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Enabling Precision Medicine for TP53 Wild Type Endometrial Carcinoma Patients Through Validation of a Companion Diagnostic for Selinexor

Stephanie Castagliuolo

A Thesis in the Field of Biology

for the Degree of Master of Liberal Arts in Extension Studies

Harvard University

February 2025

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Abstract

Prevalence of endometrial cancer (EC) in women has risen globally over the past several decades. EC is a major health concern due to risk factors spanning across common stages within a woman's lifespan, such as hormonal birth control usage, pregnancy complications, obesity, aging, polycystic ovary syndrome (PCOS), tumors, chemotherapy treatment, and menopausal hormone therapy. Prognosis is poor for patients who develop advanced and recurrent EC; therefore, prompt and dynamic clinical intervention is crucial for positive patient outcomes. One such clinical intervention technique is the use of comprehensive genomic profiling (CGP) to provide molecular insight into the underlying oncogenic drivers of each individual's unique cancer. Companion diagnostic tests utilize CGP to generate a medical data report that provides clinically actionable information about a patient's genomic profile, enabling oncologists to develop a personalized, more targeted treatment strategy for each patient. The following research provides evidence that FoundationOne®CDx (F1CDx®), Foundation Medicine's FDA-approved qualitative next-generation sequencing-based in vitro CGP assay for solid tumors, is a safe and reliable companion diagnostic for tumor protein p53 (TP53) wild type (WT) EC patients as a means to qualify for selinexor administration. Through analytical validation studies, F1CDx did not produce false-positive TP53 results, demonstrated reproducibility for each targeted TP53 variant with ≥95% confidence, and maintained precision in identifying TP53 alterations across differing variables. These

results therefore demonstrate that F1CDx is safe and effective to facilitate improved treatment decisions for EC patients by enabling access to selinexor.

Dedication

I dedicate this body of work to women past, present, and future who are diagnosed with endometrial cancer. My hope is that advancements in care, like the ones described within this thesis, continue to provide hope and a path to healing for those who suffer. I wish all endometrial cancer patients health, happiness, and remission.

Acknowledgments

I would like to extend my deepest gratitude to Dr. Shannon Bailey and Kimberly Winnick. Their time, effort, and invaluable insight made the creation of this thesis possible. I would like to thank Foundation Medicine, Inc., for making it feasible for me to continue my education. It is a gift to be able to complete a graduate degree while pursuing my career dreams. I hope this thesis serves to highlight a fraction of the amazing work FMI is doing for the betterment of cancer care.

Thank you to my family; through your love and prayers, anything feels possible. Thank you, mom and dad, for your endless support, your unconditional love, and for teaching me the value of hard work. Thank you, Tom, for always believing in me. Your encouragement and strength over the past decade have meant the world to me.

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Chapter I.

Introduction

Endometrial cancer (EC) occurs when abnormal cells of the endometrial lining within the uterus divide uncontrollably. The World Cancer Research Fund lists EC as the sixth most common cancer in women worldwide. Statistics gathered by the American Cancer Society (ACS) conclude that endometrial carcinomas are the most common gynecologic malignancies in the United States, with about 67,800 new cases diagnosed in 2024. The Surveillance, Epidemiology, and End Results (SEER) database, maintained by the National Cancer Institute (NCI), estimates the overall 5-year relative survival rate for EC is 80.8%, based on the number of women diagnosed between 2014 and 2020. Patients may recognize the presence of EC through a variety of symptoms, the most common being atypical uterine bleeding (Bagaria et al., 2017). Other symptoms include abnormal discharge, pelvic pain, sudden weight loss, and detection of a mass through physical manipulation of the lower abdomen (Jeppesen et al., 2017). It is critical to note that EC can also be asymptomatic for 5-20% of patients, often leading to disease progression over time and an increased risk of metastasis (Bagaria et al., 2017). Asymptomatic patients have an increased likelihood of being in the advanced stages of EC at the time of diagnosis. Prognosis is poor for patients with advanced and recurrent EC, serving as a reminder for how crucial it is for women, particularly those who are post-menopausal, to visit their gynecologist on a routine basis.

Root Cause of EC

A growing number of risk factors has put a majority of women in danger of developing EC during their lifetime. Risk factors include hormonal birth control usage, pregnancy complications, obesity, aging, polycystic ovary syndrome (PCOS), tumors, chemotherapy treatment, and menopausal hormone therapy (Morice et al., 2016). These risk factors all have a singular characteristic in common, they impact female reproductive hormone levels. Normally, the female body requires estrogen to grow the endometrium during the follicular phase of menstruation (Rodriguez et al., 2019). Estrogen levels then decrease, and progesterone levels increase to limit endometrial growth during the luteal phase (Rodriguez et al., 2019). This balance between estrogenic growth of the endometrium, shedding, and progesterone inhibition during menstruation helps maintain a healthy uterus for pregnancy. When estrogen levels are too high and progesterone levels are too low, hyperplasia occurs. Hyperplasia is an abnormally thick endometrial lining, a major hallmark of EC due to the increased proliferation of endometrial cells (Sanderson et al., 2017). This unregulated endometrial cell growth, driven by endogenous hormones, can lead to abnormal tissue and increase the risk of tumorigenesis (Henderson et al., 2000).

One of the most common risk factors for type I endometrioid tumors is obesity and type 2 diabetes (MacKintosh et al., 2018). Recent studies have found evidence that an increase in body mass index (BMI) correlates directly to an increase in EC risk for women (MacKintosh et al., 2018). Research has shown that an obese woman possessing a BMI >30 has up to a 10-fold risk of developing EC compared to women of healthy weight who maintain a BMI between 18-25 (MacKintosh et al., 2013). Obesity puts

women at risk of EC due to insulin resistance, hyperestrogenemia, and inflammation (MacKintosh et al., 2013). Increased circulating levels of insulin increase the bioavailability of IGF-1, a growth factor that when unregulated and in high abundance is responsible for tumor growth (MacKintosh et al., 2013). Obesity causes hyperestrogenemia due to adrenal androgens being converted to estrogen by adipocytes within adipose tissue (MacKintosh et al., 2013). Hyperestrogenemia also lowers levels of sex hormone binding globulin (SHBG), which results in an increase in the bioavailability of free estrogen (MacKintosh et al., 2013). These molecular transformations caused by obesity increase the 3% lifetime risk of EC to 9-10% (MacKintosh et al., 2013).

Breastfeeding and multiple healthy full-term pregnancies during a woman's lifetime can lower the risk of developing EC due to prolonged periods of an advantageous shift in hormonal balance where estrogen is reduced, progesterone is elevated, and ovulation ceases (Liu et al., 2021). Research has demonstrated that elevated progesterone levels during pregnancy inhibits estrogen-driven endometrial cell proliferation, promoting differentiation and apoptosis, thus lowering the risk of EC (Kim et al., 2010). Conversely, pregnancy complications, such as gestational diabetes mellitus (GDM), preeclampsia, multiple miscarriages, preterm birth, and delivery of a large for gestational age (LGA) infant can lead to EC (Liu et al., 2021). These complications related to pregnancy induce hormonal changes within the female that give rise to malignant transformation, such as an increase in estrogen that can cause DNA damage, chromosomal damage, gene mutations, and microsatellite instability (MSI) (Roy et al., 1999).

Estrogen and progesterone levels are not only affected by obesity and pregnancy complications but also by birth control pills, chemotherapy, menopausal hormone therapy, tumors, and polycystic ovary syndrome (PCOS), putting a wide variety of women at risk of EC and attributing to its prevalence (MacKintosh et al., 2018). A study conducted by Lortet-Tieulent et al. in 2018 found that the global incidence of EC has significantly increased from 1978 to 2013, which can be attributed to the popularity of birth control, a rise in obesity, and an increase in other cancers and subsequent chemotherapy treatment. Furthermore, even if a female does not encounter the risk factors associated with EC during their lifetime, the potential to develop the disease is ever present due to the process of aging. All women undergo menopause in their lifetime, putting all women at risk of developing EC. According to the ACS, 75% of EC patients are postmenopausal with the average age of women with EC being >60. The risk of EC increases for every year older a woman is at menopause beginning around 46.5 years old (Zhang et al., 2019). Conversely, a woman who goes through menopause at a younger age has a decreased risk of EC. Aging and menopausal timeframe impacts the chance of developing EC due to the length of estrogen exposure (Zhang et al., 2019).

Ultimately, the commonality between all risk factors is the impact on hormone levels; it can be surmised that the root cause of EC is hormonal imbalances within the patient, leading to increases in cell proliferation and corresponding oncogenic transformation.

