TECHNICAL MANUAL

OncoMate® MSI Dx Analysis System

Instructions for Use of Product
MD2140
In Vitro Diagnostic

This Technical Manual (TM543) for the OncoMate® MSI Dx Analysis System is formatted into two parts based on the intended use. Both parts of this Technical Manual are for use with the Applied Biosystems® 3500 Dx Genetic Analyzer.

Part 1 (Sections 1–14): Instructions for Use as a companion diagnostic test. Refer to Section 1.5 for approved product use(s).

For use with:

MD2140 OncoMate® MSI Dx Analysis System

MD4130 OncoMate® MSI Dx Assay Installer

MD4850 OncoMate® 5C Matrix Standard

MD4160 OncoMate® MSI Dx Analysis Software

Part 2 (Sections 15–26): Instructions for Use as an aid to identify candidates for further testing for Lynch syndrome

For use with:

MD2140 OncoMate® MSI Dx Analysis System

MD4150 OncoMate® MSI Dx Assay Installer

MD4850 OncoMate® 5C Matrix Standard

MD4140 OncoMate® MSI Dx Interpretive Software

INSTRUCTIONS FOR USE OF PRODUCT
MD2140

IVD

PROMEGA
2800 Woods Hollow Rd.
Madison, WI
USA

Rev2
TM543

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OncoMate® MSI Dx Analysis System (Part 1)

Part 1 (Sections 1–14) is for use as a companion diagnostic test. Refer to Section 1.5 for approved product use(s).

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1. Introduction

1.1 Product Name

OncoMate® MSI Dx Analysis System

Cat.# MD2140, 100 reactions 

Common Name
Fluorescent, multiplex PCR reagents

1.2 About This Guide

This guide describes the OncoMate® MSI Dx Analysis System and is the primary source for information about its intended use as a companion diagnostic test, components, limitations, protocol, troubleshooting and more. The assay workflow comprises several components, which are used together to analyze microsatellite instability in formalin-fixed, paraffin-embedded (FFPE) solid tumor samples:

- A DNA extraction system for normal and tumor FFPE tissue samples such as the Maxwell® CSC Instrument (Cat.# AS6000) and Maxwell® CSC DNA FFPE Kit (Cat.# AS1350)
- **Optional:** A DNA extraction system for matched normal blood samples such as the Maxwell® CSC Instrument and Maxwell® CSC Blood DNA Kit (Cat.# AS1321)
- A dye-based DNA quantification system such as the QuantiFluor® Dx dsDNA System (Cat.# E5900)
- OncoMate® MSI Dx Analysis System (Cat.# MD2140) for amplification
- OncoMate® MSI Dx Assay Installer (Cat.# MD4130)
- OncoMate® 5C Matrix Standard (Cat.# MD4850)
- Applied Biosystems® 3500 Dx Genetic Analyzer (Thermo Fisher Scientific Cat.# A46344) with 3500 Dx Series Data Collection Software 3 IVD, Version 3.2 and higher
- OncoMate® MSI Dx Analysis Software (Cat.# MD4160)

While this guide provides an overview of the entire assay workflow, emphasis is given to the keystone component of the assay: the OncoMate® MSI Dx Analysis System amplification kit. Section 4, Assay Protocol, includes the procedures required for DNA extraction and quantification, and step-by-step instructions for amplifying DNA using the OncoMate® MSI Dx Analysis System. Detailed instructions are also provided in Section 4 to analyze amplified products by capillary electrophoresis. Sections 5 and 6 are dedicated to analysis of capillary electrophoresis data using the OncoMate® MSI Dx Analysis Software. Information on specific functions and capabilities of the OncoMate® MSI Dx Analysis Software are found in the *OncoMate® MSI Dx Analysis Software Technical Manual* #TM680.

The following technical manuals contain instructions for the use of associated assay components.

- *Maxwell® CSC Instrument Operating Manual* #TM457
- *Maxwell® CSC DNA FFPE Kit Technical Manual* #TM395
- *Maxwell® CSC Blood DNA Kit Technical Manual* #TM374
- *QuantiFluor® Dx dsDNA System Technical Manual* #TM676
- *OncoMate® 5C Matrix Standard Technical Manual* #TM542
- *OncoMate® MSI Dx Analysis Software Technical Manual* #TM680
- *Applied Biosystems® 3500 Dx Genetic Analyzer and 3500xL Dx Genetic Analyzer IVD User Guide* (Thermo Fisher Scientific Part #100070881)

1.3 Abbreviations and Definitions

Allele, a DNA sequence at a specific chromosome location. Multiple allele variants may exist within a population, but a single allele is present for each chromosome in each individual.

Bleedthrough, capillary electrophoresis artifact where peaks from one dye channel are detected in a second dye channel at approximately the same size (bp); also known as “spectral pull-up”

bp, base pair

CSV, comma-separated values

DCS, data collection software for the Applied Biosystems® 3500 Dx Genetic Analyzer

dMMR, deficient-mismatch-repair

dsDNA, double-stranded DNA

ESMO, European Society for Medical Oncology

FFPE, formalin-fixed, paraffin-embedded

FSA, fragment analysis data file extension

ICI, immune checkpoint inhibitor

LOH, loss of heterozygosity

MMR, mismatch repair

MSI, microsatellite instability

MSI-H, microsatellite instability high; sample result when two or more markers are unstable

MSS, microsatellite stable; sample result when four or more markers are stable

not MSI-H, an MSS result generated by the OncoMate® MSI Dx Analysis Software

PCR, polymerase chain reaction

PDF, portable document format

RFU, relative fluorescence unit

QC, quality control

UDF1, user-defined field 1

DNA marker or locus, a sequence of interest within the genome (plural: markers, loci)

Heterozygous, a gene (DNA marker) that has two different alleles

Homozygous, a gene (DNA marker) that has two identical alleles

Mononucleotide repeat, DNA structure where the same nucleotide base is repeated

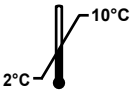
Quasi-monomorphic, a DNA marker for which most individuals are homozygous for the same common allele

Stable, microsatellite instability was not detected for the marker

Stutter peak, an inherent PCR artifact resulting from slippage of the polymerase during in vitro DNA replication. Stutter peaks are one or more repeat units shorter or longer than the true allele peak.

Unstable, microsatellite instability was detected for the marker

1.4 Key to Symbols Used

Symbol	Explanation	Symbol	Explanation
	For In Vitro Diagnostic Use		Protect from light
	Store at 2°C to 10°C		Manufacturer
	Caution		Irritant
	Use by		Contains sufficient for <n> tests
	Do not reuse		Warning. Biohazard
	Consult instructions for use		Catalog number
	Lot number		Serial number

1.5 Intended Use

The OncoMate® MSI Dx Analysis System is a qualitative multiplex polymerase chain reaction (PCR) test intended to detect the deletion of mononucleotides in five microsatellite loci (BAT-25, BAT-26, NR-21, NR-24 and MONO-27) for the identification of microsatellite instability (MSI) using DNA obtained from formalin-fixed, paraffin-embedded (FFPE) solid tumor specimens, and DNA isolated from matched normal FFPE specimen or whole blood. The OncoMate® MSI Dx Analysis System is for use with the Applied Biosystems® 3500 Dx Genetic Analyzer and OncoMate® MSI Dx Analysis Software.

The OncoMate® MSI Dx Analysis System is indicated for use as a companion diagnostic test to identify patients who may benefit from treatment with the targeted therapies listed in Table 1 in accordance with the approved therapeutic product labeling.

Table 1 OncoMate® MSI Dx Analysis System Companion Diagnostic Indications.

Indication	Biomarker	Therapy
Endometrial Carcinoma	MSS; defined as not MSI-high [not MSI-H]	KEYTRUDA® (pembrolizumab) in combination with LENVIMA® (lenvatinib)
Solid Tumors	MSI-H	KEYTRUDA® (pembrolizumab)

1.6 Summary and Explanation

Microsatellites are short, repetitive DNA sequences consisting of repeat units of one to six nucleotides [e.g., (A)n, (CA)n, (AAAAG)n]. Microsatellite sequences are distributed throughout the human genome and are prone to copying errors during DNA replication. Normally, copying errors are repaired by the cellular DNA mismatch-repair (MMR) system. Microsatellite instability (MSI) is observed when MMR function is deficient and DNA replication errors are therefore not repaired (1–6). Among microsatellites, mononucleotide repeats are the most likely to show instability, resulting in microsatellite alleles that are typically shorter in MMR-deficient tissue versus matched normal samples. Microsatellite instability may result from several underlying mechanisms, including somatic and germline mutation of MMR genes and alterations to MMR gene promoter regions(6).

The OncoMate® MSI Dx Analysis System is a qualitative multiplexed polymerase chain reaction (PCR) test to determine tumor MSI status (Figure 1). Five mononucleotide-repeat microsatellite markers are evaluated to assess MSI status (BAT-25, BAT-26, NR-21, NR-24 and MONO-27) and two pentanucleotide-repeat microsatellite markers are evaluated for quality control (QC) purposes (Penta C and Penta D). Stability is assessed at each of the five mononucleotide-repeat microsatellite markers by comparing allelic profiles from a normal (i.e., noncancerous) and a tumor sample collected from the same patient. MSI is indicated by a reduction in the length of microsatellites within the tumor sample. The stability results for the mononucleotide-repeat marker panel are then collectively interpreted to determine the tumor MSI status. If two or more of the five mononucleotide-repeat markers are unstable, the tumor is classified as microsatellite instability-high (MSI-H); if four or more mononucleotide-repeat markers are stable, the tumor is classified as microsatellite stable (MSS). Because samples with valid results that do not meet the test criteria for MSI-H are considered MSS (one or no unstable markers), the OncoMate® MSI Dx Analysis System MSS test result is a not-MSI-H result. The pentanucleotide-repeat markers are analyzed to help confirm shared identity between the normal and tumor DNA samples. Comparison to a normal sample mitigates incorrect interpretation of instability in individuals exhibiting a) locus heterozygosity and polymorphisms at mononucleotide-repeat markers, and b) subtle differences indicative of instability that are difficult to identify without the matched normal sample.

OncoMate® MSI Dx Analysis System data when used as a companion diagnostic test are analyzed to determine tumor MSI status using the OncoMate® MSI Dx Analysis Software.

MSI is a well-accepted, predictive, tissue-agnostic biomarker for the efficacy of immune checkpoint inhibitors (ICIs) (7). MSI can affect a wide range of genes and regulatory sequences, increasing the rate of impactful mutations and subsequent presentation of neoantigens. As a result, MSI-H tumors typically show high levels of infiltration by lymphocytes and other immune cells, whereas MSS tumors usually have low immune cell infiltration. Since ICI therapies target immune suppression receptors or their ligands, immune infiltration creates an environment in which, with treatment, an individual's immune cells can be more effective at combating the cancer. MSI has been observed in many tumor types, and numerous clinical investigations across multiple solid tumor types have confirmed the durable responses observed in MSI-H patients treated with ICIs as well as in not-MSI-H endometrial carcinomas with combination treatments (6, 8-11)



Isolate DNA from FFPE tumor samples and blood or FFPE normal samples using nucleic acid extraction reagents and instruments.

Quantitate DNA using fluorescent DNA quantitation reagents and instruments.

Amplify DNA using the OncoMate® MSI Dx Analysis System.

Calibrate the Dye Spectrum using the OncoMate® 5C Matrix Standard and OncoMate® MSI Dx Assay Installer.

Separate and Detect Fragments using the Applied Biosystems® 3500 Dx Genetic Analyzer.

Data Analysis and Reporting using the OncoMate® MSI Dx Analysis Software.

Workflow Technical Manuals or Reference Documents

Maxwell® CSC DNA kits FFPE Cat.# AS1350, TM395 Blood Cat.# AS1321, TM374 **Maxwell® CSC Instruments** Cat.# AS6000, TM475 Cat.# AS8000, TM623	**QuantiFluor® Dx dsDNA System** Cat.# E5900, TM676	**OncoMate® MSI Dx Analysis System** Cat.# MD2140, TM543	**OncoMate® 5C Matrix Standard** Cat.# MD4850, TM542 **OncoMate® MSI Dx Assay Installer** Cat.# MD4130, TM543	Applied Biosystems® 3500 Dx Genetic Analyzer and 3500xL Dx Genetic Analyzer IVD User Guide	**OncoMate® MSI Dx Analysis Software** Cat.# MD4160, TM680

Figure 1. OncoMate® MSI Dx Analysis System assay workflow.

1.7 Principles of the Procedure

The OncoMate® MSI Dx Analysis System workflow involves DNA extraction from FFPE tissue samples (and optionally blood), quantification of double-stranded DNA (dsDNA) using a fluorescent dsDNA-binding dye, amplification of specific microsatellite markers via multiplex PCR, analysis of amplified DNA fragments by capillary electrophoresis and automated analysis of capillary electrophoresis fragment data using an analysis software (Figure 1). This section reviews the technical foundation for each of these steps in the assay workflow.

Prior to DNA extraction the FFPE tissue section is typically manually preprocessed to deparaffinize the sample, digest proteins and release nucleic acids. Subsequent steps release nucleic acids crosslinked to each other and to proteinaceous components. RNase treatment digests RNA from the sample. Samples are separated into aqueous and mineral oil/paraffin phases, and the aqueous phase is further processed to capture the DNA. Typically, commercially available DNA extraction systems process the sample through nucleic acid binding, washing and elution into water or an elution buffer. When blood is used as the matched normal sample, the extraction process is similar except that deparaffination involving mineral oil is not required. Once eluted, the extracted dsDNA is ready for analysis.

DNA extracts are then quantified using fluorescent DNA-binding dyes. Fluorescent DNA-binding dyes enable sensitive quantitation of small amounts of purified DNA. These dyes are selective for dsDNA, and the signal is linear over a wide range of DNA inputs. Prior to analysis, fluorescent dyes are diluted and mixed with either sample DNA or control DNA, and DNA binding occurs within minutes. A standard curve is prepared from the control DNA and analyzed in parallel with the dye-stained sample DNA, and fluorescence is measured using a compatible fluorometer. The results of this analysis determine DNA concentration and inform the DNA volume requirement for subsequent PCR amplification.

PCR is an enzymatically driven and temperature-dependent in vitro method to make many copies or amplify specific, targeted regions of a DNA template. During PCR, short DNA sequences (primers) bind to flanking regions of the targeted DNA sequence and initiate amplification. Tightly controlled temperature variations programmed into the thermal cycler promote 1) denaturation of double-stranded DNA, 2) primer annealing and 3) synthesis of the complementary DNA strand by a DNA polymerase enzyme. Temperature cycling is repeated many times, resulting in an exponential increase in the abundance of the targeted DNA sequence. During multiplex PCR, several distinct DNA targets are copied in parallel within the same reaction. When primers are conjugated with a fluorescent dye molecule, the PCR products generated are also dye-labeled, permitting their downstream detection via fluorescence.

