelaCarta panels detected a KRAS G13R mutation. The G13L is not commonly found in melanoma tissue and it is therefore suspected that the previous Sanger sequencing results for this cell line contained an error. It would be ideal to confirm this mutation using another technology.

3.10.5 RCPA Quality Assurance Program

The Liggins Sequenom Facility (Liggins Institute, University of Auckland) participated in EGFR, KRAS and BRAF testing in The Royal College of Pathologists of Australasia Quality Assurance (RCPA QAP) (New South Wales, Australia) program for 4 years with 100% concordance. These reports were available and are summarised in table 12. Results from the RCPA samples show 100% concordance across 3 genes represented in the OncoFocus panel EGFR, BRAF & KRAS, NRAS was not covered by the RCPA QAP program. These samples are received blinded and run with no prior knowledge of the mutation status of the sample. All steps in the process are identical to how a diagnostic sample would be processed. Because samples are received as unfixed FFPE slides with a corresponding haematoxylin and eosin (H&E) stained slide the laboratory process is assessed from beginning to end. The FFPE slides received for testing came in batches of approximately 6 samples and 2 sets per year, these had been received each year over a 4 year period 2012 – 2015 inclusive.

To evaluate the OncoFocus Panel for mutation detection, the RCPA-QAP samples are an unbiased way to ensure the assay performs as expected. The RCPA QAP samples are sent out and tested across most molecular diagnostic laboratories in New Zealand and Australia. As a consequence these samples are tested by a range of testing methodologies.

In total between the cross panel validation study samples and the RCPA QAP study samples sixty nine clinical formalin-fixed paraffin embedded tissue samples with blinded results and 10 cell lines with known mutation signatures were assayed. The entire set of samples were previously shown to carry 41 mutations detectable by the OncoFocus panel, of which 100% of these were confirmed with the OncoFocus Panel (N=79). In the samples evaluated, 16 unique mutations were identified. The overall mutation status for the sample set was analysed (Figure 13) in which the number of observations for each unique mutation ranged from zero to eight.

In lung cancer there is often not a lot, if any, tissue available for molecular testing and what tissue is available is usually of poor quality. This is due largely to ~20% of patients presenting with lung cancer receiving surgery, leaving only biopsy or no tissue available. The RCPA sample cohort is of a limited number and does not only focus on the EGFR gene as with the 532 sample retested study population where there were 32 samples were non concordant between testing methodologies. It appears that the larger real world cohort indicates the RCPA samples may be too good and not a true reflection of real world molecular samples.

3.11 Conclusion

The idea of an in-house control seemed feasible and in turn worked out well. The resulting control sample with its predetermined mutation frequency is suitable for continued use to verify the sensitivity of the assay. Unlike many commercial controls the mutation frequency is very close to the clinical cut-off for this assay allowing the actual assay sensitivity to be monitored.

The master mix stability data showed that pre made master mixes subjected to no more than 1 freeze thaw cycle retain their activity and ability to detect a low abundance control sample for the full 2 month study period. This stability is well suited to the clinical environment and can easily be replicated in the diagnostic laboratory.

To ensure reliable results are produced a standardised worksheet was required, the sheet designed as part of this study allows all critical reagents and volumes to be checked off as they are used to make up batches of master mix for the assay. Additionally there is space for recording lot numbers and expiry dates of all reagents and made up master mixes.

The cross panel validation show good concordance, even detecting an anomaly in a previously categorised cell line.

The RCPA samples are pre-selected and while results are not revealed until after testing the level of concordance compared with that of the full study data indicates the samples may be too good. It would be the recommendation of the study that a low abundance control sitting on the lower level of detection be included as an additional sample released with each RCPA QAP set, failing this, that a low abundance control is established locally for each testing laboratory and that the control is then assayed alongside the RCPA samples and at every batch or lot change over. This will ensure not only that the assay can detect specific mutations but can also detect the mutations down to a clinically significant level.