Diagnosing EC

Various testing is required for disease confirmation prior to a healthcare provider giving an official EC diagnosis. Transvaginal ultrasound (TVUS) imaging can detect

tumors and polyps within the uterus, distinguish if the uterine lining has thickened, and help determine the health of the endometrium and myometrium (Bagaria et al., 2017). Any abnormalities observed within the uterus during imaging are selected as regions of interest (ROI) and become candidates for tissue sampling. Tissue sampling is performed through endometrial biopsy, hysteroscopy, or dilation and curettage (D&C) (Matteson et al., 2018). Tissue samples undergo microscopic evaluation to determine whether the ROI is benign or cancerous. According to data gathered by the ACS, EC presents in different histological variations during microscopic evaluation, including adenocarcinoma, uterine carcinosarcoma, squamous cell carcinoma, small cell carcinoma, transitional carcinoma, serous carcinoma, adenoacanthoma, adenosquamous carcinoma, secretory carcinoma, ciliated carcinoma, villoglandular adenocarcinoma, clear-cell carcinoma, mucinous adenocarcinoma, undifferentiated carcinoma, dedifferentiated carcinoma, and serous adenocarcinoma. If the ROI is cancerous, a pathologist-generated report is often able to classify cancer type and grade. According to the ACS, pathologists utilize the TNM system to assess the primary tumor (T), if the cancer has spread to lymph nodes (N), and whether the cancer has metastasized to other areas of the body (M). Stage of cancer is then determined from TNM categories and grade (ACS, 2024). Stage I indicates that the cancer has not spread to lymph nodes or other areas of the body; stage II-III indicates that the cancer has grown in the localized area and spread to lymph nodes; stage IV indicates that the cancer has metastasized (ACS, 2024).

EC is categorized into two separate groups based on estrogen dependance: endometrioid (type I) and non-endometrioid (type II) (Setiawan et al., 2013). An oncologist can determine if a patient has type I or II EC by assessing biopsy results for

histological type, grade, and gene expression. A total of 65-70% of all EC cases are classified as type I estrogen dependent, and are typically endometrioid adenocarcinomas attributed to endometrial hyperplasia (Feinberg et al., 2019). Type I EC has a typical histological grading of 1-2, exhibiting less aggressive traits compared to type II estrogen-independent EC (Feinberg et al., 2019). Type II EC tumors, usually serous or clear cell carcinomas, derive from precancerous intraepithelial carcinoma lesions of the atrophic endometrium and account for 40% of patient deaths with a typical histological grading of 3 (Setiawan et al., 2013). It is critical to understand the type, grade, and stage of EC through pathology review to ensure that patients receive the most prompt and dynamic clinical intervention after diagnosis.

Treating EC

The ACS estimates that 13,250 women will succumb to EC in 2024. This fact, along with rising disease prevalence, serves as a crucial reminder of how important it is to continue making advancements in EC treatment and prevention. Current EC treatment options align with how the majority of cancers are treated at present i.e., through surgery, radiation, chemotherapy, targeted therapy, immunotherapy, and hormone therapy. Treatment selection depends on EC type, stage, location, recurrence, and metastasis. An evolving field in cancer care, and one that will be focused on exclusively within the following research, is the use of targeted therapies. Targeted therapies are the cornerstone of precision medicine because they are designed to target the specific oncogenic drivers of interest within the patient's DNA. According to the NCI, targeted therapies can induce the immune system to destroy cancer cells, inhibit cancer cells from proliferating by

interrupting growth signaling pathways, inhibit angiogenesis, and induce apoptosis, thus improving patient survival outcomes (NCI, 2024).

Targeted therapies include drugs consisting of either small molecule inhibitors or monoclonal antibodies that target the genomic alterations responsible for influencing growth, proliferation, metabolism, and survival of malignant cells (Padma, 2015). This targeted technique of treatment is beneficial to the patient because unlike chemotherapy and radiation, it minimizes the risk of damaging healthy cells by only targeting abnormalities within the cancer cells. Side effects are caused by damage to a patient's healthy cells, therefore when treated with targeted therapies there are fewer side effects and an improved quality of life for the patient (Padma, 2015). There are several Food and Drug Administration (FDA)-approved targeted therapies on the market to combat common genomic alterations that cause EC, such as TP53, PTEN, PIK3CA, PIK3R1, CTNNB1, ARID1A, KRAS, CTCF, RPL22, FGFR2, and ARID5B mutations (Bianco et al., 2020). Comprehensive genomic profiling (CGP) can be utilized to determine if an EC patient possesses one of these treatable mutations within their DNA and when utilized as a companion diagnostic (CDx) can help connect patients to a corresponding targeted therapy.

Companion Diagnostics

CGP provides molecular insight into the unique underlying oncogenic drivers of an individual's cancer. According to Foundation Medicine, Inc. (FMI), a world-leader in molecular insights, CDx assays can detect genomic alterations that are known to promote cancer, such as base substitutions, insertions, deletions, copy number alterations, rearrangements, and fusions (Milbury et al., 2022). CDx assays can also detect important

biomarkers that can impact a patient's treatment plan, such as microsatellite instability (MSI) and tumor mutational burden (TMB) (Milbury et al., 2022). CDx assays utilize CGP to generate a medical data report that provides clinically actionable information about a patient's distinct genomic profile, enabling oncologists to develop a more personalized treatment strategy (Milbury et al., 2022). This educated treatment selection based on genomics is known as precision medicine. Precision medicine is comprised of targeted therapies, immunotherapies, tumor-agnostic treatments, and clinical trial options (Milbury et al., 2022). Precision medicine enables improved treatment decisions for patients by providing oncologists data backed targeted treatment options rather than the generalized approach of surgery, radiation, and chemotherapy.

Furthermore, CDx results may often be the only data source to diagnose and identify a patient's eligibility for a therapy and can redefine clinical trial design, expanding accessibility for patient enrollment (Milbury et al., 2022). Biopharmaceutical (biopharma) partners seeking FDA approval for a new drug often require CDx assays to identify the right patients to enroll in their clinical trial and to help determine drug efficacy as said trial progresses. The relationship between biopharma partners and CGP providers is mutually beneficial because the CGP provider is then able to expand its CDx claims and provide the new drug as a treatment option in its medical data reports. Companies like FMI will partner with a biopharma company for the clinical trial of interest. The CGP provider will then define the CDx biomarker rules with the biopharma partner and begin designing validation studies that meet regulatory body expectations. A pre-submission (pre-sub) with said validation design and regulatory strategy is submitted to a regulatory agency, such as the FDA, for feedback (FDA, 2018). Regulatory agency

involvement is critical because a CDx next-generation sequencing (NGS)-based test is considered a medical device that provides essential information for the safe and effective use of a corresponding therapy and is therefore highly regulated to ensure the assay is safe and reliable for patients (FDA, 2018). With pre-sub approval, the CGP provider will prepare and perform analytical validation (AV) and clinical validation (CV) protocols, plan statistical analyses, analyze the results, and prepare reports to demonstrate assay performance. Reports that demonstrate performance are included in premarket approval (PMA) applications to gain initial CDx assay approval and in supplemental premarket approval (sPMA) applications for when an addition or alteration is made to the assay. sPMAs consist of the AV and CV package, demonstrating the CDx assay's safety and efficacy when used to detect the defined biomarker of interest. The CGP providers sPMA is often submitted concurrently with the new drug application (NDA) by the biopharma partner. Documentation then undergoes regulatory review and feedback is addressed by the relevant parties. Once approved, the CDx claim, and new drug can be launched commercially for medical reporting.

An FDA approval for a CDx test claim is the result of thorough and successful AV and CV studies to prove safety and efficacy. AV establishes experimental evidence that a process consistently delivers reproducible, precise, and accurate results (Kanagal-Shamanna et al., 2016). AV studies include limit of blank (LoB), limit of detection (LoD), precision, and orthogonal concordance. CVs are performed with the biopharma partner to evaluate patient response in populations identified by the CDx assay from clinical trial data through clinical bridging and efficacy analysis. The following AV studies provide evidence that FoundationOne®CDx (F1CDx®), FMI's FDA-approved

qualitative NGS-based in vitro CGP assay for solid tumors, is a safe and reliable CDx for tumor protein p53 (TP53) wild type (WT) EC patients as a means to qualify for selinexor administration.

Research Problem

FMI has partnered with Karyopharm Therapeutics to aid in their phase III clinical trial for selinexor (FMI, 2023). Patients known to have TP53 WT EC must undergo F1CDx testing to enter the study. Selinexor, also known as Xpovio, is a selective inhibitor of nuclear export (SINE) (Mo et al., 2023). Selinexor is a small molecule inhibitor that plays a vital role in the p53 regulatory pathway by inducing apoptosis in malignant cells to treat cancer (Mo et al., 2023). Selinexor is successful in the treatment of advanced and recurrent EC when mutations in the TP53 gene are not present. For patients to qualify for selinexor administration they must retain the normal function of their TP53 gene and complementary p53 protein. The TP53 gene provides instructions for the production of the p53 protein (Petitjean et al., 2007). The p53 protein, commonly referred to as the guardian of the genome, acts as a tumor suppressor in healthy cells by regulating proliferation and repairing damaged DNA (Petitjean et al., 2007). Selinexor causes an influx of p53 into the nucleus of malignant cells by blocking exportin 1 from transporting growth proteins to the cytoplasm (Bogani et al., 2023). p53 binds to the promoter and enhancer regions of damaged DNA where it either activates DNA repair proteins or signals the cell to undergo apoptosis (Petitjean et al., 2007). When the TP53 gene is mutated, DNA damage goes unchecked and can lead to cancer growth. Therefore, it is crucial for EC patients taking selinexor to have a functional, unmutated TP53 gene.