The OncoMate® MSI Dx Analysis System is a fluorescent, multiplex PCR-based test to detect DNA sequence length changes in microsatellite regions of tumor cell DNA relative to the same regions from the patient's normal cells. Fluorophore-labeled primers are used to co-amplify seven microsatellite markers: five mononucleotide repeat markers evaluated for MSI status (BAT-25, BAT-26, NR-21, NR-24 and MONO-27) and two pentanucleotide repeat markers, Penta C and Penta D (Table 2 and Figure 2), which are evaluated for QC purposes. The mononucleotide-repeat markers are analyzed to determine MSI status and were selected for high sensitivity and specificity to alterations in repeat lengths in samples containing mismatch repair defects. These markers are quasi-monomorphic; in other words, most individuals are homozygous for the same common alleles. The pentanucleotide-repeat markers were selected for their high level of polymorphism and low degree of MSI. These markers are analyzed as an identity check between the normal and tumor samples comprising each pair (2).

Following PCR, dye-labeled OncoMate® MSI Dx Analysis System amplification products from control and patient samples are separated and analyzed using capillary electrophoresis. The double-stranded amplified DNA is heat-denatured in formamide alongside fluorescently labeled DNA fragments of known size, the Size Standard 500. The resulting single-stranded DNA is electrokinetically injected into a capillary of the Applied Biosystems® 3500 Dx Genetic Analyzer, where the DNA fragments are separated based on size and detected through the incorporated fluorescent label.

Table 2. Expected Amplified Size Ranges and Detection Channels for the Markers Included in the OncoMate® MSI Dx Analysis System.

Mononucleotide Markers	Repeat Structure	Detection Channel	Amplified Size Range
BAT-26	$A_{(26)}$	Blue	83 to 121bp
NR-21	$A_{(21)}$	Green	83 to 108bp
BAT-25	$A_{(25)}$	Green	110 to 132bp
MONO-27	$A_{(27)}$	Green	134 to 168bp
NR-24	$A_{(24)}$	Yellow	103 to 138bp

Pentanucleotide Markers	Repeat Structure	Detection Channel	Amplified Size Range
Penta D	$AAAGA_{(2–17)}$	Blue	123 to 253bp
Penta C	$AAAAC_{(4–17)}$	Yellow	140 to 228bp

Tumor MSI status is determined using the OncoMate® MSI Dx Analysis Software, which analyzes microsatellite fragment data (.fsa files) generated during capillary electrophoresis. Microsatellite DNA fragments are sized with reference to the Size Standard 500 fragments using the Local Southern method (36). Following fragment sizing, the software analyzes the required positive and negative amplification controls to verify expected PCR and capillary electrophoresis performance and applies additional QC checks to evaluate patient data quality (see Table 8 for a description of all QC tests performed by the software).

The OncoMate® MSI Dx Analysis Software requires data from a paired normal sample to determine tumor MSI status. To help confirm correct sample pairing, the software analyzes pentanucleotide-repeat markers; if all Penta C and Penta D alleles detected in the normal sample are also present in the tumor sample, the sample identity QC passes. The software then evaluates mononucleotide-repeat loci for evidence of nucleotide deletions indicative of microsatellite instability.

PCR amplification of a mononucleotide-repeat locus results in a central allele peak that is flanked on both sides by PCR stutter peaks. For heterozygous loci, two peak distributions are observed for a locus. Microsatellite instability at a locus is detected as changes in the size (bp) and abundance, represented by fluorescent intensity (RFU), of these DNA fragments in the tumor sample relative to the same patient's normal sample. These changes are produced when a new shorter-length allele and corresponding stutter products are present in the tumor sample. Depending on the size of the deletion and tumor content of the sample, these new DNA fragment peaks may be distinct from or overlap with the cluster of peaks amplified from the patient's normal allele (Figure 3).

The OncoMate® MSI Dx Analysis Software detects deletions ≥2bp in microsatellites when specimen requirements are met (implemented in the software as ≥1.75bp to account for the sizing precision of capillary electrophoresis). A locus is "Unstable" when a new, smaller allele is detected in the tumor sample. Otherwise, the locus is interpreted as "Stable" when no deletion is detected, "No Call" when peak intensities are too low or too divergent from its normal reference to make a confident stability assessment, or "Invalid" when other quality control failures are observed. Stability results from the five mononucleotide-repeat loci are collectively interpreted to assign an overall tumor MSI status:

- A tumor sample is interpreted as "MSI-H" when two or more loci are unstable.
- A tumor sample is interpreted as "MSS" when four or more loci are interpreted as stable (2). A sample interpreted as "MSS" by the software is a not-MSI-H result.
- A sample is assigned an MSI result of "Invalid" when the sample identity check fails, any locus-level Invalid results are observed, or No Call results preclude sample interpretation (see Section 7, Assay Quality Controls, for additional information).

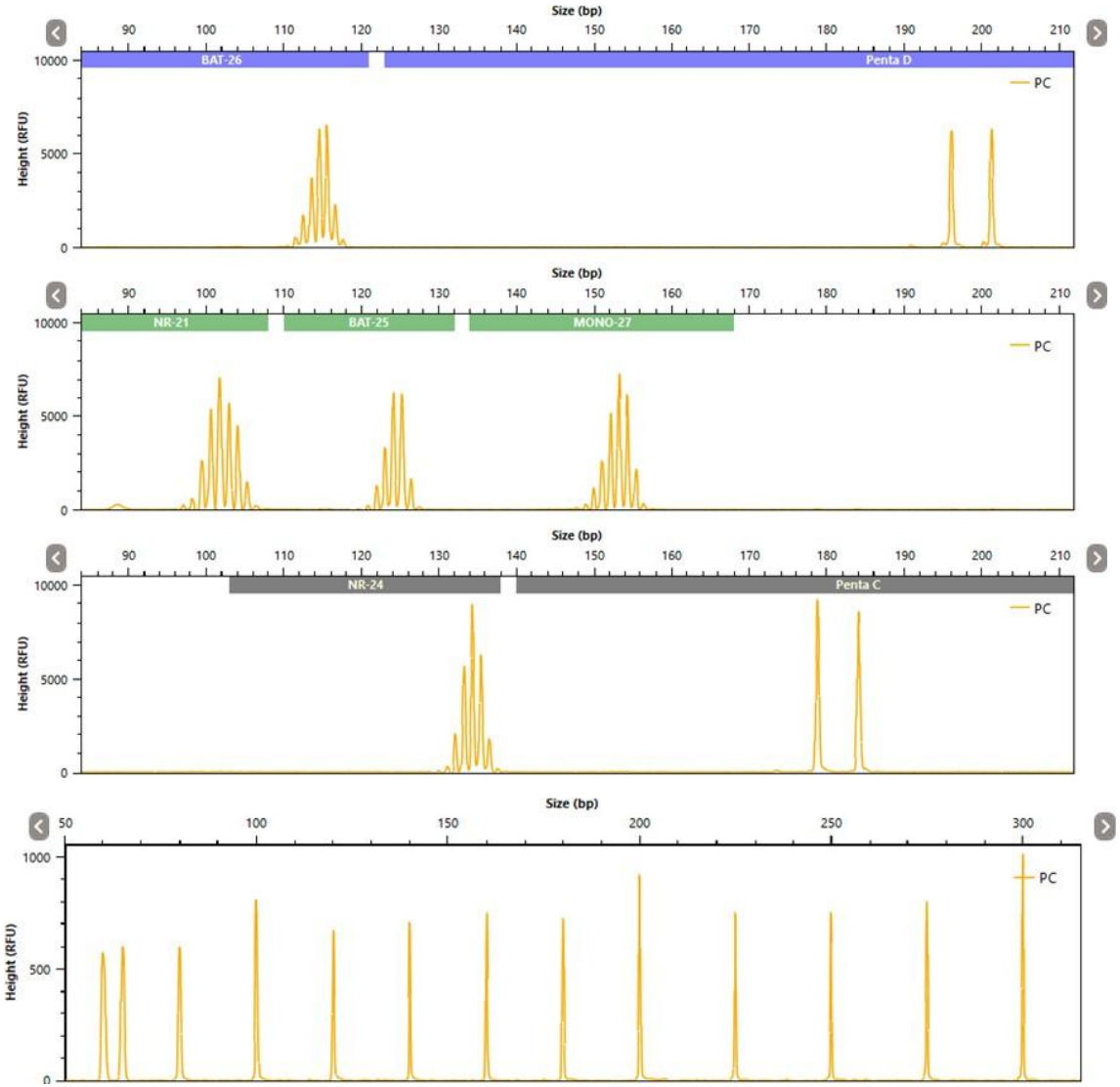


Figure 2. OncoMate® MSI Dx Analysis System data example. A single genomic DNA template (1ng of 2800M positive amplification control, –PC) was amplified using the OncoMate® MSI Dx Analysis System, and the PCR products were analyzed using the Applied Biosystems® 3500 Dx Genetic Analyzer with POP-7® polymer and 50cm capillary array. The OncoMate® MSI Dx Analysis Software displays microsatellite data and the Size Standard 500 by detection channel for easier interpretation.

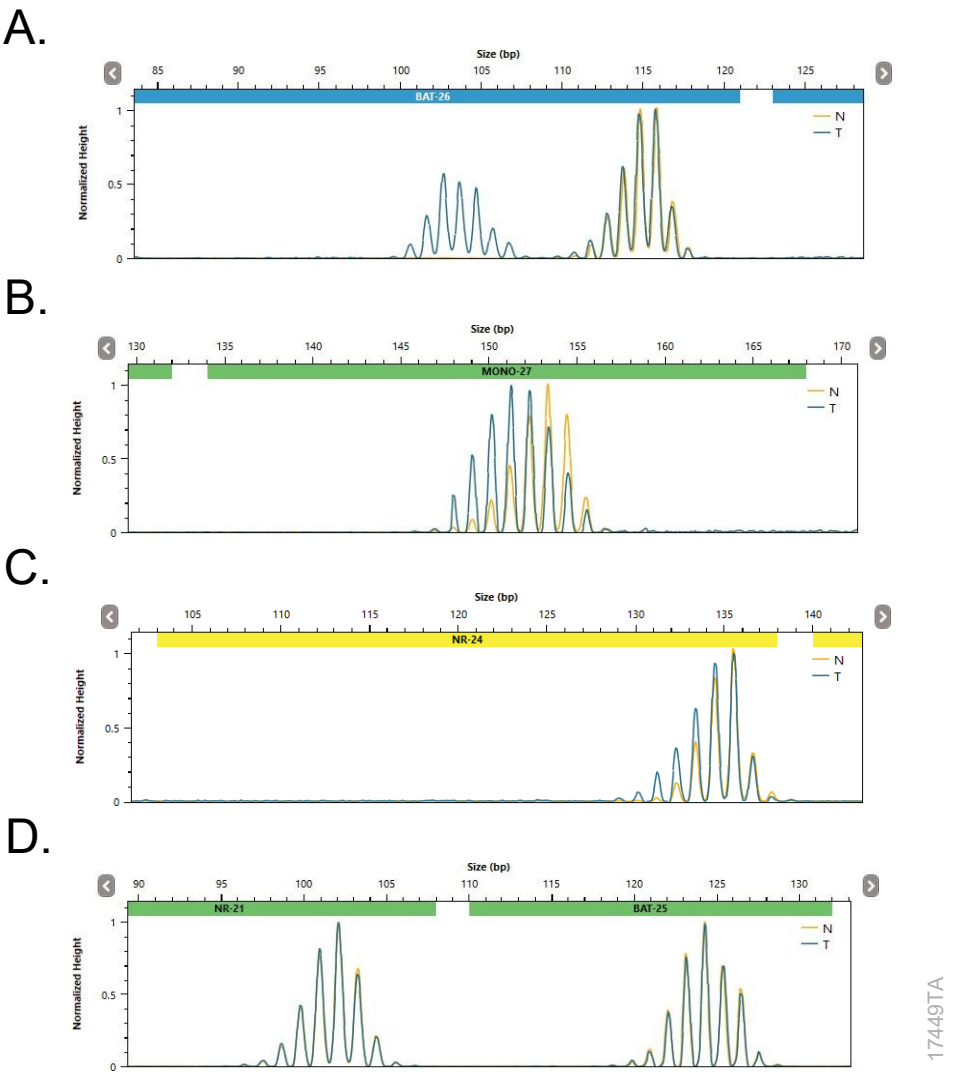


Figure 3. Examples of unstable and stable mononucleotide-repeat markers identified by the OncoMate® MSI Dx Analysis Software. Normalized peak height data are displayed. Depending on the deletion size for a given marker and the tumor content in an MSI-H sample, allele and stutter peaks that indicate instability may be distinct from, or overlapping with, the cluster of peaks amplified from the patient's normal allele. Panel A. New allele and stutter peaks indicating instability in the tumor sample (blue, –T) are distinct from the normal allele peaks amplified in the normal (yellow, –N) and tumor samples. Panels B and C. New allele and stutter peaks amplified from the shorter, unstable allele in the tumor sample partially overlap with the normal allele peaks. Panel D. Unlike MSI-H tumors, MSS tumors show minimal differences between allelic profiles for normal and tumor samples amplified using the OncoMate® MSI Dx Analysis System.

1.8. Assay Limitations

1. For in vitro diagnostic use
2. For professional use only.
3. The OncoMate® MSI Dx Analysis System is intended for use with DNA extracted from formalin-fixed, paraffin-embedded (FFPE) solid tumor tissue samples from cancer patients. The system is not intended for use with DNA from fresh cancer tissues or tissues prepared using other types of fixatives.
4. Normal DNA and tumor tissue DNA from the same patient must be tested at the same time, and data from both samples must be available to generate a result.
5. This assay is validated for use on the Applied Biosystems® 3500 Dx Genetic Analyzer.
6. This assay is not validated to assess expansion of repeats in the target loci.
7. The assay is validated for a DNA input of 1ng and a minimum tumor content (TC) of 30%. The LOD for colorectal, gastric and small bowel solid tumors was validated for a DNA input of 1ng and a minimum tumor content (TC) of 20%. Using less than this amount of DNA or tumor content may lead to quality control failures or false negative results.
8. This assay is validated for use with the OncoMate® 5C Matrix Standard.
9. For tumor samples exhibiting instability at a single locus (1/5 alleles unstable), assess the tumor content and examine electropherograms. Based on this review, consider repeat testing after enriching specimen tumor content or orthogonal testing to rule out an incorrect MSI result.
10. Performance of the OncoMate® MSI Dx Analysis System was validated using the procedures described in the instructions for use. Modifications to these procedures may alter the performance of the assay.
11. Test results obtained using the product must be interpreted by healthcare professionals in conjunction with other clinical findings and laboratory data.