Overall the Agena Mass Array OncoFocus method has been optimised for clinical use and proven to detect clinically relevant oncogenic driver mutations in 4 key genes EGFR, BRAF, KRAS & NRAS when DNA is extracted from FFPE tissue. The method has also been validated against the low abundance control and external quality control samples. Further application of this analytical assay to clinical oncogenic studies is warranted.

References

Angμlo, B., Conde, E., Suárez-Gauthier, A., Plaza, C., Martínez, R., Redondo, P., . . . Hidalgo, M. (2012). A comparison of EGFR mutation testing methods in lung carcinoma: direct sequencing, real-time PCR and immunohistochemistry. *PloS one, 7*(8), e43842.

Arcila, M., Lau, C., Nafa, K., & Ladanyi, M. (2011). Detection of KRAS and BRAF mutations in colorectal carcinoma: Roles for high-sensitivity locked nucleic acid-PCR sequencing and broad-spectrum mass spectrometry genotyping. *The Journal of Molecμlar Diagnostics, 13*(1), 64-73.

Biocartis. Idylla EGFR mutation assay. Retrieved Febuary 20 2017, from https://www.biocartis.com/idylla-egfr-mutation-assay

Cappuzzo, F., Bemis, L., & Varella-Garcia, M. (2006). HER2 mutation and response to trastuzumab therapy in non–small-cell lung cancer. *New England Journal of Medicine, 354*(24), 2619-2621.

Corrie, P. G. (2008). Cytotoxic chemotherapy: clinical aspects. *Medicine, 36*(1), 24-28.

Dias-Santagata, D., Akhavanfard, S., David, S. S., Vernovsky, K., Kuhlmann, G., Boisvert, S. L., . . . Kwak, E. L. (2010). Rapid targeted mutational analysis of human tumours: a clinical platform to guide personalized cancer medicine. *EMBO Molecμlar Medicine, 2*(5), 146-158.

Drilon, A., Wang, L., Hasanovic, A., Suehara, Y., Lipson, D., Stephens, P., . . . Zakowski, M. F. (2013). Response to Cabozantinib in patients with RET fusion-positive lung adenocarcinomas. *Cancer Discovery, 3*(6), 630-635.

Eikuch. (2007). Epidermal growth factor receptor (EGFR) signaling pathway. *In Wikimedia. Retrieved January 12, 2017, from https://commons.wikimedia.org/wiki/File:EGFR_signaling_pathway.png*

Ellison, G., Zhu, G., Moμlis, A., Dearden, S., Speake, G., & McCormack, R. (2013). EGFR mutation testing in lung cancer: a review of available methods and their use for analysis of tumour tissue and cytology samples. *Journal of Clinical Pathology, 66*(2), 79-89.

Garraway, L. A. (2013). Genomics-driven oncology: framework for an emerging paradigm. *Journal of Clinical Oncology, 31*(15), 1806-1814.

Gatzemeier, U., Pluzanska, A., Szczesna, A., Kaukel, E., Roubec, J., De Rosa, F., . . . Serwatowski, P. (2007). Phase III study of erlotinib in combination with cisplatin and gemcitabine in advanced non–small-cell lung cancer: The Tarceva Lung Cancer Investigation Trial. *Journal of Clinical Oncology, 25*(12), 1545-1552.

Giaccone, G., Herbst, R. S., Manegold, C., Scagliotti, G., Rosell, R., Miller, V., . . . Pluzanska, A. (2004). Gefitinib in combination with gemcitabine and cisplatin in advanced non–small-cell lung cancer: A phase III trial—INTACT 1. *Journal of Clinical Oncology, 22*(5), 777-784.

Goodsell, D. S. (2001). The molecµlar perspective: the src oncogene. *The Oncologist, 6*(5), 474-476.

Herbst, R. S., Giaccone, G., Schiller, J. H., Natale, R. B., Miller, V., Manegold, C., . . . Reeves, J. A. (2004). Gefitinib in combination with paclitaxel and carboplatin in advanced non–small-cell lung cancer: A phase III trial—INTACT 2. *Journal of Clinical Oncology, 22*(5), 785-794.