Selinexor is in the phase III clinical trial stage of development to determine safety and efficacy for TP53 WT EC patients. Selinexor is currently FDA-approved to treat multiple myeloma and relapsed or refractory diffuse large B-cell lymphoma (Syed, 2019). Researchers found that selinexor will also benefits patients with advanced and recurrent EC who are unresponsive to other therapies due to selinexor's ability to block the action of nuclear transport protein exportin 1 (Bogani et al., 2023). When exportin 1 is blocked, various proteins involved in cancer cell growth remain in the nucleus instead of being transported to the cytoplasm (Bogani et al., 2023). This inhibition causes several tumor suppressor proteins, including p53, to gather in the nucleus and ultimately arrest the cell cycle, leading to the apoptosis of malignant cells (Bogani et al., 2023). It is crucial that patients with EC receiving selinexor are TP53 WT because treatment with this drug leads to an increase in the p53 protein, which results in an increase in its tumor suppressor effects. Patients with mutations in TP53 would not respond to selinexor treatment due to potential p53 loss of function. F1CDx will confirm that TP53 gene mutations are not present in TP53 WT EC samples as a means for patients to qualify for selinexor administration (FMI, 2023).

The FDA requires a CDx for the NDA associated with the phase III clinical trial of selinexor administration to TP53 WT EC patients. The following thesis aims to verify that F1CDx is accurate, reliable, and safe for patients to be used as a companion diagnostic for selinexor. F1CDx will be used to verify that the patients enrolled in the clinical trial do not possess TP53 mutations within their DNA. In order to verify that F1CDx accurately detects the absence of TP53 alterations within EC DNA samples, FMI must perform AV and CV studies for submission to the FDA as part of a sPMA

application. An sPMA is required because the addition of this new claim to the F1CDx assay is considered a medical device change. The following research encompasses three AV studies; LoB, confirmation of LoD, and precision. LoB is the highest apparent analyte concentration expected to be found when replicates of a blank sample containing no analyte are tested, ensuring no false positives appear for the genomic biomarker of interest (Armbruster et al., 2008). LoD is the lowest analyte concentration likely to be reliably distinguished from the LoB and at which detection is feasible (Armbruster et al., 2008). LoD is determined by utilizing both the measured LoB and test replicates of a sample known to contain a low concentration of analyte, ensuring that the genomic marker can be reliably detected with 95% confidence (Armbruster et al., 2008). Precision evaluates two aspects of assay performance: reproducibility and repeatability per CLSI guidelines Evaluation of precision of Quantitative Measurement Procedures (EP05-A3). Reproducibility is evaluated by processing sample replicates from the same source sample under different conditions by varying one factor at a time, and repeatability is evaluated by processing replicates from the same sample under the same condition. The following precision study will evaluate the following sources of variability: location, reagent lots, and time.

To develop my hypothesis the following research questions will be addressed:

1. Will the AV studies prove that F1CDx is a reliable and safe companion diagnostic for selinexor?
2. Will F1CDx produce false-positive TP53 results when sequencing EC samples that do not harbor TP53 alterations?
3. Will LoD be reliably confirmed?

4. Will the F1CDx assay be precise in identifying the presence or lack of TP53 alterations in EC samples when experiencing degrees of variability?

I hypothesize that F1CDx will be a reliable and safe companion diagnostic for selinexor. I hypothesize that F1CDx will not produce false-positive TP53 results. I hypothesize that F1CDx will demonstrate reproducibility for each targeted TP53 variant with ≥95% confidence. Lastly, I hypothesize that F1CDx will maintain precision in identifying TP53 alterations across differing variables. To test this hypothesis, various TP53 WT EC DNA samples extracted from formalin-fixed, paraffin-embedded (FFPE) tumor tissue specimens will undergo F1CDx testing. F1CDx is a Clinical Laboratory Improvement Amendments (CLIA), College of American Pathologists (CAP), and FDA-regulated laboratory assay that employs whole-genome shotgun library construction and hybridization-based capture of 324 genes related to cancer (Milbury et al., 2022). Using the Illumina® NovaSeq platform, hybrid capture selected libraries will be sequenced to high uniform depth (Milbury et al., 2022). Sequence data will then be processed using a customized analysis pipeline designed to detect all classes of genomic alterations (Milbury et al., 2022).

The goal of the following research is to generate successful AV study results so that FMI may gain FDA approval for their claim that F1CDx is a reliable form of detection for TP53 mutations in EC DNA specimens. F1CDx will also serve as a means for EC patients to qualify for selinexor administration during the Karyopharm phase III clinical trial. FDA approval that F1CDx is a safe and reliable companion diagnostic for selinexor would be impactful to TP53 WT EC patients. Selinexor is expected to make a significant improvement in the lives of patients suffering from advanced and recurrent

EC who have developed resistance to platinum-based chemotherapy by restoring tumor suppressor pathways and improving disease outcome.

Chapter II.

Methods

All samples were processed according to the F1CDx assay procedure. Extracted DNA was quantified via fluoroscopy, where concentrations were measured to ensure that only samples meeting the established NGS acceptance criteria continue to normalization. Quantified samples were then normalized to 50-1,000 ng to ensure the yield of high-quality sequencing data. After normalization, samples were prepared for library construction. After purification, the DNA libraries were constructed and amplified. Libraries underwent a quality control (QC) assessment prior to proceeding to hybrid capture (HC). Then, the baits were hybridized to target regions within the DNA and underwent a purification phase where everything except for the ROI was washed away. After another QC assessment, samples were then sequenced using the Illumina NovaSeq sequencing by synthesis platform. Sequencing data then entered the analysis pipeline in an automated fashion. Curation was then completed post-pipeline analysis by FMI-trained bioinformatic analysts.

Limit of Blank Study Design Overview

The objective of this AV protocol was to determine the LoB of the F1CDx assay when detecting alterations in the TP53 gene from DNA derived from FFPE EC tissue samples. This study aimed to demonstrate that the F1CDx assay would not produce false-positive TP53 results when sequencing EC samples that do not harbor TP53 alterations. The F1CDx assay was expected to confirm the LoB as zero for TP53 alterations. Sample processing with the F1CDx assay was performed in the FMI patient testing laboratory in

Cambridge, Massachusetts. The study is IRB approved by the New England Institutional Review Board (NEIRB) IRB#:120160955. I confirmed that the Harvard University Area Institutional Review Board Committee on the Use of Human Subjects did not need to conduct an independent review because the human material used was de-identifiable residual specimen, which does not meet their definition of human subjects.

The LoB experimental design reflects in part the guidance in CLSI EP17-A2 (Evaluation of Detection Capability for Clinical Laboratory Measurement Procedures). The EC FFPE tissue samples were derived from previously tested patient specimens. These individuals gave prior consent for research use. Samples were de-identified prior to research following Health Insurance Portability and Accountability (HIPAA) guidelines. Researchers only had access to information such as gender, age, cancer type, stage, and genomic mutation, which is not considered enough information to be able to reidentify an individual. A minimum of ten replicates of each DNA sample were processed using a range of DNA inputs representing the maximum input, the median input (determined from historical commercial samples run on F1CDx), and the minimum input. The proposed replicate numbers were adjusted based on the quantity of DNA available per unique sample or to supplement processing failures.

LoB Sample Selection and Preparation

Six unique female uterus endometrial adenocarcinoma samples were processed with a minimum of 10 replicates per sample using a range of DNA inputs, producing a total of 60 sample replicates processed (Table 1 and 2). The selected FFPE EC samples were absent of TP53 alterations as defined in Table 3. A minimal remaining mass of 14,650 ng was required to achieve DNA inputs of approximately 50 ng, 550 ng, and

1,000 ng for each sample. DNA was prepared based on initial sample processing concentrations to support ~550 ng and 1,000 ng inputs, and a portion of the samples were diluted to a working stock concentration of 10 ng/µL to support ~50 ng input. Intermediate DNA quantification was then performed to assess the DNA concentration and remaining mass. Following DNA quantification, DNA was further diluted to a working target stock concentration to support DNA input at 50 ng per sample replicate. For each source sample, 4 replicates were run close to ~1,000 ng input DNA for library construction, 3 replicates at ~550 ng, and 3 replicates at ~50 ng. Samples underwent normal F1CDx assay process with current clinical reagents utilizing the Illumina NovaSeq platform (Figure 1).

Table 1. Source Samples, Interim Quantification, and Dilutions.

Sample Biopsy Site	Remaining Mass (ng)	Conc. (ng/ul)	Volume Check (ul)	Estimated Remaining Mass (ng)	Int. Quant Conc. (ng/ul)	Aliquot (DNA) ul (60 ul at 10 ng/ul)	Elution Buffer (ul)	Int. Quant Conc. (ng/ul)	Aliquot (DNA) ul (185 ul at 27 ng/ul)	Elution Buffer (ul)
Uterus	31,381.6	58.9	516	30,435.2	65.2	10.2	49.8	9.2	76.6	108.4
Lung	27,121.1	51.2	520	26,635.4	43.3	11.7	48.3	7.0	115.3	69.7
Uterus	27,022.6	51.0	510	26,031.9	43.7	11.8	48.2	7.1	114.3	70.7
Uterus	27,413.8	51.7	506	26,188.5	52.2	11.6	48.4	9.0	95.5	89.5
Uterus	31,449.3	59.1	508	30,025.8	49.1	10.2	49.8	6.7	101.5	83.5
Uterus	31,373.0	58.9	504	29,719.3	58.7	10.2	49.8	8.2	85.1	99.9

Table 2. Final Sample Preparation of Replicates.