2. Product Components and Storage Conditions

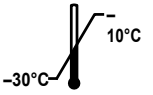
2.1 Materials Provided with the OncoMate® MSI Dx Analysis System (Cat.# MD2140)

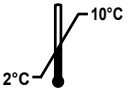
Σ 100 This product contains sufficient reagents to perform 100 amplification reactions.

The following materials are included:

COMPONENT	SIZE	PART #
OncoMate® MSI 5X Primer Mix	**200µl**	**MD705A**

Includes: Fluorophore-labeled and unlabeled primers for BAT-26, Penta D, NR-21, BAT-25, MONO-27, NR-24 and Penta C in a buffered solution.

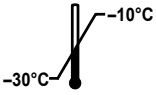
Storage Conditions: Pre-amplification area; 

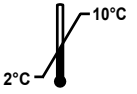
prior to use; 

following first use. Protect from light.

COMPONENT	SIZE	PART #
OncoMate® MSI 5X Master Mix	**200µl**	**MD280A**

Includes: GoTaq® MDx Hot Start DNA Polymerase, dNTPs, magnesium chloride and salts in a buffered solution with stabilizers.

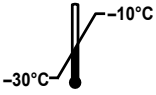
Storage Conditions: Pre-amplification area; 

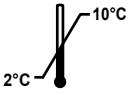
prior to use; 

following first use.

COMPONENT	SIZE	PART #
2800M Control DNA, 10ng/µl	**25µl**	**MD810A**

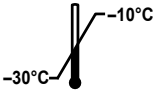
Includes: Cell-line derived male genomic DNA standard in a buffered solution.

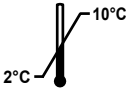
Storage Conditions: Pre-amplification area; 

prior to use; 

following first use.

COMPONENT	SIZE	PART #
Water, Amplification Grade	**1.25ml**	**MD193A**

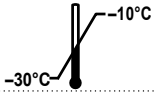
Storage Conditions: Pre-amplification area; 

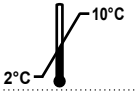
prior to use; 

following first use.

COMPONENT	SIZE	PART #
Size Standard 500	**100µl**	**MD500A**

Includes: Fluorophore-labeled DNA fragments in a buffered solution.

Storage Conditions: Post-amplification area; 

prior to use; 

following first use. Protect from light.

2.2 Storage and Handling of the OncoMate® MSI Dx Analysis System



Upon receipt, store all components at –30°C to –10°C in a nonfrost-free freezer. Before first use, store the 2800M Control DNA at 2°C to 10°C for at least 8 hours. After the first use, store the OncoMate® MSI Dx Analysis System at 2°C to 10°C for up to 3 months. Do not refreeze. Store the OncoMate® MSI 5X Primer Mix and Size Standard 500 protected from light. Store pre-amplification and post-amplification reagents in separate areas and use with dedicated pipettes, tube racks, etc. Components stored under conditions other than those stated on the labels may not perform properly and may adversely affect results.

2.3 Materials Not Provided

Laboratory Reagents

- DNA extraction systems for FFPE or FFPE and blood, such as Maxwell® CSC DNA FFPE Kit (Cat.# AS1350) and Maxwell® CSC Blood DNA Kit (Cat.# AS1321)
- fluorescent-dye-based dsDNA quantification reagents (e.g., QuantiFluor® Dx dsDNA System, Cat.# E5900)
- Nuclease-Free Water (Cat.# MC1191)
- OncoMate® MSI Dx 5C Matrix Standard (Cat.# MD4850)
- Hi-Di™ Formamide 3500 Dx Series (Thermo Fisher Scientific Cat.# 4404307)

  Note: Using high-quality Hi-Di™ Formamide is critical. Freeze Hi-Di™ Formamide in aliquots at –30°C to –10°C. Multiple freeze-thaw cycles or long-term storage at 2–10°C may cause formamide breakdown. Poor-quality formamide may contain ions that compete with DNA during injection, resulting in lower peak heights and reduced sensitivity.

Laboratory Equipment

Note: The following laboratory equipment is required in two distinct areas of the laboratory: one for pre-amplification procedures and one for post-amplification procedures.

- set of calibrated precision pipettes capable of delivering 1µl to 1,000µl
- aerosol-resistant pipette tips (10µl to 1,000µl)
- 1.5ml microcentrifuge tubes
- MicroAmp® Optical 96-Well Reaction Plate with Barcode (Thermo Fisher Scientific Cat.# 4306737)
- MicroAmp® 8-Cap Strip, clear (Thermo Fisher Scientific Cat.# N8010535) (pre-amplification only)
- personal microcentrifuge (“mini centrifuge”)
- centrifuge compatible with 96-well plates (e.g., “mini plate spinner centrifuge”)
- microcentrifuge tube racks
- vortex mixer
- nonfrost-free freezer at –30°C to –10°C
- refrigerator at +2°C to +10°C
- crushed ice (post-amplification only)

Instruments and Accessories

- fluorometer compatible with fluorescent-dye-based dsDNA quantification reagents
- thermal cycler compatible with 96-well plates or reaction tubes
- Applied Biosystems® 3500 Dx Genetic Analyzer (Thermo Fisher Scientific Cat.# A46344)
- 3500 Dx Capillary Array 50cm (Thermo Fisher Scientific Cat.# 4404684)
- 3500 Dx Series Septa 96-Well (Thermo Fisher Scientific Cat.# 4410700)
- POP-7® Performance Optimized Polymer 3500 Dx Series (Thermo Fisher Scientific Cat.# 4393709, 4393713)
- Anode Buffer Container 3500 Dx Series (Thermo Fisher Scientific Cat.# 4393925)
- Cathode Buffer Container 3500 Dx Series (Thermo Fisher Scientific Cat.# 4408258)
- 3500 Dx Series Septa Cathode Buffer Container (Thermo Fisher Scientific Cat.# 4410716)
- Conditioning Reagent 3500 Dx Series (Thermo Fisher Scientific Cat.# 4409543)
- **optional:** Automation to support DNA extraction, such as Maxwell® CSC Instrument (Cat.# AS6000, AS8000)

Software

- OncoMate® MSI Dx Assay Installer (Cat.# MD4130)
- OncoMate® MSI Dx Analysis Software (Cat.# MD4160)

3. Before You Begin

3.1 Warnings and Precautions

Chemical Safety Warning: Some reagents used with fragment analysis are potentially hazardous. Handle and dispose of hazardous materials according to the guidelines established by your institution. Formamide is an irritant and a teratogen; avoid inhalation and contact with skin. Read the warning label and take appropriate precautions when handling this substance. Always wear gloves and safety glasses when working with formamide. Consult the safety data sheet for formamide, available from the Thermo Fisher Scientific Technical Services Department, prior to use.

Safety Data Sheet Statement: Important information regarding the safe handling, transport and disposal of this product is contained in the Safety Data Sheet (SDS). SDSs for all reagents provided in the kits are available online at: **www.promega.com/resources/msds/** or upon request from Promega Technical Services at: **genetic@promega.com**

Biosafety Precautions: Formalin-fixed paraffin-embedded tissues and blood are used in the OncoMate® MSI Dx Analysis System. Follow the guidelines established by your institution for the handling and disposal of these biological specimens.

PCR Precautions and Good Laboratory Practices: The quality of purified DNA, small changes in buffers, ionic strength, primer concentrations, reaction volume, choice of thermal cycler and thermal cycling conditions can affect PCR success. Therefore, the OncoMate® MSI Dx Analysis System requires strict adherence to the recommended procedures for amplification and fluorescence detection described in this manual.

PCR-based microsatellite analysis is subject to contamination by very small amounts of human DNA. Extreme care should be taken to avoid cross-contamination when preparing template DNA, handling kit components, assembling amplification reactions and analyzing amplification products. Store and use reagents and materials used prior to amplification (OncoMate® MSI 5X Master Mix, OncoMate® MSI 5X Primer Mix, 2800M Control DNA and Water, Amplification Grade) in a separate room from those used following amplification (Size Standard 500). Prepare amplification reactions in an area dedicated for reaction setup. Use equipment and supplies dedicated for amplification setup. Always include a negative control reaction (i.e., no template) to detect reagent contamination and a positive control reaction using 2800M Control DNA to verify reagent and instrument performance. Wear gloves and use aerosol-resistant pipette tips to prevent
cross-contamination.

3.2 Specimen Requirements

The OncoMate® MSI Dx Analysis System is intended for use with separate tumor and normal samples collected from the same cancer patient. FFPE tumor tissue is appropriate, and blood or FFPE normal tissue may be used as the required matched normal sample.

Prepare FFPE tissue samples using 10% neutral-buffered formalin following standard pathology practices. Complete a pathology review on prepared tissue sections to confirm that the material is appropriate for downstream use. Tissue suitable for use in the assay contains a tumor content ≥30% tumor cells and sufficient nucleated cells to yield adequate DNA for quantification and amplification with the OncoMate® MSI Dx Analysis System. Tumor specimens may be macrodissected to reach the 30% tumor-cell requirement. The LOD for colorectal, gastric and small bowel solid tumors was validated for a minimum tumor content (TC) of 20%. Stability of FFPE tissue sections mounted on glass slides for the assay was demonstrated for up to 24 months when stored at +20°C to +25°C.

The OncoMate® MSI Dx Analysis System was validated for use with whole blood collected in BD Vacutainer® K_2EDTA tubes. For this assay, blood stability was demonstrated for up to 2 weeks at +2°C to +10°C or +18°C to +23°C and up to 1 month at –10°C to –30°C.

The OncoMate® MSI Dx Analysis System requires 1ng of input DNA and can accommodate up to 6µl of DNA eluate.

Notes:

a. Tumors are heterogeneous in terms of both genotype and phenotype. Mutation-positive tumors can contain wildtype DNA.

b. Obtaining sufficient high-quality DNA from FFPE tissues can be problematic. DNA may be degraded due to prolonged or unsuitable fixation of the tissue sample before embedding in paraffin. Performance of OncoMate® MSI Dx Analysis System amplification reactions may be affected by using insufficient or poor-quality DNA. Accordingly, adhere to best practices to fix and process FFPE tissues.

3.3 DNA Extraction System Requirements

The OncoMate® MSI Dx Analysis System requires DNA purified from FFPE solid tumor tissue and a matched normal sample, consisting of FFPE tissue or blood. Suitable DNA extraction systems provide DNA of sufficient quantity for quantification and amplification and sufficient purity for PCR.

- The OncoMate® MSI Dx Analysis System requires a 1ng DNA input for PCR. Therefore, DNA extracts must have a minimum concentration ≥0.17ng/µl to provide 1ng of DNA for PCR when amplification reactions (10µl total) are formulated to accept the maximum DNA eluate volume (6µl).
- DNA must be eluted in a no-EDTA or low-EDTA solution (e.g., nuclease-free water, 10mM Tris [pH 8.0], 0.1mM EDTA). Amplification may be inhibited by changes in pH (due to added Tris) and/or available magnesium concentration (due to chelation by EDTA) if DNA solutions containing >0.5mM EDTA or >10mM Tris are used for PCR.

DNA eluate stability was demonstrated for up to 18 months when stored at –30°C to –10°C.

The OncoMate® MSI Dx Analysis System is compatible with multiple DNA extraction methods when the above requirements are met. DNA isolated using the Maxwell® CSC DNA FFPE Kit (Cat.# AS1350) or Maxwell® CSC Blood DNA Kit (Cat.# AS1321) with the Maxwell® CSC Instrument (Cat.# AS6000) is suitable for use with the OncoMate® MSI Dx Analysis System. (See Section 10.1.6)

3.4 DNA Quantification System Requirements

The OncoMate® MSI Dx Analysis System is intended for use with purified DNA that was quantified by fluorescence using dsDNA-binding dyes. Suitable quantification systems must reproducibly quantitate DNA eluates with concentrations ≥0.17ng/µl. Assay performance may be negatively affected by using less-sensitive reagents and instrumentation. When selecting a fluorometer, follow the recommendations of the fluorescent reagent manufacturer to ensure compatibility.

The QuantiFluor® Dx dsDNA System (Cat.# E5900) is suitable for use with the OncoMate® MSI Dx Analysis System.

Note: UV-absorbance measurements are unreliable for determining dsDNA concentration in DNA extracts from FFPE tissue samples.

3.5 Thermal Cycler Requirements for OncoMate® MSI Dx Analysis System Amplification Reactions

The OncoMate® MSI Dx Analysis System was developed and tested using thermal cyclers that meet the following specifications:

Maximum Block Ramp Rate: 3.9°C/second to 5°C/second
Temperature Accuracy: ±0.25°C (at ≥90°C)

Temperature Uniformity: <0.5°C (at ≥90°C)

Heated lid capable of reaching 103°C to 105°C

The performance of this assay may be negatively affected by using thermal cyclers with specifications outside of the indicated ranges. Thermal cyclers should be maintained and calibrated according to the instrument manufacturer's instructions.

After confirming that the thermal cycler selected meets the required performance criteria, preprogram the instrument with the protocol provided in Section 4.6.

3.6 Capillary Electrophoresis Requirements

3.6.1 Instrument Configuration and Requirements

OncoMate® MSI Dx Analysis System amplification products are analyzed by capillary electrophoresis using the Applied Biosystems® 3500 Dx Genetic Analyzer in "Diagnostic Mode" using POP-7® 3500 Dx Series Polymer and a 3500 Dx Series Capillary Array, 50cm. This instrument must be regularly maintained according to its instructions for use.

The Applied Biosystems® 3500 Dx Genetic Analyzer conforms to the following technical specifications:

- Number of dyes detected: ≥5
- Excitation wavelength (approximate): 480nm to 520nm
- Detection optics: OncoMate® MSI Dx Analysis System dyes require emission capture from approximately 500nm to 630nm
- Fragment resolution: Single-base resolution through an optimized combination of separation matrix, array length and run conditions
- Resolution range: 1bp resolution from 60bp to ≥300bp
- Sizing precision (repeatability, expressed as standard deviation): ≤0.15bp across a range from 60bp to ≥300bp

3.6.2 Assay Installation

OncoMate® MSI Dx Analysis System amplification products are analyzed using the capillary electrophoresis run settings provided with the OncoMate MSI Dx Assay v2.0. To run in Diagnostic Mode, the OncoMate MSI Dx Assay v2.0 must be installed prior to first analysis on the Applied Biosystems® 3500 Dx Genetic Analyzer using the OncoMate® MSI Dx Assay Installer (Cat.# MD4130). OncoMate® File Name Convention and Results Group files also are installed for your convenience. However, a laboratory can create customized File Name Convention and Results Group files to meet its specific needs. Refer to Section 14.1 for complete assay installation instructions.