Herbst, R. S., Prager, D., Hermann, R., Fehrenbacher, L., Johnson, B. E., Sandler, A., . . . Li, X. (2005). TRIBUTE: A phase III trial of erlotinib hydrochloride (OSI-774) combined with carboplatin and paclitaxel chemotherapy in advanced non–small-cell lung cancer. *Journal of Clinical Oncology, 23*(25), 5892-5899.

Huang, S.-F., Liu, H.-P., Li, L.-H., Ku, Y.-C., Fu, Y.-N., Tsai, H.-Y., . . . Kuo, H.-P. (2004). High frequency of epidermal growth factor receptor mutations with complex patterns in non–small cell lung cancers related to gefitinib responsiveness in Taiwan. *Clinical Cancer Research, 10*(24), 8195-8203.

J McKeage, M., Shepherd, P., Yozu, M., & R Love, D. (2013). Tumour Mutation Profiling with High-throughput Mµltiplexed Genotyping: A Review of its Use for Guiding Targeted Cancer Therapy. *Current Cancer Therapy Reviews, 9*(4), 236-244.

Keedy, V. L., Temin, S., Somerfield, M. R., Beasley, M. B., Johnson, D. H., McShane, L. M., . . . Giaccone, G. (2011). American Society of Clinical Oncology provisional clinical opinion: Epidermal growth factor receptor (EGFR) mutation testing for patients with advanced non–small-cell lung cancer considering first-line EGFR tyrosine kinase inhibitor therapy. *Journal of Clinical Oncology, 29*(15), 2121-2127.

Kosaka, T., Yatabe, Y., Endoh, H., Kuwano, H., Takahashi, T., & Mitsudomi, T. (2004). Mutations of the epidermal growth factor receptor gene in lung cancer. *Cancer Research, 64*(24), 8919-8923.

Kriegsmann, M., Arens, N., Endris, V., Weichert, W., & Kriegsmann, J. (2015a). Detection of KRAS, NRAS and BRAF by mass spectrometry-a sensitive, reliable, fast and cost-effective technique. *Diagnostic Pathology, 10*(1), 132.

Kriegsmann, M., Arens, N., Endris, V., Weichert, W., & Kriegsmann, J. (2015b). Detection of KRAS, NRAS and BRAF by mass spectrometry - a sensitive, reliable, fast and cost-effective technique. *Diagnostic Pathology, 10*, 132. 10.1186/s13000-015-0364-3

Kris, M. G., Johnson, B. E., Berry, L. D., Kwiatkowski, D. J., Iafrate, A. J., Wistuba, I. I., . . . Su, P.-F. (2014). Using mµltiplexed assays of oncogenic drivers in lung cancers to select targeted drugs. *Jama, 311*(19), 1998-2006.

Laine, S. (2013), *FDA approves first companion diagnostic to detect gene mutation associated with a type of lung cancer* [Press release]. Retrieved from http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm352230.htm

Lovly, C., Horn, L., & Pao, W. (2015a). BRAF. Retrieved January 4 2017, from https://www.mycancergenome.org/content/disease/lung-cancer/braf/?tab=0

Lovly, C., Horn, L., & Pao, W. (2015b). BRAF in NSCLC. Retrieved January 4 2017, from https://www.mycancergenome.org/content/disease/lung-cancer/braf/

Lovly, C., Horn, L., & Pao, W. (2015c). EGFR in NSCLC Retrieved January 4 2017, from https://www.mycancergenome.org/content/disease/lung-cancer/egfr/

Lovly, C., Horn, L., & Pao, W. (2015d). KRAS. Retrieved January 4 2017, from https://www.mycancergenome.org/content/disease/lung-cancer/kras/?tab=0

Lynch, T. J., Bell, D. W., Sordella, R., Gurubhagavatµla, S., Okimoto, R. A., Brannigan, B. W., . . . Haluska, F. G. (2004). Activating mutations in the epidermal growth factor receptor underlying responsiveness of non–small-cell lung cancer to gefitinib. *New England Journal of Medicine, 350*(21), 2129-2139.