Patient	DNA Dil. (ul)	Dil. Buffer (ul)	Patient	DNA Dil. (ul)	Dil. Buffer (ul)	Patient	DNA Dil. (ul)	Dil. Buffer (ul)	Patient	DNA Dil. (ul)	Dil. Buffer (ul)	Patient	DNA Dil. (ul)	Dil. Buffer (ul)	Patient	DNA Dil. (ul)	Dil. Buffer (ul)
	55	-		55	-		55	-		55	-		55	-		55	-
	55	-		55	-		55	-		55	-		55	-		55	-
	55	-		55	-		55	-		55	-		55	-		55	-
1	55	-	2	55	-	3	55	-	4	55	-	5	55	-	6	55	-
	76.6	108.4		115.3	69.7		114.3	70.7		95.5	89.5		101.5	83.5		85.1	99.9
	19.5	160.5		25.8	154.2		25.3	154.7		20.1	159.9		26.7	153.3		21.9	158.1

Table 3. Biomarker Eligibility Requirements.

Gene	Variant class	Biomarker rules
TP53	Short variant	Any missense, frameshift, nonsense, non-frameshift, or splice site alteration
	Copy number	Homozygous deletion of one or more exons
	Rearrangement	Any rearrangement

Samples meet biomarker eligibility requirements when the following alterations are not detected.

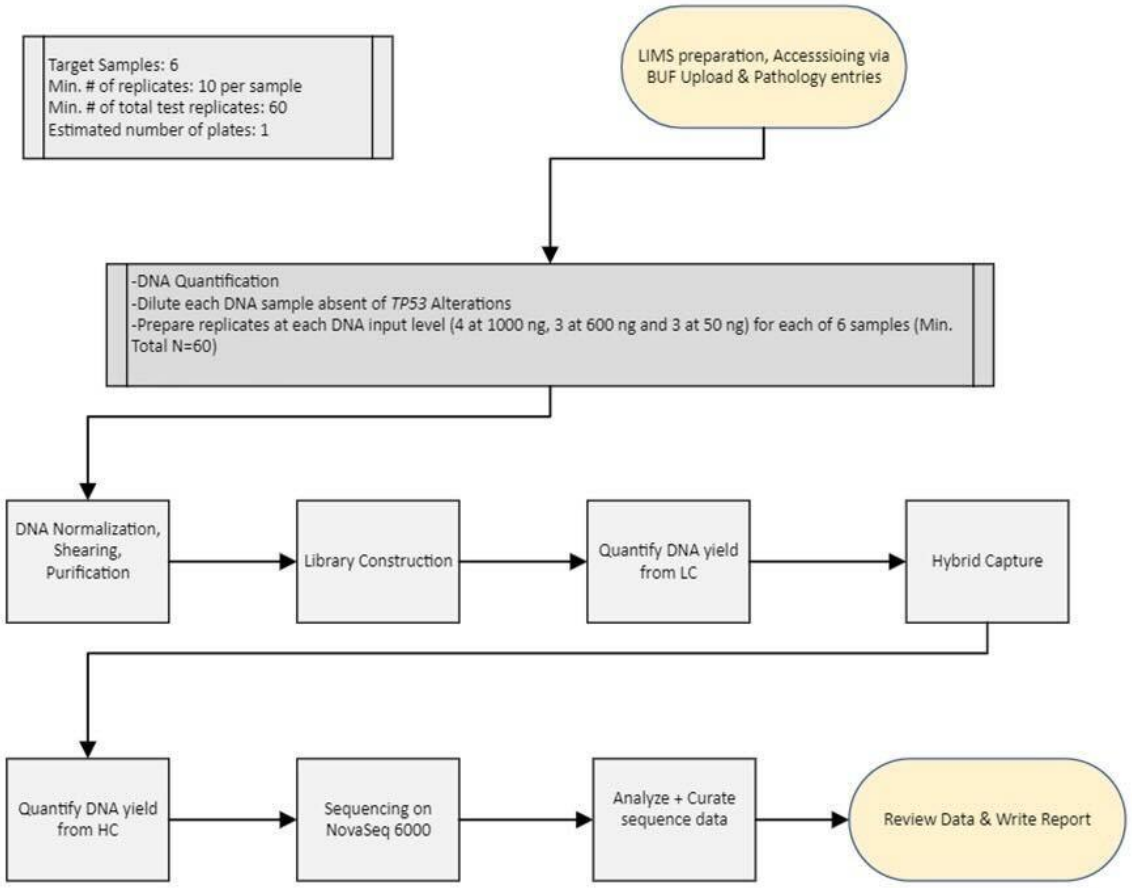

Figure 1. LoB Sample Processing Procedure.

LoB Analysis

The false positive rate (FPR) for the detection of TP53 alterations was calculated across all valid replicates of all source samples. For the i^{th} sample, n_i was the total number of valid replicates, and x_{ij} was the detection status for the j^{th} replicate for j = 1, 2, …, ni. If any biomarker-positive variant per the biomarker rules in Table 3 was detected in a replicate, then x_{ij} = 1, otherwise x_{ij} = 0. The formula for calculating the FPR for TP53 alterations across all valid replicates of all source samples was as follows:

$$FPR = \frac{\sum_{i=1}^{N} \sum_{j=1}^{n_i} x_{ij}}{\sum_{i=1}^{N} n_i} * 100\%$$

where N is the total number of source samples. The LoB for TP53 alterations was assessed for each variant type: short variants (SVs), rearrangements (REs), and copy number alterations (CNAs). The formula for calculating the FPR for TP53 variant types across all valid replicates of all source samples was as follows (using SVs as an example):

$$FPR\ for\ TP53\ SV = \frac{\sum_{i=1}^{N} \sum_{j=1}^{n_i} x_{ij}}{\sum_{i=1}^{N} n_i} \times 100\%$$

where x_{ij} = 1 indicates there is at least one TP53 SV that meets the biomarker rules defined in Table 3 detected in the j^{th} replicate of the i^{th} sample. The FPR for RE and CNA variants was obtained in a comparable way. The study was accepted if the primary endpoint FPR is less than or equal to 5% (i.e., type I error = 0.05) so that LoB was confirmed as zero. Curation of sequencing data was completed by FMI-trained computational bioinformatic analysts, who generated a report summarizing the findings.

Precision and Confirmation of LoD Study Design Overview

The objective of this study was to evaluate the site-to-site precision and to confirm the LoD of the F1CDx assay in identifying TP53 rearrangements, short variants, and homozygous deletions in DNA derived from FFPE EC samples. Additionally, this study evaluated the precision of the F1CDx assay in identifying samples absent of TP53 alterations in DNA derived from FFPE EC samples by assessing reproducibility and repeatability. The variables utilized to test reproducibility and repeatability were different

sites, reagent lots, and library construction (LC) start dates (Table 5). For confirmation of LoD, 1x-1.5x the median platform LoD determined in Table 4, was targeted for each variant subcategory. The sites included in this study were the three main FMI facilities that run the F1CDx assay: Cambridge, MA (CAM), Research Triangle Park, NC (RTP), and Penzberg, Germany (PNZ).

The study is IRB approved by the New England Institutional Review Board (NEIRB) IRB#: 120160955. I confirmed that the Harvard University Area Institutional Review Board Committee on the Use of Human Subjects did not need to conduct an independent review because the human material used was de-identifiable residual specimen, which does not meet their definition of human subjects. The site-to-site precision experimental design reflects in part the guidance in CLSI EP12- A2; User Protocol for Evaluation of Qualitative Test Performance and CLSI EP05-A3E; Evaluation of Precision of Quantitative Measurement Procedures. The study design evaluated 8 EC samples harboring TP53 alterations (4 SVs, 2 REs, and 2 HDs) and 1 sample absent of TP53 alterations in a total of 36 replicates (2 reagent lots x 3 sites x 3 LC start dates x 2 replicates) for each sample. An overview of the study design is shown in Table 5 and study logistics are presented in Figure 2.

Table 4. Median LoD of Variants by Variant Category.

Variant Category	Subcategory	Median LoD
Base Substitutions	Known	3.20%
Indels at non-homopolymer context, including insertions up to 42 bp and deletions up to 276 bp	Other	8.20%
Indels at homopolymer context	6 bp repeat	13.70%
CNA: HDs	NA	33.40%
Genomic REs (reads based)	NA	17.23

Table 5. Precision Design Overview.