3.6.3 Spectral Calibration

Prior to use, spectral calibration of the Applied Biosystems® 3500 Dx Genetic Analyzer using the OncoMate® 5C Matrix Standard (Cat.# MD4850) is required. Spectral calibration is performed using the 'OncoMate_MSI' dye set, which is installed on the instrument using the OncoMate® MSI Dx Assay Installer. Perform a new spectral calibration after any major maintenance on the system, such as changing the laser, calibrating or replacing the CCD camera, or changing the polymer type or capillary array. In addition, perform a new spectral calibration after the instrument is moved to a new location or is serviced by the manufacturer. In some instances, a software upgrade may also necessitate generation of a new spectral calibration. Refer to the *OncoMate® 5C Matrix Standard Technical Manual* #TM542 for detailed protocols and additional information on spectral calibration.

3.6.4 Use of Conditioning Reagent 3500 Dx Series

Conditioning Reagent 3500 Dx Series (Thermo Fisher Scientific Cat.# 4409543) is used during routine maintenance of the 3500 Dx Genetic Analyzer and when changing the polymer type on the instrument. During execution of the 3500 Dx Genetic Analyzer "Change Polymer Type" and "Wash Pump and Channels" wizards, complete the optional bubble purge steps ('bubbles are observed before' and 'bubbles are observed after') and the Fill Array step when installing or reinstalling polymer.

Refer to the *Applied Biosystems® 3500 Dx Genetic Analyzer and 3500xL Dx Genetic Analyzer IVD User Guide* for additional information on instrument preparation and maintenance.

3.7 OncoMate® MSI Dx Analysis Software Requirements

Data analysis is performed using the OncoMate® MSI Dx Analysis Software, which automates data quality control evaluation and sample MSI determination. Refer to the *OncoMate® MSI Dx Analysis Software Technical Manual* #TM680 for comprehensive information on its installation and use. Sample analysis using the software (as described in Section 5) requires the creation of one or more user accounts by the software administrator. Each user is assigned a role with specific permissions to perform workflow tasks. The workflow described in Section 5 requires the creation of at least one user with the Lab Director role to review, approve and release test results.

In general, the OncoMate® MSI Dx Analysis Software conforms to the following technical specifications:

- Accepts fragment data (.fsa) files
- Calculates fragment size using the Local Southern method
- Attributes fragments to a marker by size range and fluorescent label (i.e., color)
- Tabulates and displays relative fluorescence data from a mixture of fragment sizes (60–300bp) and colors (≥5)
- Displays routine data quality information (e.g., off-scale peaks) captured during capillary electrophoresis
- Provides visual and tabular data to enable determination of assay-specific data quality parameters (see Section 6.3, Data Quality Requirements) required for accurate data analysis and interpretation.

3.7.1 Data Requirements for Analysis with the OncoMate® MSI Dx Analysis Software

The OncoMate® MSI Dx Analysis Software groups samples and controls into batches for analysis. A batch is defined by the individual capillary electrophoresis plate from which the test samples and controls were injected. To import and analyze data using the analysis software, the following minimum requirements must be met:

- Capillary electrophoresis must be performed using the Applied Biosystems® 3500 Dx Genetic Analyzer in Diagnostic Mode using the OncoMate MSI Dx Assay v2.0.
- Samples must exist as matched normal and tumor sample pairs (see Section 7.2), including when troubleshooting QC issues.
- During capillary electrophoresis run setup, information for paired samples (sample name and normal/tumor designation) must be entered into the Applied Biosystems® 3500 Dx Genetic Analyzer Data Collection Software (DCS) following prescribed conventions (see Section 4.9) and each sample must be identified as "Samples" in the 'Sample Type' field.
- The batch must have both a positive amplification control and a negative amplification control, and these controls must be identified as "Positive Control" and "Negative Control" in the Applied Biosystems® 3500 Dx Genetic Analyzer DCS 'Sample Type' field. (See Sections 4.9 and 7.3.)
- The software will only import and analyze one positive and one negative amplification control per batch even if more than one of a control type is analyzed during capillary electrophoresis.

4. Assay Protocol

Note: Do not transfer volumes less than 1.0µl when quantifying DNA, performing DNA dilutions, setting up amplification reactions or preparing amplification products for capillary electrophoresis. Pipetting volumes less than 1.0µl may affect assay performance.

4.1 DNA Extraction from FFPE Tissue Sections and Blood

Once the sample is confirmed to meet the OncoMate® MSI Dx Analysis System assay requirements (Section 3.2), extract DNA from blood and/or FFPE samples using the selected DNA extraction system. Store the purified DNA at –30°C to –10°C for up to 1 year.

The OncoMate® MSI Dx Analysis System is compatible with multiple DNA extraction methods when the requirements in Section 3.3 are met. The Maxwell® CSC Blood DNA Kit (Cat.# AS1321) and Maxwell® CSC DNA FFPE Kit (Cat.# AS1350) using the Maxwell® CSC Instrument (Cat.# AS6000) provide DNA eluates suitable for use with the OncoMate® MSI Dx Analysis System.

Note: Residual resin from the Maxwell® CSC DNA FFPE Kit commonly carries over into the final DNA eluate. The resin will not interfere with downstream analyses.

4.2 Quantification of Purified DNA

Following DNA extraction, quantify dsDNA using a method based on fluorescent dsDNA-binding dyes.

Prior to quantification, vortex the purified DNA three times for 5 seconds each at maximum speed, and centrifuge briefly (1 to 2 seconds) in a microcentrifuge. When quantifying DNA, use ≥1µl sample to improve accuracy. The OncoMate® MSI Dx Analysis System amplification reaction can accommodate up to 6µl of sample when the amplification mix is assembled with less Water, Amplification Grade. Therefore, purified DNA samples with concentrations ≥0.17ng/µl are acceptable for use (i.e., are of sufficient concentration to provide 1ng of DNA for amplification).

The QuantiFluor® Dx dsDNA System is suitable for use with the OncoMate® MSI Dx Analysis System.

Note: For highly concentrated DNA samples, particularly those purified from whole blood, accuracy may be improved by diluting the sample. Do not accept results that fall outside of the linear range of the fluorescence assay. Dilute the sample and repeat the fluorescence measurement.

4.3 Dilution of Purified DNA

The OncoMate® MSI Dx Analysis System was developed with a dsDNA input of 1ng, delivered in a volume of 1µl to 6µl. We recommend diluting DNA to a constant concentration across samples so that a 1ng DNA input is added to each reaction in a constant volume. During method validation of the OncoMate® MSI Dx Analysis System, all DNA templates were diluted to 0.5ng/µl and 2µl of diluted template DNA was added to each reaction. Complete the following steps for DNA samples that require dilution prior to amplification:

1. Vortex DNA sample three times for 5 seconds each at maximum speed, and centrifuge briefly (1 to 2 seconds), if necessary, in a microcentrifuge to collect the sample at the bottom of the tube.

   Note: Failure to adequately mix DNA samples may result in heterogeneous DNA concentration and inaccurate dilutions.

2. Dilute DNA sample in Nuclease-Free Water (Cat.# MC1191) or Water, Amplification Grade, so that 1ng of dsDNA is added to each amplification reaction in the desired template volume (see Section 4.5). To maximize accuracy, pipet volumes of ≥1µl when diluting sample DNA and mix dilutions well. Extended storage of diluted sample DNA for future use may result in poor amplification and assay failure.

   Note: PCR amplification efficiency can be greatly altered by changes in pH (due to added Tris HCl) or available magnesium concentration (due to chelation by EDTA) in the amplification reaction when Tris or Tris-EDTA-based diluents are used. DNA dilutions should not be performed in a 96-well plate unless thorough mixing is ensured. Incomplete mixing may result in non-uniform DNA concentration and inaccurate DNA input for PCR, which can lead to assay QC failures or incorrect results.

4.4 2800M Control DNA Dilution

Store the 2800M Control DNA, 10ng/µl, at 2°C to 10°C for a minimum of 8 hours before first use. We recommend diluting the control DNA to the same constant concentration as the test samples. During method validation of the OncoMate® MSI Dx Analysis System, 2800M Control DNA was diluted to 0.5ng/µl and 2µl of diluted control DNA was added to each reaction.

1. Vortex the 2800M Control DNA three times for 10 seconds each at maximum speed.
2. Dilute 2800M Control DNA in Nuclease-Free Water (Cat.# MC1191) or Water, Amplification Grade, so that 1.0ng is added to the positive control reaction in the desired volume (1–6µl). See Table 3 for example dilutions. To ensure accuracy, pipet volumes ≥1µl when preparing 2800M Control DNA dilutions.

Table 3. Diluting the 2800M Control DNA.

Volume of DNA Template Per Reaction	Volume of 2800M Control DNA (10ng/µl)	Volume of Water[1]
1.0µl	2.0µl	18µl
2.0µl	2.0µl	38µl
3.0µl	2.0µl	58µl
4.0µl	2.0µl	78µl
5.0µl	2.0µl	98µl
6.0µl	2.0µl	118µl

[1]Nuclease-Free Water or Water, Amplification Grade

Notes:

- Prepare a fresh 2800M Control DNA dilution for each experiment. Storing diluted 2800M Control DNA for future use may result in poor amplification of the positive control and assay failure.
- The OncoMate® MSI Dx Analysis Software requires one 2800M Control DNA positive control reaction to be amplified and analyzed per plate (“batch”). Failure to include a positive control reaction or use of a DNA other than 2800M Control DNA as the positive control will result in batch failure and invalid results for all samples in that batch.

4.5 Preparation of OncoMate® MSI Dx Analysis System Amplification Reactions

Keep all pre-amplification and post-amplification reagents in separate areas. Prepare amplification reactions in a room dedicated for reaction setup. Use equipment and supplies dedicated for amplification setup. Wear gloves and use aerosol-resistant pipette tips to prevent DNA cross-contamination. Use a fresh pipette tip when adding each DNA sample, the 2800M Control DNA and Water, Amplification Grade (for negative controls), to amplification reactions.

1. If necessary, dilute the template DNA to the desired DNA concentration. See Section 4.3 for more information.
2. At the first use, thaw Water, Amplification Grade, OncoMate® MSI 5X Primer Mix and OncoMate® MSI 5X Master Mix completely. After the first use, store the reagents at +2°C to +10°C.

   Note: A precipitate may form in the OncoMate® MSI 5X Master Mix. Presence of the precipitate will not affect DNA amplification using the OncoMate® MSI Dx Analysis System.
3. Centrifuge tubes briefly (1 to 2 seconds) in a microcentrifuge to bring contents to the bottom, and vortex reagents three times for 3 seconds each at maximum speed. Do not centrifuge after vortexing, as this may cause the reagents to form a concentration gradient in the tube.
4. Label a new MicroAmp® Optical 96-Well Reaction Plate.
5. Determine the number of reactions to be assembled. This must include at least one positive amplification control and one negative amplification control reaction for each plate processed. Add additional reactions to the calculation to compensate for pipetting error. While this approach consumes a small amount of each reagent, it ensures that sufficient PCR amplification mix is available for all samples.
6. Assemble the PCR amplification mix as described in Table 4. Add the final volume of Water, Amplification Grade, OncoMate® 5X Master Mix and OncoMate® 5X Primer Mix to a clean, 1.5ml tube. The template DNA will be added to each reaction well individually at Step 8.

Table 4. Assembly of PCR Amplification Mix.

PCR Amplification Mix Component[1]	Volume Per Reaction	×	Number of Reactions	=	Final Volume
Water, Amplification Grade[2]	to a final volume of 10µl	×		=	
OncoMate® MSI 5X Master Mix[3]	2µl	×		=	
OncoMate® MSI 5X Primer Mix[4]	2µl	×		=	
DNA (1.0ng)	up to 6µl				
Total Reaction Volume	10µl				

[1]Combine Water, Amplification Grade, OncoMate® 5X Master Mix and OncoMate® 5X Primer Mix in a new 1.5ml tube. The template DNA will be added to each reaction well individually at Step 8.

[2]The volume of Water, Amplification grade added to the amplification mix equals 6µl minus the Template DNA volume (e.g., if using 2µl of DNA template, assemble the mix using 4µl of Water, Amplification Grade).

[3]A precipitate may form in OncoMate® MSI 5X Master Mix. Presence of the precipitate will not affect DNA amplification using the OncoMate® MSI Dx Analysis System.

[4]The OncoMate® MSI 5X Primer Mix is light sensitive and must be protected from light.

7. Vortex the PCR amplification mix three times for 3 seconds each at maximum speed, and then pipet the PCR amplification mix into each well of the reaction plate(s) used for samples and controls.

   Note: Failure to vortex the PCR amplification mix sufficiently can result in poor amplification or marker-to-marker imbalance. Add the PCR amplification mix to the wells of the reaction plate as soon as the mix is prepared. Proceed promptly with Steps 8 through 11, followed immediately by thermal cycling.

8. Vortex the diluted FFPE template DNA (prepared in Section 4.3) three times for 5 seconds each at maximum speed, and centrifuge briefly (1 to 2 seconds) in a mini centrifuge to collect the liquid at the bottom of the tube. Pipet 1.0ng of each sample into the designated well containing PCR amplification mix. Mix by pipetting several times.

9. Vortex the diluted 2800M Control DNA (prepared in Section 4.4) three times for 10 seconds each at maximum speed, and centrifuge briefly (1 to 2 seconds) in a mini centrifuge to collect the liquid at the bottom of the tube. Pipet 1.0ng of the 2800M Control DNA dilution into the well reserved for the positive control reaction. Mix by pipetting several times.

10. For the negative amplification control, pipet Water, Amplification Grade, (instead of template DNA) into the well reserved for the negative control reaction. Mix by pipetting several times.

    Note: Failure to amplify and analyze a negative control reaction will result in batch failure and 'Invalid' results for all patient samples during data analysis using the OncoMate® MSI Dx Analysis Software.

11. Cap the wells with MicroAmp® 8-Cap Strips, and centrifuge briefly in a mini plate centrifuge to bring contents to the bottom of the wells and to remove air bubbles.