Maemondo, M., Inoue, A., Kobayashi, K., Sugawara, S., Oizumi, S., Isobe, H., . . . Kinoshita, I. (2010). Gefitinib or chemotherapy for non–small-cell lung cancer with mutated EGFR. *New England Journal of Medicine, 362*(25), 2380-2388.

Marchetti, A., Del Grammastro, M., Filice, G., Felicioni, L., Rossi, G., Graziano, P., . . . Iacono, M. (2012). Complex mutations & subpopµlations of deletions at exon 19 of EGFR in NSCLC revealed by next generation sequencing: potential clinical implications. *PloS One, 7*(7), e42164.

Ministry of Health. (2015). *Cancer patient survival 1994 to 2011*. Retrieved from http://www.health.govt.nz/publication/cancer-patient-survival-1994-2011

Ministry of Health. (2016). *Cancer: new registrations and deaths 2013*. Retrieved from http://www.health.govt.nz/nz-health-statistics/health-statistics-and-data-sets/cancer-new-registrations-and-deaths-series

Mitsudomi, T., Morita, S., Yatabe, Y., Negoro, S., Okamoto, I., Tsurutani, J., . . . Hirashima, T. (2010). Gefitinib versus cisplatin plus docetaxel in patients with non-small-cell lung cancer harbouring mutations of the epidermal growth factor receptor (WJTOG3405): an open label, randomised phase 3 trial. *The Lancet Oncology, 11*(2), 121-128.

Mitsudomi, T., & Yatabe, Y. (2010). Epidermal growth factor receptor in relation to tumor development: EGFR gene and cancer. *The FEBS Journal, 277*(2), 301-308.

Mok, T. S., Wu, Y.-L., Thongprasert, S., Yang, C.-H., Chu, D.-T., Saijo, N., . . . Ichinose, Y. (2009). Gefitinib or carboplatin–paclitaxel in pµlmonary adenocarcinoma. *New England Journal of Medicine, 361*(10), 947-957.

Network, C. G. A. R. (2014). Comprehensive molecµlar profiling of lung adenocarcinoma. *Nature, 511*(7511), 543-550.

Oda, K., Matsuoka, Y., Funahashi, A., & Kitano, H. (2005). A comprehensive pathway map of epidermal growth factor receptor signaling. *Molecµlar Systems Biology, 1*(1)

Ou, S.-H. I., Kwak, E. L., Siwak-Tapp, C., Dy, J., Bergethon, K., Clark, J. W., . . . Bang, Y.-J. (2011). Activity of crizotinib (PF02341066), a dual mesenchymal-epithelial transition (MET) and anaplastic lymphoma kinase (ALK) inhibitor, in a NSCLC patient with de novo MET amplification. *Journal of Thoracic Oncology, 6*(5), 942-946.

Paez, J. G., Jänne, P. A., Lee, J. C., Tracy, S., Greµlich, H., Gabriel, S., . . . Boggon, T. J. (2004). EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science, 304*(5676), 1497-1500.

Pao, W., Miller, V., Zakowski, M., Doherty, J., Politi, K., Sarkaria, I., . . . Fµlton, L. (2004). EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. *Proceedings of the National Academy of Sciences of the United States of America, 101*(36), 13306-13311.

Planchard, D., Mazieres, J., Riely, G. J., Rudin, C. M., Barlesi, F., Quoix, E. A., . . . Ma, B. (2013). Interim resµlts of phase II study BRF113928 of dabrafenib in BRAF V600E mutation–positive NSCLC (NSCLC) patients: American Society of Clinical Oncology.