Site	Reagent Lot #	LC Start Day #	# of Replicates Per Run
CAM	1	1	2
CAM	1	2	2
CAM	1	3	2
CAM	2	1	2
CAM	2	2	2
CAM	2	3	2
RTP	1	1	2
RTP	1	2	2
RTP	1	3	2
RTP	2	1	2
RTP	2	2	2
RTP	2	3	2
PNZ	1	1	2
PNZ	1	2	2
PNZ	1	3	2
PNZ	2	1	2
PNZ	2	2	2
PNZ	2	3	2

Precision and Confirmation of LoD Sample Selection and Preparation

Deidentified residual samples containing TP53 alterations as defined by the biomarker definition (Table 3) and with at least 2,000 ng remaining DNA mass were selected for testing. Some samples were titrated with diluent DNA extracted from EC FFPE samples that are absent of TP53 alterations to achieve the desired target of 1x-1.5x median platform LoD. Samples harbor TP53 alterations (including SVs, REs, and HDs) when an alteration in any of the variant classes listed in Table 3 was detected. Samples were absent of TP53 alterations when none of the alterations listed in Table 3 were detected. Nine EC samples were included in this study. Eight EC samples harboring TP53 alterations including four SVs, two HDs, and two REs were selected. In addition, one sample absent of TP53 alterations was selected to evaluate the precision for absence of TP53 alterations. Each sample was tested in duplicate on each plate at three sites. Three runs on different LC start dates were performed with each of two reagent lots (2 replicates x 3 sites x 3 LC start days x 2 reagent lots) for a total of 36 replicates for each of the 9 samples. A total of 324 replicates were included in this study design. Samples that required titration were titrated with diluent DNA absent of TP53 alterations to achieve the target value based on the median platform LoD for each alteration type. DNA replicates for samples that required titration, and samples that did not require titration were aliquoted to tubes, scanned into the laboratory information management system (LIMS) individually, and assigned to plates. A mock quantification file containing the newly diluted DNA concentrations (1 ng/µL) was uploaded to LIMS. Samples then underwent normal F1CDx assay processing, utilizing the Illumina NovaSeq platform with precision design specifications as shown in Figure 2.

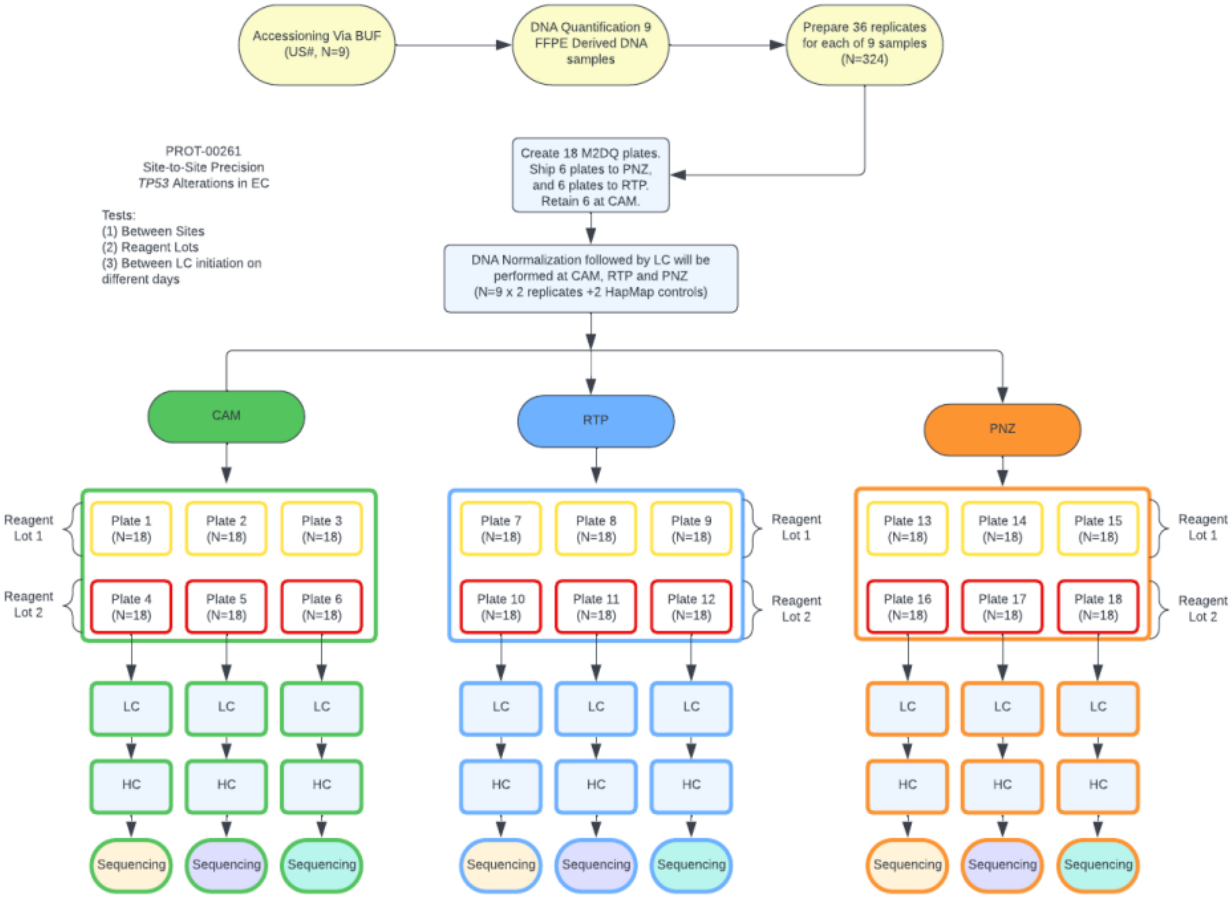

Figure 2. Precision and Confirmation of LoD Study Logistics.

Precision and Confirmation of LoD Analysis

To evaluate reproducibility of targeted variants, the point estimate of agreement for the targeted variant detection status among all valid replicates in each sample was computed and reported with corresponding 95% 2-sided score CIs if the corresponding observed average measurement (variant allele frequency (VAF) for SVs, chimeric reads for REs, test performance (TP) for HDs) among all valid replicates of the source sample was ≥ 1x LoD. For the $i\ th$ sample, let ni be the total number of valid replicates (up to 36

valid replicates), and xij be the detection status for the $j\ th$ replicate for j = 1, 2, … , ni . If the variant was detected in the $j\ th$ replicate, then xij = 1, otherwise xij = 0. The observed average metric is denoted as Mij in the $j\ th$ replicate of the $i\ th$ sample. For VAF and chimeric reads, Mij was assigned as zero if xij = 0. Note that if a variant had an observed average metric < 1x LoD, it was analyzed under exploratory analysis, and no acceptance criteria were applied. The formula for the reproducibility of each targeted variant in each sample was as follows:

$$Reproducibility\ of\ the\ targeted\ variant\ in\ the\ i^{th}\ sample = \frac{\sum_{j=1}^{n_i} x_{ij}}{n_i} \times 100\%$$

The formula for the observed average metrics for each targeted variant in each of the samples was as follows:

$$Observed\ average\ measurements\ of\ the\ targeted\ variant\ in\ the\ i^{th}\ sample = \frac{\sum_{j=1}^{n_i} M_{ij}}{n_i} * 100\%$$

To assess repeatability of targeted variants above LoD according to the study design, two replicates per sample were run under identical measurement conditions (same LC start day, same reagent lot, and same site). A comparison was made between the two replicates under the identical measurement conditions for a total of up to 3 LC start dates x 2 reagent lots x 3 sites = 18 pairs of evaluable comparisons. Let mi denote the total number of valid pairs (up to 18 pairs) for the $i\ th$ sample. The detection status of the $j\ th$ pair of replicates under identical measurement conditions were denoted by aij, bij respectively, where j = 1, 2, … , mi . For each targeted variant, an “agreed” pair was defined as the two replicates having the same calls, i.e., $aij = bij$ = 1, $else$ 0.

Repeatability of the targeted variant in each sample was assessed using the formula below, with 95% 2-sided score CIs.

$$Repeatability\ of\ the\ targeted\ variant\ in\ the\ i^{th}\ sample = \frac{\sum_{j=1}^{m_i} I(a_{ij} = b_{ij})}{m_i} \times 100\%$$

Where $I(\cdot)$ is the indicator function, which means $I(A) = 1$ if event A occurs. Otherwise, $I(A) = 0$.