4.6 Thermal Cycling

1. Ensure that the heated lid has reached the programmed temperature and place the reaction plate in a thermal cycler. Close the thermal cycler lid.
2. Select and run the specified protocol in Figure 4. Ensure that the reaction volume is set to 10µl. The total cycling time, including ramping, is approximately 1 hour and 15 minutes.

Thermal Cycling Protocol[1]
96°C for 1 minute, then:
96°C for 10 seconds
58°C for 1 minute
72°C for 30 seconds
for 29 cycles, then:
60°C for 10 minutes, then:
4°C hold

[1]Reaction volume: 10µl; Heated lid: 103° to 105°C

Figure 4. Thermal cycling protocol for the OncoMate® MSI Dx Analysis System.

3. After completion of the thermal cycling protocol, proceed with fragment analysis, or store amplification products protected from light for up to 7 days at 2°C to 10°C or for up to 30 days at –30°C to –10°C.

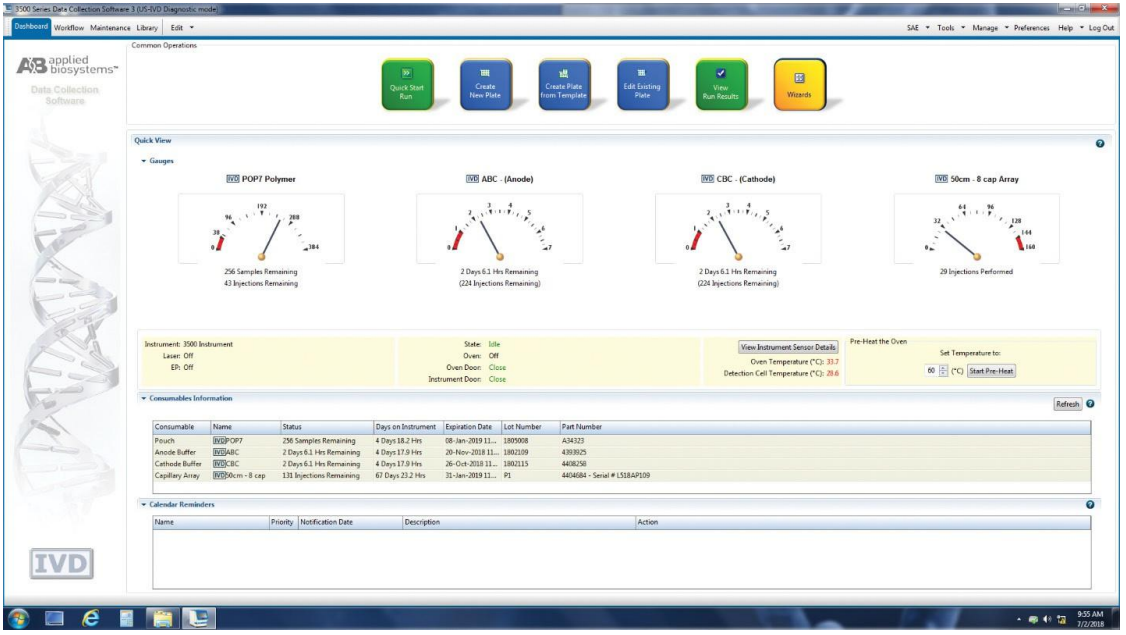
4.7 Preparation of Applied Biosystems® 3500 Dx Genetic Analyzer

1. Open the 3500 Series Data Collection Software and select **Diagnostic Mode** upon login. Navigate to the 'Dashboard' screen (Figure 5).
2. Complete any instrument maintenance required under Calendar Reminders. Under Consumables Information, ensure that consumables are not expired and that a sufficient number of samples or injections are available to complete the planned analysis. Within the instrument, inspect the consumables to ensure that buffer levels are at their fill lines. Check the pump assembly for bubbles and run the Remove Bubble wizard if needed.

Refer to the *Applied Biosystems® 3500 Dx Genetic Analyzer and 3500xL Dx Genetic Analyzer IVD User Guide* for additional information on instrument preparation and maintenance.

3. Set the oven temperature to 60°C and then select **Start Pre-Heat**. Preheat the oven for at least 30 minutes before starting a run.

   Note: The oven will turn off after 2 hours of instrument inactivity.



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Figure 5. Dashboard on the Applied Biosystems® 3500 Dx Genetic Analyzer, installed with POP-7® polymer, a 50cm array and Data Collection Software Version 3.2.

4.8 Preparation of OncoMate® MSI Dx Analysis System Amplified Fragments for Capillary Electrophoresis

1. If amplified samples were stored at –30°C to –10°C, thaw them completely before proceeding. Vortex for 5 seconds and centrifuge the plate for 5 to 10 seconds in a mini plate centrifuge to collect contents at the bottom of wells.
2. Determine the number of wells required to analyze all amplified samples, including the positive and negative control reactions. Add to this number any unused wells from which an injection will be initiated plus additional wells to compensate for pipetting error.
3. Vortex the Size Standard 500 three times for 3 seconds each at maximum speed, and prepare the capillary electrophoresis loading cocktail as directed in Table 5.

Note: Formamide is an irritant and a teratogen; avoid inhalation and contact with skin. Read the warning label and take appropriate precautions when handling this substance. Always wear gloves and safety glasses when working with formamide.

Table 5 Capillary Electrophoresis Loading Cocktail Preparation.

Loading Cocktail	Volume Per Well	×	Number of Wells	=	Final Volume
Hi-Di™ 3500 Dx Series Formamide	9.5µl	×		=	
Size Standard 500	0.5µl	×		=	
Total Volume	10µl				

4. Vortex the loading cocktail three times for 3 seconds each at maximum speed, and centrifuge briefly (1 to 2 seconds) in a microcentrifuge to collect the reagents at the bottom of the tube.
5. Pipet 10µl of loading cocktail into each required well of a MicroAmp® Optical 96-Well Reaction Plate.

   Note: Loading cocktail or Hi-Di™ formamide must be added to every well from which an injection is initiated, whether amplified products are also added to the well or not. Failure to add loading cocktail or Hi-Di™ formamide to a well that is injected may result in damage to the capillary array and run failure.
6. Add 1µl of amplified sample or control reaction to each designated well.
7. Cover wells with 3500 Dx Series Septa.
8. Centrifuge the plate for 5 to 10 seconds in a mini plate centrifuge to bring the formamide-sample mixture to the bottom of each well and to remove air bubbles.
9. Denature samples at 95°C for 3 minutes in a thermal cycler, and then immediately chill the plate on crushed ice for at least 3 minutes. Denature samples just prior to loading the plate onto the Applied Biosystems® 3500 Dx Genetic Analyzer.

   Note: Do not close the heated lid of the thermal cycler, as this may melt the plate septa.
10. Place the plate in the 96-well plate base and cover with the plate retainer. Load the plate onto the Applied Biosystems® 3500 Dx Genetic Analyzer. Ensure that the oven is preheated to 60°C.

4.9 Detection of Amplified Fragments Using the Applied Biosystems® 3500 Dx Genetic Analyzer

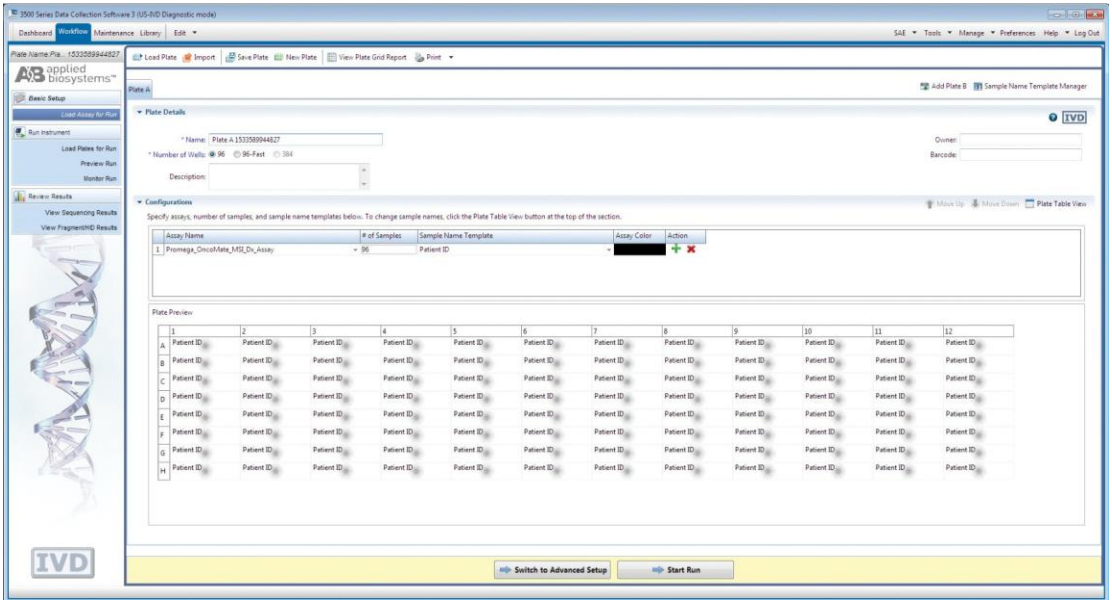
The Applied Biosystems® 3500 Dx Genetic Analyzer Data Collection Software (DCS) employs an application-specific "Assay" that defines run parameters during sample analysis. Separation and fluorescence-based detection of PCR products generated using the OncoMate® MSI Dx Analysis System amplification kit is accomplished using the conditions in the OncoMate MSI Dx Assay v2.0. If this assay is not yet installed on the Applied Biosystems® 3500 Dx Genetic Analyzer, refer to Section 14.1 for installation instructions.

The OncoMate MSI Dx Assay v2.0 is preconfigured with all necessary parameters to separate and detect amplified fragments (e.g., dye set, injection time, injection voltage). These parameters cannot be changed.

There are two methods that can be used to set up sample plates in the Applied Biosystems® 3500 Dx Genetic Analyzer Data Collection Software, either using the instrument software interface or using the Import function. The steps below use the instrument software interface.

Note: Sample and run information assigned in Steps 4–6, below, also can be entered for the entire plate from a text file using the Import function in the 3500 Dx Series Data Collection Software. Refer to the *Applied Biosystems® 3500 Dx Genetic Analyzer and 3500xL Dx Genetic Analyzer IVD User Guide* for information on how to create and use a plate import template for importing plate information.

1. In the Applied Biosystems® 3500 Dx Genetic Analyzer Data Collection Software, navigate to the 'Workflow' tab. Select **Switch to Advanced Setup** if Basic Setup is displayed under the Applied Biosystems logo in the navigation pane (Figure 6).



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Figure 6. 'Workflow' tab, Load Assay for Run screen.

2. Select **Define Plate Properties** in the navigation pane (Figure 7). Under Plate Details, assign the plate a unique Name, and set the Number of Wells to **96** and the Plate Type to **Fragment**. Verify that the Capillary Length and Polymer are set to **50cm** and **POP7**, respectively.

   Note: The plate Name assigned at this step is used by the OncoMate® MSI Dx Analysis Software to name the sample batch.

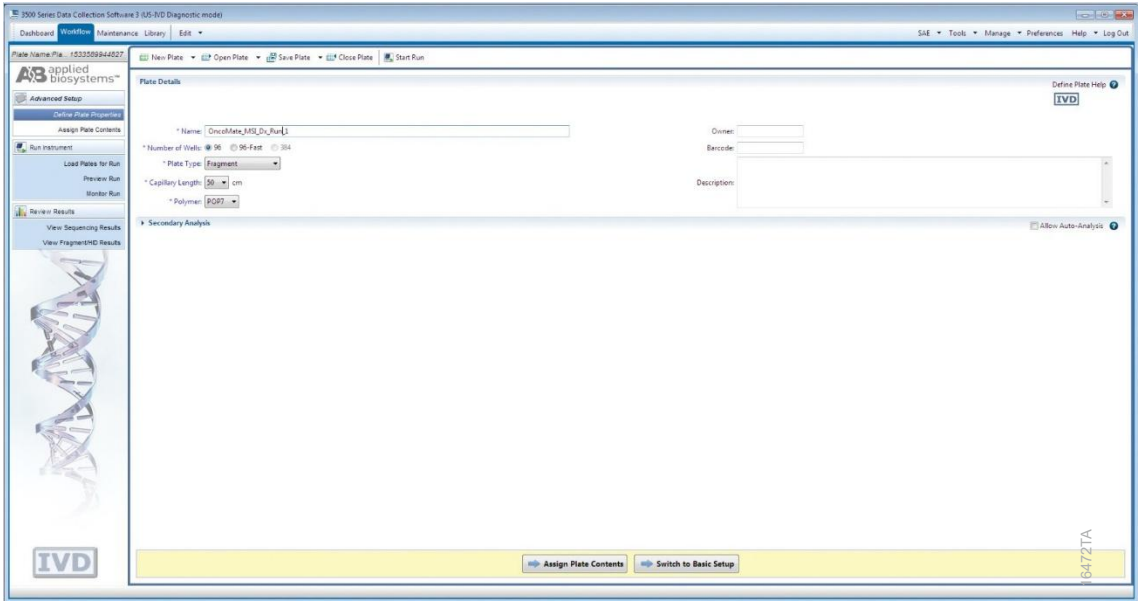


Figure 7. 'Define Plate Properties' screen.

3. Select **Assign Plate Contents** in the navigation pane or at the bottom of the screen (Figure 7).
4. With the 'Plate View' tab selected (Figure 8), use the three **Add from Library** links to load the OncoMate® Assay (OncoMate MSI Dx Assay v2.0), file name convention (OncoMate_MSI_DX) and results groups (OncoMate_MSI_DX) files.
5. Assign these protocols to the batch by selecting the wells in the plate containing samples or controls and adding a checkmark to each of the three check boxes next to the file names.

   Note: The OncoMate_MSI_DX File Name Convention and Results Group files are provided for convenience; customized files can be created and used in their place to suit the unique needs of different laboratories.