Reungwetwattana, T., Weroha, S. J., & Molina, J. R. (2012). Oncogenic pathways, molecµlarly targeted therapies, and highlighted clinical trials in non–small-cell lung cancer (NSCLC). *Clinical Lung Cancer, 13*(4), 252-266.

Roche. (2013). *cobas EGFR mutation test – FDA* Retrieved from http://www.accessdata.fda.gov/cdrh_docs/pdf12/P120019c.pdf

Rosell, R., Carcereny, E., Gervais, R., Vergnenegre, A., Massuti, B., Felip, E., . . . Sanchez, J. M. (2012). Erlotinib versus standard chemotherapy as first-line treatment for European patients with advanced EGFR mutation-positive non-small-cell lung cancer (EURTAC): a mµlticentre, open-label, randomised phase 3 trial. *The Lancet Oncology, 13*(3), 239-246.

Sawyers, C. (2004). Targeted cancer therapy. *Nature, 432*(7015), 294-297.

Sharma, S. V., Bell, D. W., Settleman, J., & Haber, D. A. (2007). Epidermal growth factor receptor mutations in lung cancer. *Nature Reviews Cancer, 7*(3), 169-181.

Shaw, A. T., Kim, D.-W., Mehra, R., Tan, D. S., Felip, E., Chow, L. Q., . . . De Pas, T. (2014). Ceritinib in ALK-rearranged non–small-cell lung cancer. *New England Journal of Medicine, 370*(13), 1189-1197.

Shaw, A. T., Kim, D.-W., Nakagawa, K., Seto, T., Crinó, L., Ahn, M.-J., . . . Blackhall, F. (2013). Crizotinib versus chemotherapy in advanced ALK-positive lung cancer. *New England Journal of Medicine, 368*(25), 2385-2394.

Shaw, A. T., Ou, S.-H. I., Bang, Y.-J., Camidge, D. R., Solomon, B. J., Salgia, R., . . . Costa, D. B. (2014). Crizotinib in ROS1-rearranged non–small-cell lung cancer. *New England Journal of Medicine, 371*(21), 1963-1971.

Shepherd, F. A., Rodrigues Pereira, J., Ciµleanu, T., Tan, E. H., Hirsh, V., Thongprasert, S., . . . Martins, R. (2005). Erlotinib in previously treated non–small-cell lung cancer. *New England Journal of Medicine, 353*(2), 123-132.

Thomas, R. K., Baker, A. C., DeBiasi, R. M., Winckler, W., LaFramboise, T., Lin, W. M., . . . MacConaill, L. E. (2007). High-throughput oncogene mutation profiling in human cancer. *Nature Genetics, 39*(3), 347-351.

Treangen, T. J., & Salzberg, S. L. (2012). Repetitive DNA and next-generation sequencing: computational challenges and solutions. *Nature Reviews Genetics, 13*(1), 36-46.

Wang, J., Ramakrishnan, R., Tang, Z., Fan, W., Kluge, A., Dowlati, A., . . . Ma, P. C. (2010). Quantifying EGFR alterations in the lung cancer genome with nanofluidic digital PCR arrays. *Clinical Chemistry, 56*(4), 623-632.

Wu, J.-Y., & Shih, J.-Y. (2016). Effectiveness of tyrosine kinase inhibitors on uncommon E709X epidermal growth factor receptor mutations in non-small-cell lung cancer. *OncoTargets and Therapy, 9*, 6137.

Yarden, Y., & Schlessinger, J. (1987). Epidermal growth factor induces rapid, reversible aggregation of the purified epidermal growth factor receptor. *Biochemistry, 26*(5), 1443-1451.

Zhou, C., Wu, Y.-L., Chen, G., Feng, J., Liu, X.-Q., Wang, C., . . . Ren, S. (2011). Erlotinib versus chemotherapy as first-line treatment for patients with advanced EGFR mutation-positive non-small-cell lung cancer (OPTIMAL, CTONG-0802): a mµlticentre, open-label, randomised, phase 3 study. *The Lancet Oncology, 12*(8), 735-742.