For each targeted variant within each sample included in this study, the repeatability and reproducibility of the underlying measurement (VAF for SVs, chimeric reads for REs, and TP for HDs) was assessed with a variance component analysis across all valid replicates for each sample. A three-way factorial design, and analysis of variance (ANOVA) model, with site, reagent lot, and LC start day as factors, was applied to each targeted variant individually with a total of up to 36 data points per sample source:

$$Y_{ijkl} = \mu + S_i + L_j + R_{k(ij)} + \varepsilon_{ijkl}$$

For each targeted variant with observed average measurement < 1x LoD, the reproducibility and repeatability were estimated with a 95% 2-sided score CI for informational purposes only without any acceptance criteria. Using the same notation as defined above, the majority call of the targeted variant was positive (denoted as $ci = 1$) if over half of the valid replicates had the targeted variant detected (i.e., $\sum xij\ ni\ j=1 > ni$ 2). Otherwise, $ci = 0$. The formulas for calculating reproducibility and repeatability for each targeted variant with observed average measurement < 1x LoD in each of the samples were as follows:

$$Reproducibility\ of\ the\ targeted\ variant\ in\ the\ i^{th}\ sample = \frac{\sum_{j=1}^{n_i} I(x_{ij} = c_i)}{n_i} \times 100\%$$

$$Repeatability\ of\ the\ targeted\ variant\ in\ the\ i^{th}\ sample = \frac{\sum_{j=1}^{m_i} I(a_{ij} = b_{ij})}{m_i} \times 100\%$$

To evaluate the reproducibility of the absence of TP53 alterations, the point estimate of agreement for the variant non-detection status among all valid replicates in the relevant sample was computed and reported with corresponding 95% 2-sided score CIs. For the $i\ th$ sample, let ni be the total number of valid replicates (up to 36 valid replicates as mentioned in Section 6.1) and xij be the non-detection status for the $j\ th$ replicate for j = 1, 2, … , ni . If the variant was not detected in the $j\ th$ replicate, then xij = 1, otherwise xij = 0.

$$Reproducibility\ of\ the\ absence\ of\ TP53\ alterations\ in\ the\ i^{tn}\ sample = \frac{\sum_{j=1}^{n_i} x_{ij}}{n_i} \times 100\%$$

$$Repeatability\ of\ the\ absence\ of\ TP53\ alterations\ in\ the\ i^{th}\ sample = \frac{\sum_{j=1}^{m_i} I(a_{ij} = b_{ij})}{m_i} \times 100\%$$

Repeatability of the absence of TP53 alterations in the given sample was assessed using the formula above with 95% 2-sided score CIs.

For each targeted variant with observed an average measurement (VAF for SVs, chimeric reads for REs, TP for HDs) < 1x LoD in each sample, the repeatability and reproducibility of the underlying measurements was assessed by variance component analysis across all valid replicates for each sample. The variance component analysis method applied for targeted variants below LoD is the same as the method described for evaluation of continuous measurements of targeted variants above LoD.

Chapter III.

Results

There were no sample processing failures during the LoB, confirmation of LoD, and precision studies. All 60 LoB study replicates were successfully processed through LC, HC, sequencing, and post sequencing analysis. Table 6 details the processing success rates, also known as pass rates, for all sample replicates at each LoB processing step. All 324 confirmation of LoD and precision study replicates were successfully processed through LC, HC, sequencing, and post sequencing analysis, summarized in Table 7.

Table 6. LoB Sample Replicate Processing Pass Rates.

Process Step	# Total Replicates	# Pass Replicates	# Fail Replicates	Pass Rate (%)	95% 2-Sided Score CI (%)
LC	60	60	0	100.00	[93.98, 100.00]
HC	60	60	0	100.00	[93.98, 100.00]
Sequencing	60	60	0	100.00	[93.98, 100.00]
Post-Sequencing QC	60	60	0	100.00	[93.98, 100.00]
Overall	60	60	0	100.00	[93.98, 100.00]

Table 7. Confirmation of LoD and Precision Sample Processing Success Rates.

Process Step	# Total Replicates	# Pass Replicates	# Fail Replicates	Pass Rate (%)	95% 2-Sided Score CI (%)
LC	324	324	0	100.00	[98.83, 100.00]
HC	324	324	0	100.00	[98.83, 100.00]
Sequencing	324	324	0	100.00	[98.83, 100.00]
Post-Sequencing QC	324	324	0	100.00	[98.83, 100.00]
Overall	324	324	0	100.00	[98.83, 100.00]

Limit of Blank Results

Ten replicates from each of the 6 DNA source samples derived from FFPE EC tissue were processed for a total of 60 sample replicates.

Table 8. False Positive Rate Across All Valid Replicates from All Samples.

Target Variant	# False Positive Replicates	# Total Valid Replicates	False Positive Rate (%)
TP53 alterations	3	60	5.00

Among the 60 total valid replicates, 3 were found to have a TP53 alteration detected (FPR = 5.00%) (Table 8). This result met the study acceptance criteria of FPR ≤ 5%, and the LoB for TP53 alteration detection was confirmed as zero. Upon investigation, it was found that the three alterations were detected in three replicates of the same source sample, and all detected alterations were for the same TP53 SV (TP53_1083delG). All three detections were reported at a low VAF, ranging from 0.75% to 0.95%. The detail of each alteration detection is outlined in Table 9.

Table 9. Summary of Replicates with Detected TP53 Alterations.

Sample	DNA Input (ng)	Median Exon Coverage	TP53 Alteration	Supporting Reads	%VAF
A	1,000	1,043	N/A	N/A	N/A
B	1,000	2,733	TP53 SV c.1083delG	1,676	0.95
D	1,000	2,779	TP53 SV c.1083delG	1,734	0.75
E	624.17	2,571	TP53 SV c.1083delG	1,565	0.77

Additionally, it was observed that the three replicates with detected TP53 alterations had greater than 2.5x the median exon coverage of the source sample, which

may represent a source of higher sensitivity in the replicates with the detected TP53 variant (Figure 3). Furthermore, the source sample was determined to be microsatellite instability-high (MSI-H), and the detected TP53 variant was noted to be a single base deletion in a repetitive region, which is consistent with the variant type that is expected to be observed in mismatch repair deficient tumors. There is supporting evidence that the TP53 c.1083delG SV detected in the three replicates represents a sub-clonal TP53 variant present in the tumor. Justification for this conclusion includes the following:

1. A variant candidate was generated in the source sample but was not called due to insufficient supporting reads.

2. The TP53 variant is a single base deletion in a repetitive region and is consistent with the variant type that is expected to be observed in MSI-H samples.

3. Sequencing coverage in the three replicates with the TP53 variant detected was ~2.5x greater than the median exon coverage of the source sample. Samples with higher coverage are expected to have higher sensitivity.

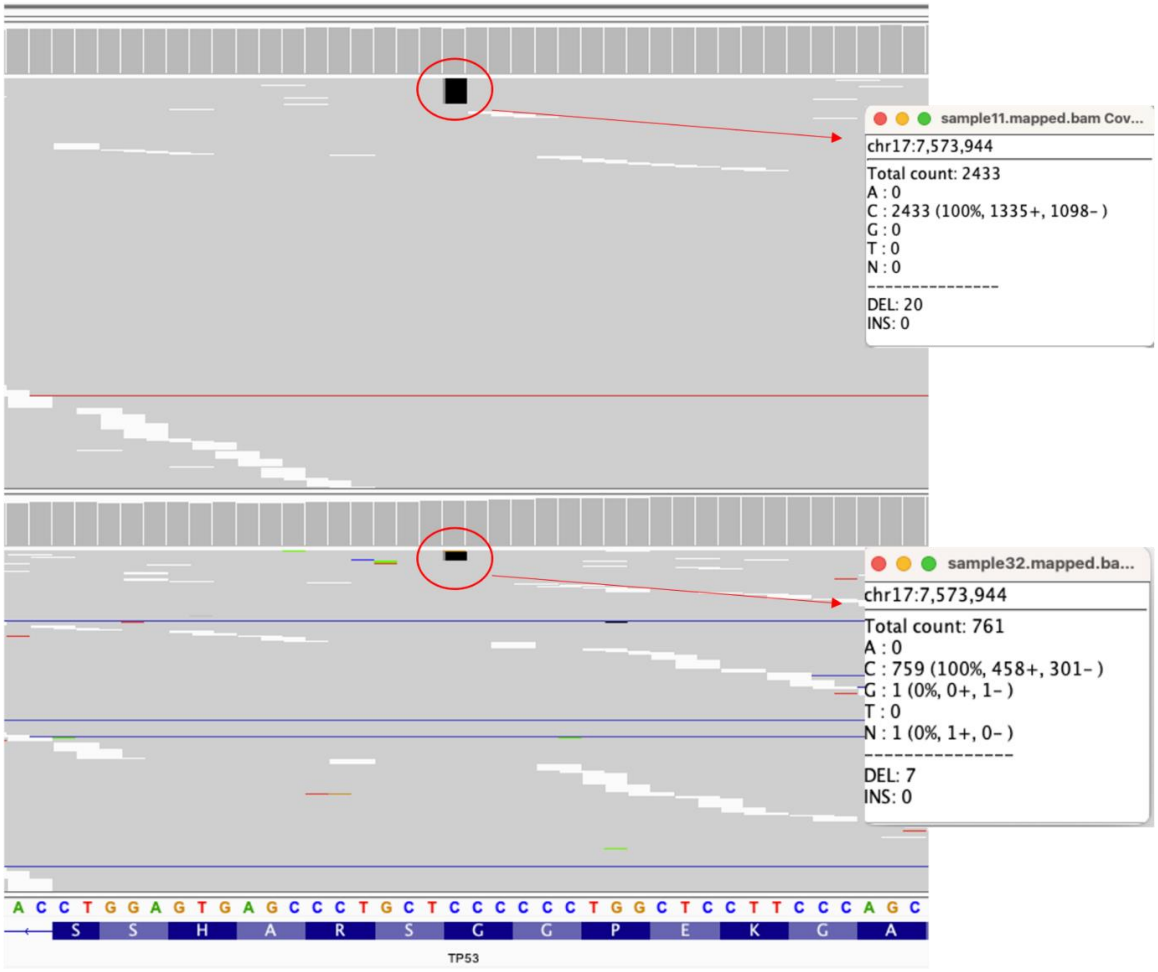

Figure 3. Integrative Genomics Viewer Comparison of TP53-Positive Replicate and TP53-Negative Source Sample.