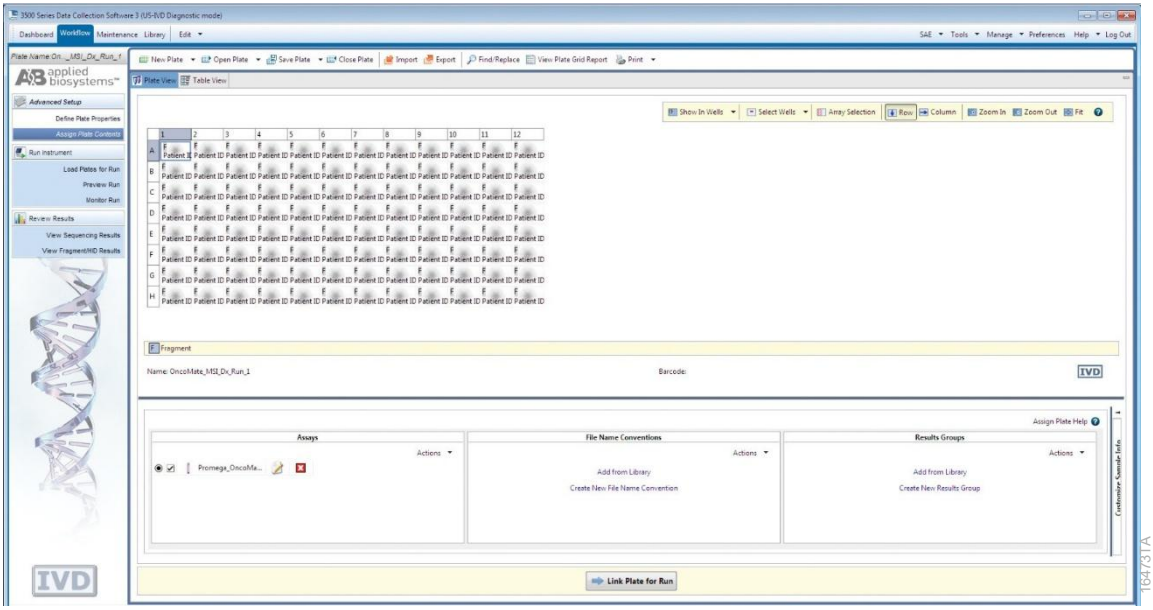


Figure 8. 'Plate View' tab, Assign Plate Contents screen.

6. Switch to the 'Table View' tab, and enter information for the samples and controls to be analyzed (Figure 9).
   - Assign a sample name and normal/tumor designation for paired samples. Ensure patient privacy when labeling or naming samples. Do not include any protected health information, such as names, dates of birth, medical record numbers or other identifiers that could directly or indirectly be used to identify an individual. A single data (.fsa) file is created for each well assigned as a sample or control during capillary electrophoresis setup. The OncoMate® MSI Dx Analysis Software will correctly identify and import normal and tumor sample pairs when either of the following conventions is used:

     Option 1: Enter required sample information into the Sample Name field and User Defined Field 1 (UDF1).

     - Assign matched normal and tumor samples identical sample names in the Sample Name field.
     - Place the letter N or T in UDF1 to indicate the normal or tumor sample, respectively. The N or T designation is case insensitive and should not be used for amplification controls. This convention is demonstrated in Figure 9.

Option 2: Enter all required sample information into the Sample Name field in three parts (separated by underscores): [Part 1]_[Part 2]_[Optional Part 3]. See the *OncoMate® MSI Dx Analysis Software Technical Manual* #TM680 for a comprehensive description of using option 2 for sample identification, including examples.

- Part 1 is the sample name that will be displayed in the analysis software; it must be identical for the normal and tumor samples.
- Part 2 is an N or T; this field is case-insensitive.
- Part 3 is optional and contains additional sample information (e.g., specimen name) desired by the laboratory. Part 3 can be different for the normal and tumor samples. Using this option, the user may also include other sample metadata in UDF1. When using this option, sample import will fail if N or T (case insensitive) is entered in the UDF1 field.

- Assign names for the control samples in the Sample Name field according to your laboratory's preferences.
- Assign the Sample Type as "Sample", "Positive Control" or "Negative Control". The Sample Type field must be assigned correctly to perform data analysis using the OncoMate® MSI Dx Analysis Software.

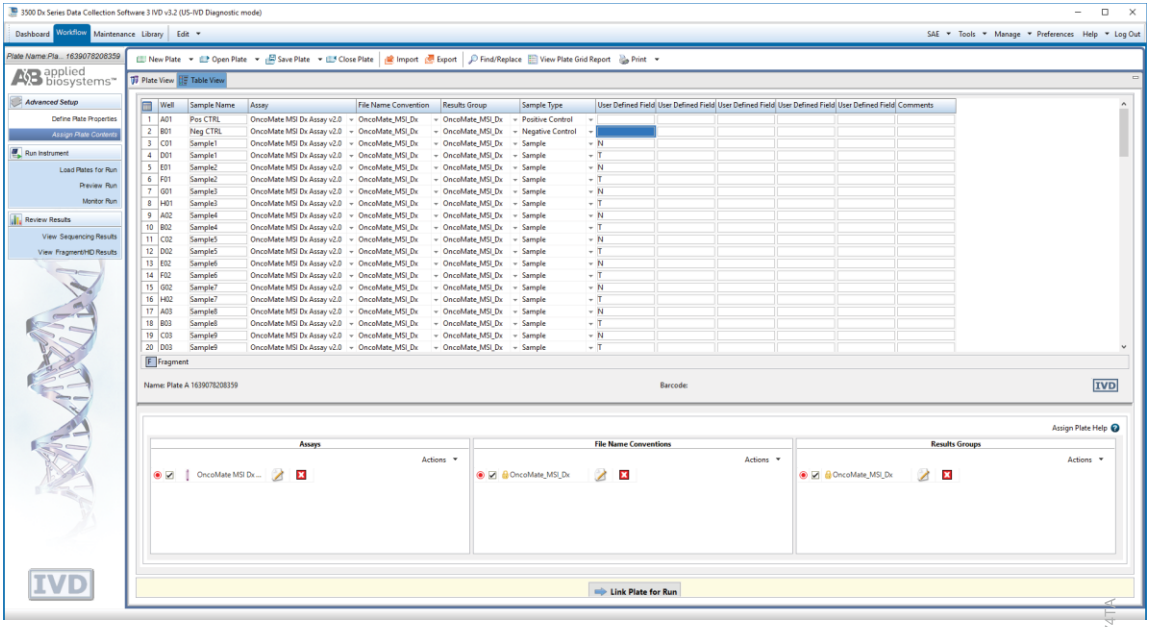


Figure 9. 'Table View' tab, Assign Plate Contents screen.

7. Select **Link Plate for Run** (Figure 9). The 'Load Plate' window will launch. Select **Yes** to acknowledge plate changes (if applicable) and **OK** to acknowledge the "Plate loaded successfully" message.

8. When the 'Run Information' screen launches (Figure 10), change the Run Name, if desired, and select **Start Run**.

   Note: A **Reinject** option is available through the Applied Biosystems® 3500 Dx Genetic Analyzer DCS while the analysis of a sample batch is in progress. If the **Reinject** option is used, the data generated are considered part of the same sample batch as the original injection.

   Data (.fsa) files created using the **Reinject** option have a "_1" suffix applied to the file name, but the Sample Name within the .fsa files is identical to the original injection. The OncoMate® MSI Dx Analysis Software uses the Sample Name to identify sample pairs, and sample names must be unique. Because data (.fsa) files created using the **Reinject** option have nonunique sample names, they are excluded during batch import by the analysis software.

   To analyze the original and re-injected data in the same batch, use the **Rename** option in the DCS to rename the samples. Samples can be renamed following capillary electrophoresis completion according to the instructions provided in the *Applied Biosystems® 3500 Dx Genetic Analyzer and 3500xL Dx Genetic Analyzer IVD User Guide*. The renamed samples must have unique matching Sample Names for the normal and tumor sample pair.

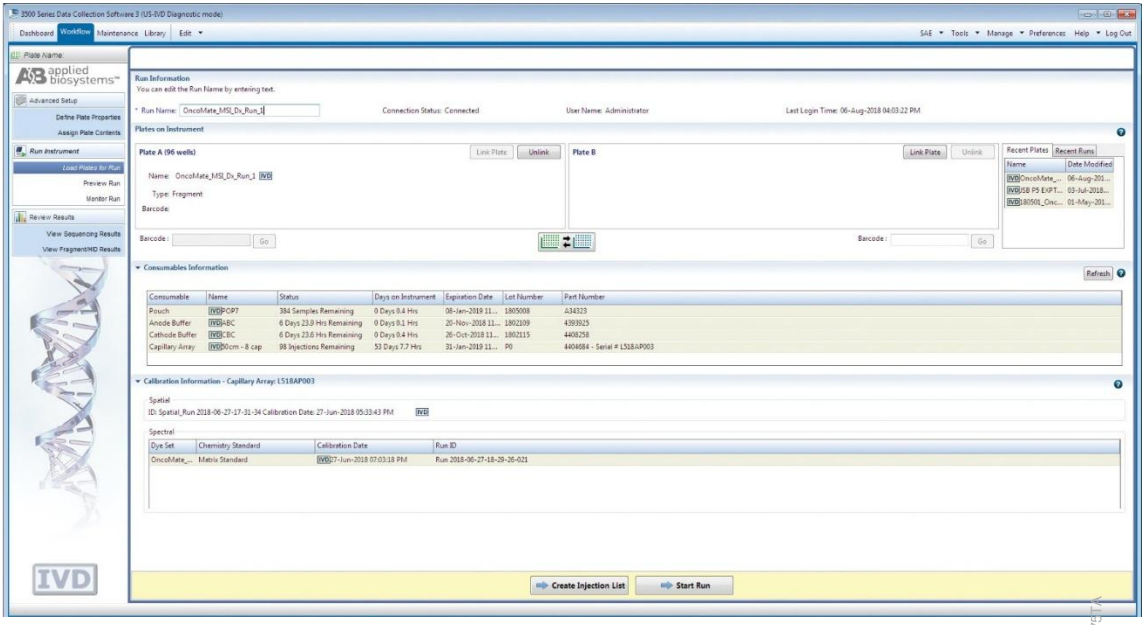


Figure 10. Run Information screen.

5. Data Analysis using the OncoMate® MSI Dx Analysis Software

5.1 Introduction to Data Analysis

Separation and detection of OncoMate® MSI Dx Analysis System amplification products by capillary electrophoresis result in data (.fsa files) that require downstream analysis to determine sample MSI status. The instructions below provide an overview of the use of OncoMate® MSI Dx Analysis Software to analyze .fsa files from matched normal and tumor sample pairs and controls. The interpretation of software results is discussed in Section 6.

Refer to the *OncoMate® MSI Dx Analysis Software Technical Manual* #TM680 for additional information about the software and comprehensive instructions describing its installation, configuration and use.

Data analysis using the OncoMate® MSI Dx Analysis Software is more sophisticated than the basic analysis performed within the Applied Biosystems® 3500 Dx Genetic Analyzer DCS. Therefore, all samples must be analyzed using the OncoMate® MSI Dx Analysis Software as the final assessment of data quality.

The data analysis workflow in the OncoMate® MSI Dx Analysis Software (Figure 11) entails:

- Starting and logging into the analysis software
- Importing controls and matched tumor and normal sample pairs to create a sample batch
- Automated fragment sizing, allele filtering and quality control verification for each sample pair and control in the batch
- Automated interpretation of MSI status for each sample pair
- Reviewing MSI results for each sample pair and control in the batch
- Approving MSI results for each sample pair in the batch
- Creating reports and export files for each batch

Within the OncoMate® MSI Dx Analysis Software, each user is assigned a role with specific permissions to perform workflow tasks. Users assigned a Lab Technician role can review sample results, while users assigned a Lab Director role can review and approve sample results. Once results are reviewed and approved, both Lab Technician and Lab Director users can generate results reports.

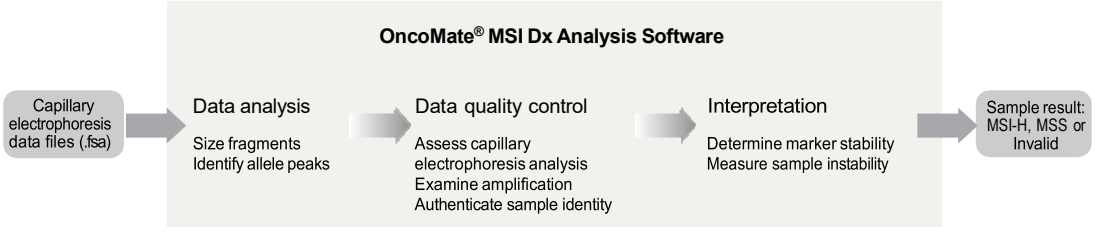


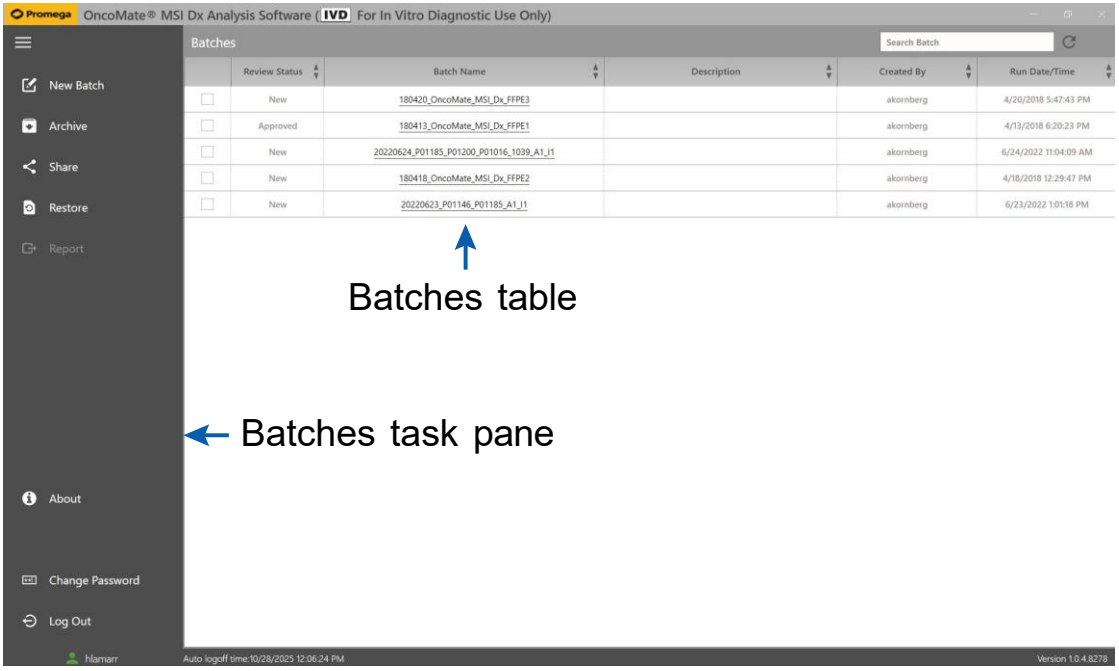
Figure 11. The OncoMate® MSI Dx Analysis Software automated workflow.

5.2 Standard Login Procedure

1. Double-click the icon for the OncoMate® MSI Dx Analysis Software on the computer desktop.
2. At the 'Login' screen, enter your user name and password (case-sensitive) to activate the **Login** button. Select **Login** to enter the OncoMate® MSI Dx Analysis Software. When successfully logged in, Lab Technician and Lab Director users are presented with the 'Batches' screen (Figure 12). To exit the application without logging in, select **X** in the upper right corner of the Login screen.

5.3 Creating a New Batch: Importing Sample Data (FSA files)

1. From the task pane of the 'Batches' screen, select **New Batch** to open the 'Import Samples' file browser.



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Figure 12. The OncoMate® MSI Dx Analysis Software 'Batches' screen.

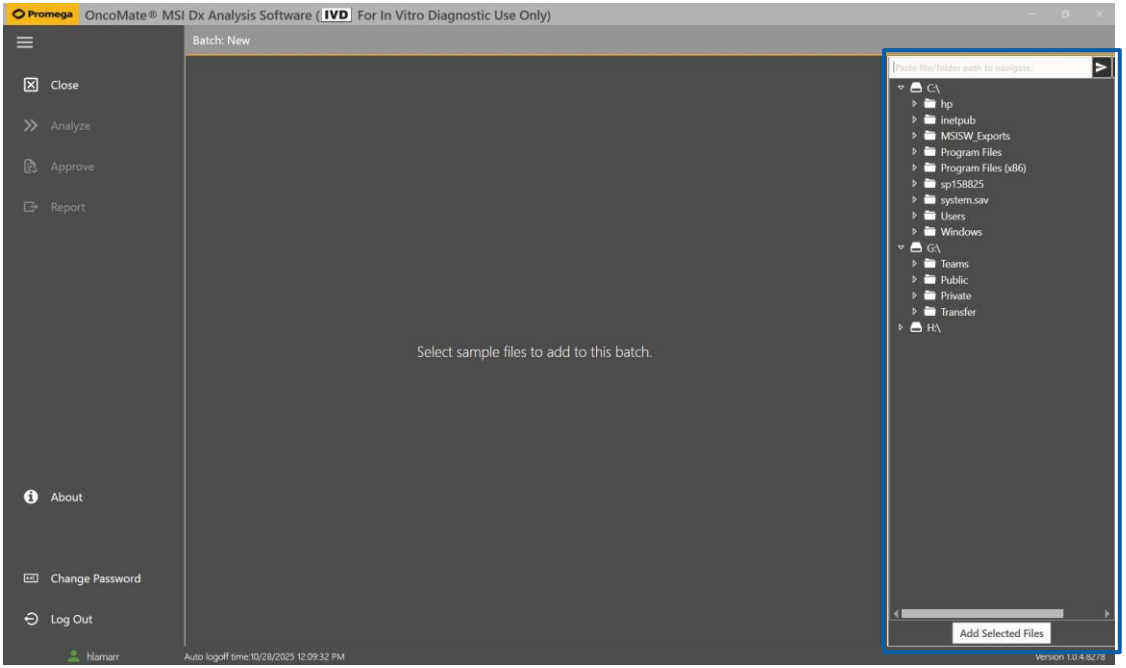
2. In the file tree window that appears (Figure 13, blue box), navigate to the desired sample data folder or individual .fsa files one of two ways:
   - Type or paste the file path of the target folder into the empty field at the top of the file tree. Select the **Go** icon . The file tree will expand to that file location.
   - Navigate to the desired folder or files using the file tree by double-clicking on the drive name where the desired sample files are saved or clicking on the small arrow to the left of the drive name. This will expand the view
     to include all folders on that drive. Continue to expand the file tree by clicking on the small arrows or double-clicking on folders within the file tree to navigate to the desired folder or .fsa files.

   Notes:

   a. When importing samples into a batch, you can select a folder containing the .fsa files or individual ,fsa files. When a folder is selected, all .fsa files within that folder will be added to the batch.

   b. Selecting the **Reset** icon  will refresh the screen and cause the file tree to revert back to the initial file tree structure.

   c. Mapped drives are visible in the folder tree. Cloud drives, such as Microsoft® OneDrive, are not accessible.



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Figure 13. Adding samples to a new batch. The file tree is outlined in blue.

3. Choose the desired sample folder or .fsa files in the file tree, being sure to include the positive and negative controls and normal and tumor samples for each patient.

4. Select **Add Selected Files** at the bottom of the file tree to initiate import. To exit this screen without selecting .fsa files for the new batch, select **Close** in the task pane to return to the 'Batches' screen.

   Notes:

   a. The OncoMate® MSI Dx Analysis Software will import the selected samples and assign them to a sample batch based on the capillary electrophoresis plate name assigned by the user.

   b. Minimally, two controls, a positive and a negative, and one sample, comprising a matched pair of normal and tumor .fsa data files, are required for successful batch creation. Import will fail for batches missing one or both controls and for samples and controls that were identified improperly in the Applied Biosystems® 3500 Dx DCS (see Section 4.9, Step 6 for acceptable naming conventions to identify samples and controls). Samples that fail import are listed in a pop-up window for user review.

   c. While samples are being imported, the software displays a message that the sample import is in progress. Once import is finished, the Samples screen (Figure 14) loads automatically and displays samples comprising the new batch.

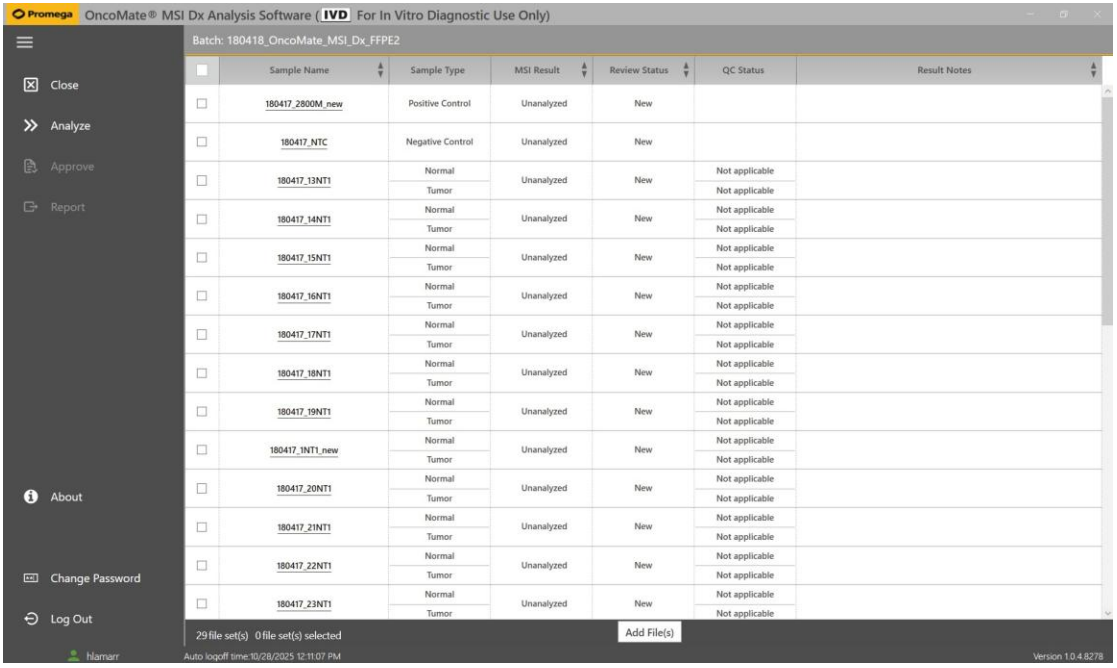


Figure 14. The Samples screen prior to data analysis.

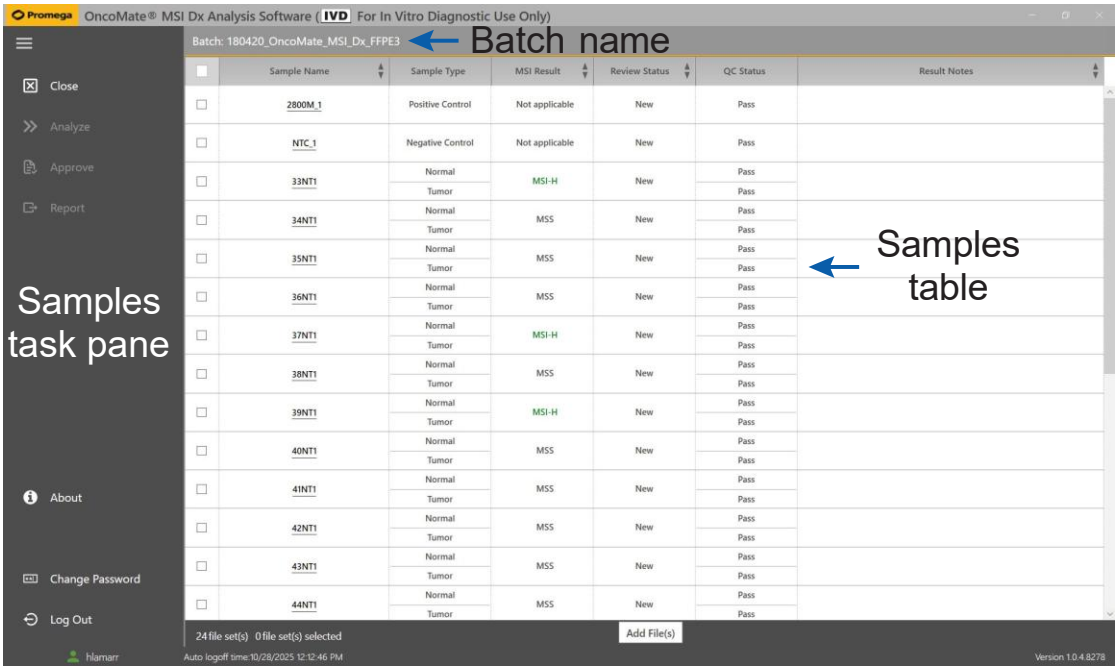
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5.4 Analyzing Sample Data

Once samples are added to a new or existing batch, these samples must be analyzed immediately by selecting **Analyze** in the task pane on the Samples screen (Figure 14). Any user with the role of Lab Technician or Lab Director can analyze samples.

Until a sample is analyzed, the sample review status on the Samples Screen is "New", the MSI result is "Unanalyzed" and the QC status is undetermined (i.e., neither "Pass" or "Fail"). Once samples are analyzed, the sample review status changes to "New" and the QC status changes to "Pass" or "Fail" (Figure 15).

During analysis, the software evaluates batch and sample quality (see Section 6.3) and assigns a result of "MSI-H", "MSS" or "Invalid" to all matched normal and tumor sample pairs. The positive and negative controls are assigned a result of "Not Applicable". Analyzed samples are ready for review and approval.



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Figure 15. The Samples screen following data analysis. The samples task pane and samples table are indicated. The Samples screen displays the names, MSI results, review status and QC status of the samples belonging to a batch. The batch name is displayed above the Samples table.

5.5 Releasing Sample Results

To release test results for reporting outside the OncoMate® MSI Dx Analysis Software, the results must be formally reviewed and approved. The software allows the two-step process to be completed by a single user or multiple users. Lab Technician users have permission to mark a result "Reviewed", and Lab Director users have permission to mark results as "Reviewed" and to approve results, releasing them for reporting. Sample reports are unavailable for export from the software until the selected sample is formally reviewed and then approved. Batch reports are unavailable for export from the software until all samples and controls are reviewed and approved.

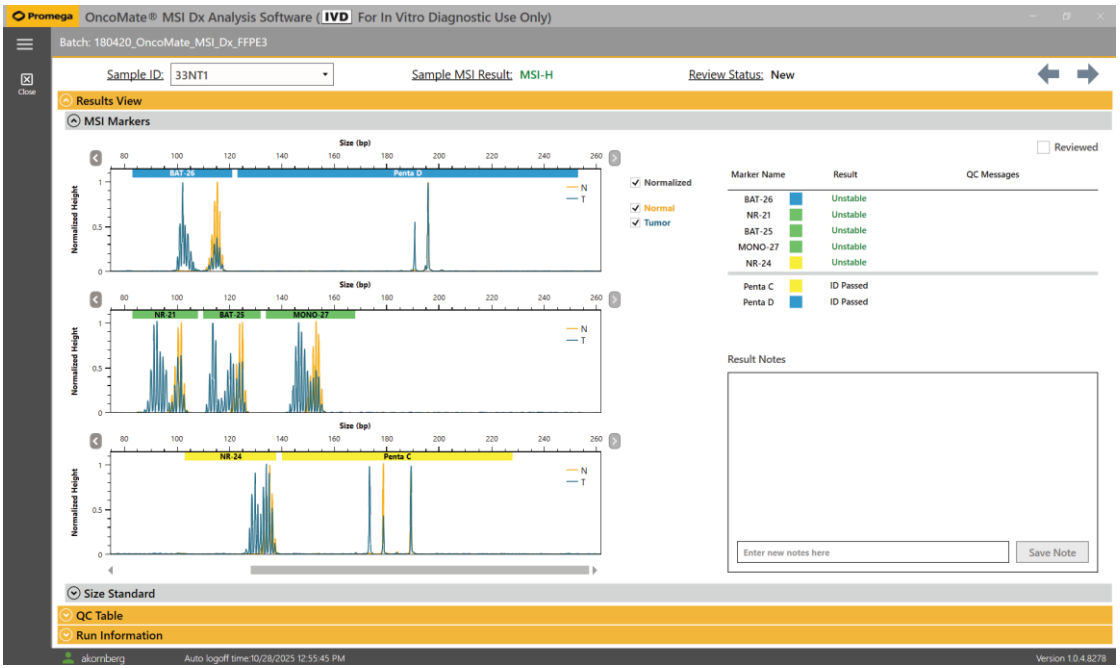
5.5.1 Reviewing Sample Results

After sample analysis, the initial reviewer must complete a formal sample review, as described below. Following sample review, the sample 'Review Status' changes from "New" to "Reviewed". All samples, including samples with an MSI result of "Invalid", must be formally reviewed before they can be approved, except when a control fails a QC test (see below).

Note: When the QC Status for a positive or negative amplification control is "Fail", the data quality of the batch is questionable and all samples in the batch will have an MSI Result of "Invalid". These samples are not subject to the standard review and approval process. For a batch that fails QC on the control reactions, it is only necessary to approve the samples; sample review is not required. Proceed to Section 5.5.2 for instructions to approve samples when a batch QC failure is observed.