Figure 3 shows the sequence read alignment for a TP53 positive replicate (top panel) and the TP53 negative source sample (bottom panel). In both cases, reads were observed with chr17:7,583,944 deleted; 20 and 7 reads in TP53 positive replicate and TP53 negative source sample respectively. Total sequence coverage at chr17:7,573,944 differed between the TP53 positive replicate and TP53 negative source sample with 2433 and 761 total coverage respectively.

The FPR was calculated across all valid replicates from all samples for each variant type (i.e., SVs, REs, CNAs). The results are shown below in Table 10.

Table 10. FPR Across All Valid Replicates from All Samples for Each Variant Type.

TP53 Variant Subtype	# False Positive Replicates	# Total Valid Replicates	False Positive Rate (%)
SV	3	60	5.00
RE	0	60	0.00
CNA	0	60	0.00

The FPR for SV detection was calculated to be 5.00%, as all three replicates with false positives had SV detections only. The FPR for both REs and CNAs was 0%.

Precision and Confirmation of LoD Results

In total, 18 plates, composed of 9 unique FFPE DNA samples in 36 sample replicates and 2 controls for each plate, were processed for a total of 324 sample replicates. Two reagent lots for each site were used for processing on three separate LC start dates. Reproducibility was evaluated for seven of the targeted variants that had observed measurements above LoD as shown below in Table 11.

Table 11. Reproducibility of Targeted Variants Above LoD.

Source Sample	Targeted Alteration	Variant Type	Observed Average TP (%), Chimeric Reads, or VAF (%)	LoD	Fold LoD (x)	# Positive Replicates	# Total Valid Replicates	Reproducibility (%)	95% Two-Sided Score CI (%)
A	*TP53_216delC*	SV	18.76	13.70	1.37	36	36	100.00	[90.36, 100.00]
B	*TP53_371_372insG*	SV	10.08	8.20	1.23	36	36	100.00	[90.36, 100.00]
C	*TP53_743G>A*	SV	4.32	3.20	1.35	36	36	100.00	[90.36, 100.00]
D	*TP53_818G>A*	SV	3.43	3.20	1.07	36	36	100.00	[90.36, 100.00]
E	*SKAP1_TP53*	RE	19.67	17.23	1.14	35	36	97.22	[85.83, 99.51]
F	*TP53_loss*	HD	45.65	33.40	1.37	36	36	100.00	[90.36, 100.00]
G	*TP53_loss*	HD	39.82	33.40	1.19	36	36	100.00	[90.36, 100.00]

All seven variants met the acceptance criteria with the four SVs and two HDs having 100.00% [90.36%, 100.00%] reproducibility and the one RE having 97.22% [85.83%, 99.51%] reproducibility. The one discordance observed for the SKAP1_TP53 RE was investigated, and evidence was found that the variant was observed but was filtered out of reporting due to the variant being labeled as a potential artifact resulting from poor read quality.

For confirmation of LoD, a hit rate was calculated for each targeted variant with results shown below in Table 12.

Table 12. Confirmation of LoD for Targeted Variants.

Source Sample	Targeted Alteration	Variant Type	Observed Average TP (%), Chimeric Reads, or VAF (%)	LoD	Fold LoD (x)	# Positive Replicates	# Total Valid Replicates	Hit Rate (%)
A	*TP53_216delC*	SV (indel)	18.76	13.70	1.37	36	36	100.00
B	*TP53_371_372insG*	SV (indel)	10.08	8.20	1.23	36	36	100.00
C	*TP53_743G>A*	SV (substitution)	4.32	3.20	1.35	36	36	100.00
D	*TP53_818G>A*	SV (substitution)	3.43	3.20	1.07	36	36	100.00
E	*SKAP1_TP53*	RE	19.67	17.23	1.14	35	36	97.22
F	*TP53_loss*	HD	45.65	33.40	1.37	36	36	100.00
G	*TP53_loss*	HD	39.82	33.40	1.19	36	36	100.00
H	*TP53_WRAP53*	RE	16.89	17.23	0.98	35	36	97.22

A discordance for the TP53_WRAP53 RE was observed. Investigation into this discordance found evidence that the variant was observed but was filtered from reporting due to insufficient supporting read evidence. The observed average measurements of all targeted variants were ≤3x the median platform LoD for their respective variant class.

The confirmed LoD for each TP53 variant subtype was claimed as the lowest observed average measurement with at least a 95% hit rate and is detailed below in Table 13.

Table 13. Summary of Confirmed LoD for TP53 Alterations by Variant Type Based on Observed Average VAF (%) for SVs, Chimeric Reads for REs, and TP (%) for HD Alterations.

Variant Type	Hit Rate (%)	Confirmed LoD
SV (indel)	100.00	10.08
SV (substitution)	100.00	3.43
RE	97.22	16.89
HD	100.00	39.82

Repeatability was assessed for the same seven targeted variants with observed average measurements above LoD with results shown below in Table 14. The four SVs and two HDs had 100.00% [82.41%, 100.00%] repeatability, and the one RE (SKAP1_TP53) had 94.44% [74.24%, 99.01%] repeatability.

Table 14. Repeatability of Targeted Variants Above LoD.

Source Sample	Targeted Alteration	Variant Type	# Agree Pairs	# Total Valid Pairs	Repeatability (%)	95% Two-Sided Score CI (%)
A	*TP53_216delC*	SV	18	18	100.00	[82.41, 100.00]
B	*TP53_371_372insG*	SV	18	18	100.00	[82.41, 100.00]
C	*TP53_743G>A*	SV	18	18	100.00	[82.41, 100.00]
D	*TP53_818G>A*	SV	18	18	100.00	[82.41, 100.00]
E	*SKAP1_TP53*	RE	17	18	94.44	[74.24, 99.01]
F	*TP53_loss*	HD	18	18	100.00	[82.41, 100.00]
G	*TP53_loss*	HD	18	18	100.00	[82.41, 100.00]

Variance component analysis (VCA) was also conducted for the same seven targeted variants with observed average measurements above LoD. Results are shown below in Table 15. In the VCA analysis, three targeted variants had reproducibility

coefficient of variation (%CV) ≤ 5.39% and repeatability %CV ≤ 5.22%, and three targeted variants had reproducibility %CV ≤ 19.75% and repeatability %CV ≤ 17.59%. Only one variant (SKAP1_TP53) had a reproducibility and repeatability %CV above 30% (30.39%).

Table 15. Precision of Continuous Measurements of Targeted Variants Above LoD.

Source Sample	Targeted Alteration	Variant Type	# Total Valid Replicates	Observed Average TP (%), Chimeric Reads, or VAF (%)	LoD	Fold LoD (x)	Reproducibility		Repeatability	
							SD	%CV	SD	%CV
A	*TP53_216delC*	SV	36	18.76	13.70	1.37	0.0101	5.39	0.0098	5.22
B	*TP53_371_372insG*	SV	36	10.08	8.20	1.23	0.0137	13.61	0.0137	13.61
C	*TP53_743G>A*	SV	36	4.32	3.20	1.35	0.0085	19.75	0.0076	17.59
D	*TP53_818G>A*	SV	36	3.43	3.20	1.07	0.0052	15.06	0.0048	13.94
E	*SKAP1_TP53*	RE	36	19.67	17.23	1.14	5.9761	30.39	5.9761	30.39
F	*TP53_loss*	HD	36	45.65	33.40	1.37	0.0072	1.59	0.0066	1.45
G	*TP53_loss*	HD	36	39.82	33.40	1.19	0.0049	1.23	0.0040	1.00

Reproducibility and repeatability were assessed for TP53_WRAP5 with observed average measurement below LoD with results shown below in Tables 16 and 17. The reproducibility for this TP53_WRAP53 RE was 97.22% [85.83%, 99.51%], and the repeatability was 94.44% [74.24%, 99.01%].

Table 16. Reproducibility of Targeted Variants Below LoD.

Source Sample	Targeted Alteration	Variant Type	Observed Average Chimeric Reads	LoD	Fold LoD (x)	# Positive Replicates	# Total Valid Replicates	Reproducibility (%)	95% Two-Sided Score CI (%)
H	*TP53_WRAP53*	RE	16.89	17.23	0.98	35	36	97.22	[85.83, 99.51]

Table 17. Repeatability of Targeted Variants Below LoD.

Source Sample	Targeted Alteration	Variant Type	# Agree Pairs	# Total Valid Pairs	Repeatability (%)	95% Two-Sided Score CI (%)
H	*TP53_WRAP53*	RE	17	18	94.44	[74.24, 99.01]

The reproducibility and repeatability were also assessed for TP53_WRAP53 for absence of TP53 alterations with results shown below in Table 18. For this sample (targeted for absence of TP53 alterations), reproducibility was 100.00% [90.36%, 100.00%], and repeatability was 100.00% [82.41%, 100.00%].

Table 18. Reproducibility and Repeatability of Absence of TP53 Alterations.

Source Sample	# Positive Replicates	# Total Valid Replicates	Reproducibility (%)	95% Two-Sided Score CI for Reproducibility (%)	# Agree Pairs	# Total Valid Pairs	Repeatability (%)	95% Two-Sided Score CI for Repeatability (%)
I	36	36	100.00	[90.36, 100.00]	18	18	100.00	[82.41, 100.00]

VCA was also performed for TP53_WRAP53 with an observed average measurement below LoD with results shown below in Table 19. In the VCA analysis, reproducibility %CV was 31.99% and repeatability %CV was 25.81%.

Table 19. Precision of Continuous Measurements of Targeted Variants Below LoD.

Source Sample	Targeted Alteration	Variant Type	# Total Valid Replicates	Observed Average Chimeric Reads	LoD	Fold LoD (x)	Reproducibility		Repeatability	
							SD	%CV	SD	%CV
H	*TP53_WRAP53*	RE	36	16.89	17.23	0.98	5.4030	31.99	4.3589	25.81

Reproducibility and repeatability were evaluated for the three non-targeted TP53 alterations as shown below in Table 20 and Table 21. Reproducibility and repeatability of the non-targeted alterations were found to be 100.00%.

Table 20. Reproducibility of Non-Targeted Alterations.

Source Sample	Non-Targeted Alteration	Variant Type	Observed Average VAF (%)	LoD	Fold LoD (x)	# Positive Replicates	# Total Valid Replicates	Reproducibility (%)	95% Two-Sided Score CI (%)
H	*TP53_844C>T*	SV	3.79	3.20	1.18	36	36	100.00	[90.36, 100.00]
H	*TP53_438G>A*	SV	11.42	3.20	3.57	36	36	100.00	[90.36, 100.00]
A	*TP53_375G>A*	SV	19.05	3.20	5.95	36	36	100.00	[90.36, 100.00]

Table 21. Repeatability of Non-Targeted Alterations.

Source Sample	Non-Targeted Alteration	Variant Type	# Agree Pairs	# Total Valid Pairs	Repeatability (%)	95% Two-Sided Score CI (%)
H	*TP53_844C>T*	SV	18	18	100.00	[82.41, 100.00]
H	*TP53_438G>A*	SV	18	18	100.00	[82.41, 100.00]
A	*TP53_375G>A*	SV	18	18	100.00	[82.41, 100.00]

Confirmation of LoD was evaluated for the three non-targeted variants with hit rate results shown below in Table 22. Although all three non-targeted variants had a 100.00% hit rate, the TP53_844C>T variant was the only non-targeted variant with an observed average VAF (%) ≤3x the median platform LoD. Therefore, the confirmed LoD for TP53 substitutions based on the non-targeted alterations would be 3.79% VAF. Because the confirmed LoD for TP53 substitutions based on the targeted variants in Table 13 (3.43% VAF) is smaller than this value, the confirmed LoD for TP53 substitutions in this study remains 3.43% VAF.

Table 22. Confirmation of LoD for Non-Targeted Variants.

Source Sample	Non-Targeted Alteration	Variant Type	Observed Average VAF (%)	LoD	Fold LoD (x)	# Positive Replicates	# Total Valid Replicates	Hit Rate (%)
H	*TP53_844C>T*	SV (substitution)	3.79	3.20	1.18	36	36	100.00
H	*TP53_438G>A*	SV (substitution)	11.42	3.20	3.57	36	36	100.00
A	*TP53_375G>A*	SV (substitution)	19.05	3.20	5.95	36	36	100.00

VCA was also performed for the three non-targeted variants with results shown below in Table 23. The reproducibility and repeatability %CV were all low for the three non-targeted variants with reproducibility %CV ≤ 17.22% and repeatability %CV ≤ 12.90%.

Table 23. Precision of Continuous Measurements of Non-Targeted Variants.

Source Sample	Non-Targeted Alteration	Variant Type	# Total Valid Replicates	Observed Average VAF (%)	LoD	Fold LoD (x)	Reproducibility		Repeatability	
							SD	%CV	SD	%CV
H	*TP53_844C>T*	SV	36	3.79	3.20	1.18	0.0065	17.22	0.0049	12.90
H	*TP53_438G>A*	SV	36	11.42	3.20	3.57	0.0127	11.11	0.0111	9.70
A	*TP53_375G>A*	SV	36	19.05	3.20	5.95	0.0143	7.49	0.0130	6.82

Chapter IV.

Conclusion

Ensuring the safety and efficacy of a laboratory developed assay, when used as a companion diagnostic, is critical because of the significant impact it can have on a patient's health. CDx results influence treatment decisions, determine treatment eligibility, and in some cases even alter the cancer diagnosis. Therefore, analytical and clinical validation studies are safety measures to protect patients and ensure they receive accurate health information. The health information provided by a CDx test can enable treatment options that maximize a patient's life expectancy, improve their quality of life, alleviate cancer symptoms, and even eliminate cancer growth to the point of remission in some cases. The safety of the F1CDx assay is critical to patients with advanced and recurrent TP53 WT EC because it provides clinical trial eligibility for selinexor now and will enable selinexor access to patients after commercialization in the future. Selinexor has demonstrated safety and clinical significance for TP53 WT EC patients by exhibiting a significant increase in progression-free survival rates with minimal side effects during Karyopharm's phase 3 SIENDO trial (Makker et al., 2024). This is a major advancement for late-stage and recurrent TP53 WT EC patients because effective treatment options prior to selinexor were limited, and prognosis is usually poor.

LoB Discussion

The LoB study evaluated the F1CDx assay in calling TP53 alterations in DNA derived from FFPE EC samples. There were no sample failures during processing; thus, all sample replicates were evaluable and assessed for LoB. The samples used were

previously tested using the F1CDx assay and proven to be TP53 negative, serving as a good candidate to determine the LoB for this study. TP53 alterations were detected in 3 of the 60 valid sample replicates evaluated; thus, the point estimate for the FPR was 5%. This result met the acceptance criteria because the primary endpoint FPR is less than or equal to 5%, as approved by the FDA. Investigation into the 3 detections for the single TP53 SVs yielded evidence that the identified variant may be a true sub-clonal TP53 SV detected in the tumor but was not called in the original final report for the source sample due to low number of reads and therefore low VAF. Therefore, the LoB for TP53 alterations in EC is confirmed as zero for the F1CDx assay.

Precision and Confirmation of LoD Discussion

The precision and confirmation of LoD study evaluated the site-to-site precision and confirmed the LoD of the F1CDx assay in identifying TP53 alterations (REs, SVs, HDs) in DNA derived from FFPE EC samples as well as evaluated the site-to-site precision of the F1CDx assay in identifying samples absent of TP53 alterations in FFPE EC samples. 3 runs were performed on different LC start dates using two reagent lots at each of the 3 sites (2 replicates x 3 LC start dates x 2 reagent lots x 3 sites) for a total of 36 replicates for each of the 9 source samples. A total of 324 replicates were evaluated in this study. For the primary analysis, the 7 targeted variants with observed average measurements above LoD met acceptance criteria for reproducibility. Additionally, LoD was confirmed for all 8 targeted TP53 alterations with the confirmed LoD values presented in Table 13. High reproducibility and repeatability were observed for the targeted variants above LoD, targeted variants below LoD, and the sample with an absence of TP53 alterations. The VCA analyses also demonstrated low %CVs for most

variants. Finally, analyses were conducted for the 3 co-occurring non-targeted variants with results demonstrating high reproducibility and repeatability and low VCA %CV for both metrics. In conclusion, this study demonstrated the F1CDx assay is precise in detecting TP53 alterations (REs, HDs, SVs) as well as the absence of TP53 alterations in FFPE EC samples.

Future Research

The research conducted in this thesis provides evidence that F1CDx, Foundation Medicine's FDA-approved qualitative next-generation sequencing based in vitro CGP assay for solid tumors, is a viable companion diagnostic for TP53 WT EC patients as a means to qualify for selinexor administration. The LoB, confirmation of LoD, and precision analyses yielded successful results, demonstrating that the F1CDx assay is a reliable and safe companion diagnostic for selinexor. Further research is required to complete the requirements for submission of an sPMA, including orthogonal concordance and clinical efficacy analysis. Orthogonal concordance will be important to continue to ensure the safety of F1CDx as a companion diagnostic for selinexor by evaluating the agreement of F1CDx results to an orthogonal third-party method. Clinical efficacy analysis will need to be conducted to determine how well patients who are TP53 WT, as identified by the F1CDx assay, respond to selinexor during the clinical trial. Lastly, progression-free survival rates and side effects will need to continue to be monitored to ensure drug safety and efficacy.

Closing Remarks

The analytical validation studies performed prove that F1CDx does not produce false-positive TP53 results, demonstrates the reproducibility for each targeted TP53 variant with ≥95% confidence, and maintains precision in identifying TP53 alterations across differing variables. These results demonstrate that F1CDx could be a safe and effective companion diagnostic for selinexor, which is expected to make a significant improvement in the lives of patients suffering from advanced and recurrent EC by increasing progression-free survival rates.

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