A user with a role of Lab Technician or Lab Director will complete the following steps to formally review samples:

1. From the Samples screen (Figure 15), choose the first sample or control in the table by selecting the underlined sample name to open the Sample Data screen (Figure 16).



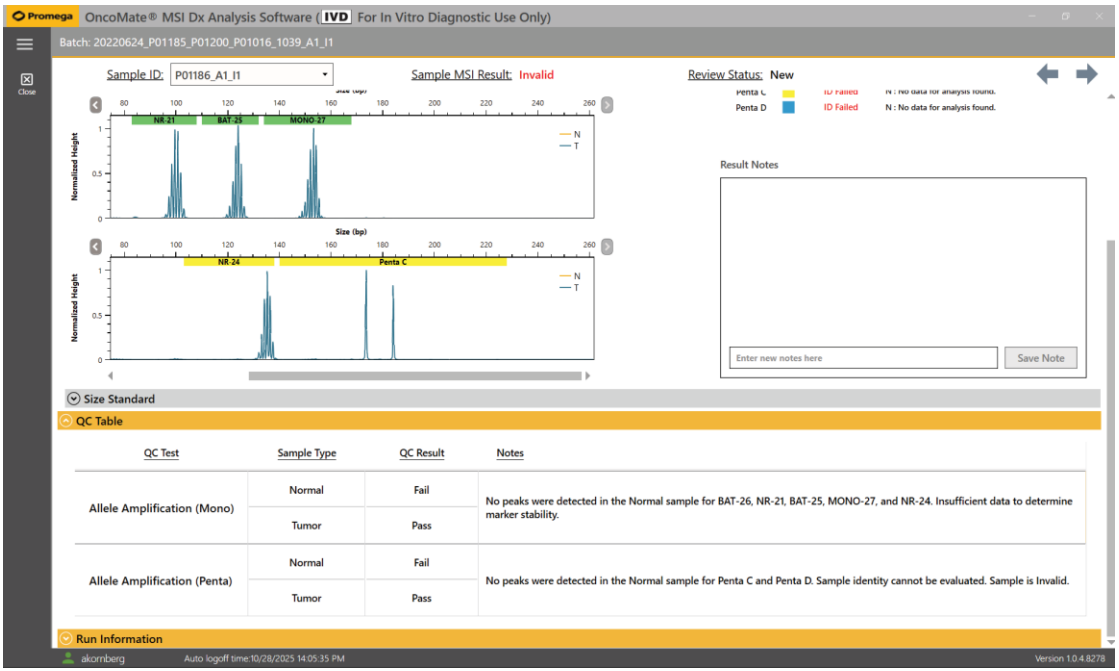
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Figure 16. The Sample Data screen. This screen contains sample and size standard electropherograms, marker stability and sample MSI results, the "Reviewed" check box and sample review status, an expandable QC Table section listing QC failures (when applicable), an expandable Run Information section describing the instrument and consumables used for capillary electrophoresis, and a text box for adding user comments.

2. Review the sample data, including sample and size standard electropherograms, results of the QC evaluation, stability calls for MSI markers, and the sample MSI result.

   Note: We recommend reviewing electropherograms with the "Normalized" check box checked and unchecked to detect peak height differences between the normal and tumor samples that may be useful for troubleshooting QC failures or of interest to the laboratory. QC failures are indicated by No Call and Invalid marker results and Invalid sample MSI results. If a QC failure was observed, consult the QC Table (Figure 17) for the Invalid sample(s) or QC failing controls and Section 11, Troubleshooting, to determine relevant troubleshooting steps.

Refer to the *OncoMate® MSI Dx Analysis Software Technical Manual* #TM680 for a full description of the information presented on the Sample Data screen.



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Figure 17. The QC Table. The selected sample exhibited amplification failures for both mononucleotide- and pentanucleotide-repeat markers in the normal sample. A patient sample identity failure was also observed because of the failed amplification for Penta C and Penta D.

3. **Optional:** Record comments about the sample review (e.g., proposed troubleshooting steps for "Invalid" samples) using the 'Enter new notes here' field of the Result Notes pane, and select **Save Note**. Notes may be added to a sample at any point during or after the review or approval.

4. Record the sample review by placing a checkmark in the "Reviewed" check box in the upper-right corner of the screen.

   Note: The check mark placed in the "Reviewed" check box is saved by navigating to a different sample (i.e., selecting the right or left arrow icon) or selecting **Close** in the task pane. Until then, you can remove the check mark from the check box and the sample review status will remain "New".

5. To review additional samples in the batch, use the right or left arrow in the upper-right corner to navigate to the next or previous sample, respectively. To return to the Samples screen, select **Close** in the task pane.

The reviewed samples are now ready for approval. For Lab Director users, the **Approve** button in the task pane is now active. When all samples in a batch are formally reviewed, the batch review status changes to "Reviewed".

5.5.2 Approving Results

Samples with a review status of "Reviewed" must be approved by a Lab Director user; sample approval is unavailable to Lab Technician users. Any Lab Director user can perform the complete review and approval workflow. Alternatively, a Lab Director user can open batches created by other Lab Director or Lab Technician users and approve samples with a status of "Reviewed".

When the positive or negative amplification control fails any QC test, the result of "Invalid" is assigned to all samples in the batch. When this occurs, the user is not required to place a checkmark in the "Reviewed" check box for samples, and the **Approve** button is active immediately for Lab Director users following sample analysis.

Perform the following steps to approve sample results:

1. Navigate to the Samples screen (Figure 15).
2. Select the samples to be approved.

   Notes:

   a. Samples may be selected by placing a checkmark in the check box in the first column of the Samples table or by clicking anywhere on the row for the sample. Select all samples by checking the check box in the title bar. Alternatively, the **Ctrl** and **Shift** keys can be held down to select multiple sample rows.

   b. If a QC failure was observed, including for the positive or negative amplification control resulting in "Invalid" results for all samples, the Lab Director user approving the sample(s) may view the 'QC Table' on the Sample Data screen (Figure 16) to identify the cause of the QC failure.

   c. Additional comments about a sample may be recorded by the approving Lab Director by using the 'Enter new notes here' field on the Sample Data screen and selecting **Save Note**.

3. From the samples task pane, select **Approve**.
4. Enter your password in the 'Approve Results(s)' pop-up window to electronically sign the sample results and finalize the approval workflow.

   Note: Sample approval requires an electronic signature. The software considers the user name and password combination to be an electronic signature.

5.6 Generating Reports and Exports from the 'Batches' Screen

Once a sample result is approved, any user may generate sample and batch reports and data export files. Reports and data export files are saved in the "Result Reports Destination Folder" set by the software administrator in the Application Settings of the software. The software saves all files for a particular batch in a batch-specific folder, which is automatically created by the software and shares the same name as the batch.

Software reports and exports may be generated individually or all together using the Report function in the 'Batches' screen task pane (see Figure 12). Sample reports for approved samples and controls may also be generated using the Report option in the Samples screen task pane. See the *OncoMate® MSI Dx Analysis Software Technical Manual* #TM680 for more information.

- Sample Reports are only generated for approved samples and controls.
- Batch Summary Reports are only generated once all samples and controls in the batch are approved.
- The Technical Support Package may be generated at any point following batch analysis.

Perform the following steps to generate reports and exports from the 'Batches' screen:

1. Select the batch(es) of interest on the 'Batches' screen and select **Report** from the task pane.

   Note: To select multiple batches, hold down the **Shift** or **Ctrl** key and select other batches in the 'Batches' screen.

2. The 'Reports' window will appear (Figure 18). Select the reports you wish to generate and then choose **Report**. The software generates the requested reports and exports, and a pop-up window informs you of the location of the batch-specific folder that contains the files.

   Select **Cancel** to return to the 'Batches' screen without generating sample reports.

   Note: If none of the samples in the selected batch have a review status of "Approved", an error message informs you that no samples are available for reporting. However, if the Technical Support Package option was selected, these reports will still be created in the destination folder. Select **OK** to return to the 'Batches' screen.

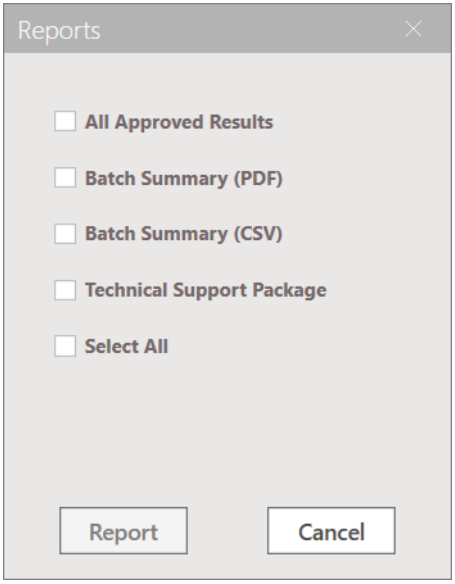


Figure 18. The 'Reports' window. This pop-up window allows users to choose the type(s) of reports and/or data export files to create.

6. Results Interpretation

This section reviews the results returned by the OncoMate® MSI Dx Analysis Software when analyzing data from matched normal and tumor sample pairs and controls. The specific QC tests performed by the software are also summarized.

See Section 1.7, Principles of the Procedure, for a description of OncoMate® MSI Dx Analysis System data and the logic employed by the OncoMate® MSI Dx Analysis Software to determine tumor MSI status.

6.1 Determining Marker Stability

The stability of five mononucleotide-repeat markers is evaluated in paired normal and tumor samples to determine tumor MSI status. The PCR amplification of mononucleotide-repeat markers produces DNA fragments comprising allele peaks and flanking PCR stutter peaks, and multiple peak distributions can exist for each marker. Microsatellite instability at a mononucleotide-repeat marker is detected by the analysis software as changes in the size (bp) and abundance, represented by signal intensity (RFU), of these DNA fragments in the tumor sample relative to the same patient's normal sample. These changes are produced when a new shorter-length allele and corresponding stutter products are present in the tumor sample. The OncoMate® MSI Dx Analysis Software detects deletions ≥2bp in microsatellites when specimen requirements are met (implemented in the software as ≥1.75bp to account for the sizing precision of capillary electrophoresis). A marker is "Unstable" when a new, smaller allele is detected in the tumor sample. Otherwise, the marker is interpreted as "Stable" when no deletion is detected, "No Call" when peak intensities are too divergent between the sample pair or too low to make a confident stability assessment, or "Invalid" when other quality control failures are observed.

Depending on the size of the deletion and tumor content of the sample, new DNA fragment peaks may be distinct from or subtly overlap with the cluster of peaks amplified from the patient's normal allele (see Figure 3). Therefore, a marker may be identified as unstable in response to obvious or very subtle differences between DNA fragments amplified in the normal and tumor samples. By default, the OncoMate® MSI Dx Analysis software presents DNA fragment intensity data for a given marker as normalized values to make differences between the normal and tumor samples easier to understand and identify for the software user.

Table 6 summarizes the marker stability calls returned by the OncoMate® MSI Dx Analysis Software.

Table 6. Marker-Level Stability Calls Provided by the OncoMate® MSI Dx Analysis Software.

Value	Description
Stable	Microsatellite instability was not detected for the marker. For the mononucleotide-repeat region analyzed, any differences identified between the normal and tumor samples did not result from a deletion of ≥2 nucleotides.
Unstable	Microsatellite instability was detected for the marker. For the mononucleotide-repeat region analyzed, the differences identified between the normal and tumor samples indicate a deletion of ≥2 nucleotides.
No Call	No Call indicates that the marker stability could not be determined due to a data quality issue (see Section 6.3). View the QC Table for information about failed QC tests for the sample. See the troubleshooting section of this manual for guidance on resolving QC failures that lead to No Call results.
Invalid	Invalid indicates that the quality of the sample data is unacceptable due to a critical QC failure (see Section 6.3). Such data cannot be used to determine a marker stability or sample MSI result. View the QC Table for information about failed QC tests affecting the sample or the amplification controls. See the troubleshooting section of this manual for guidance on resolving QC failures that lead to Invalid results.

6.2 Determining Sample MSI Status

Stability results from the five mononucleotide-repeat markers are collectively interpreted to assign an overall tumor MSI status. Table 7 summarizes the sample MSI results returned by the OncoMate® MSI Dx Analysis Software. A sample MSI result will be returned by the software when No Call marker results are observed if no Invalid marker results are present for the sample and a) two or more mononucleotide-repeat markers were identified as unstable (MSI-H), or b) four or more mononucleotide-repeat markers were identified as stable (MSS). When these criteria are not met, a sample result of Invalid is returned because the sample interpretation is ambiguous. Because the MSS test result encompasses all valid test results except for the MSI-H result, the MSS result is a not-MSI-H result.

Table 7. Sample-Level Results Provided by the OncoMate® MSI Dx Analysis Software for Solid Tumors.

Value	Description
MSI-H	MSI-H (MSI high) indicates that two or more mononucleotide-repeat markers were identified as unstable.
MSS	MSS (MSI stable) indicates that four or more mononucleotide-repeat markers were identified as stable. Because samples with valid results that do not meet the test criteria for MSI-H are considered MSS (one or no unstable markers), the OncoMate® MSI Dx Analysis System MSS test result is a not-MSI-H result.
Invalid	Invalid indicates that the quality of the sample data is unacceptable (see Section 6.3). A sample is assigned an MSI result of "Invalid" when the sample identity check fails, any marker-level Invalid results are observed, or No Call results preclude sample interpretation (see Section 7, Assay Quality Controls, for additional information). When a batch is marked QC Failed due to an issue with a control sample, all samples within that batch are marked as "Invalid". View the 'QC Details' tab for the sample or for the controls for information about failed quality attributes. See the troubleshooting section of this manual for guidance on resolving QC failures that lead to Invalid results.

6.3 Data Quality Requirements

This section provides an overview of data quality tests and potential QC failures that may be observed following data analysis using the OncoMate® MSI Dx Analysis Software. See the *OncoMate® MSI Dx Analysis Software Technical Manual* #TM680 for a comprehensive discussion of quality control measures implemented in the analysis software.

The OncoMate® MSI Dx Analysis Software evaluates several quality attributes associated with control and patient samples to determine whether the batch and sample pairs are of sufficient quality for MSI interpretation. Data quality checks performed by the software and the consequences of QC failures are summarized here and detailed in Table 8.

Any QC failure observed for an amplification control will result in a batch QC failure, and all patient samples within the batch will have a sample result of Invalid. QC failures for patient samples can have the following outcomes:

- Most QC failures detected by the software are considered severe; marker and sample results of Invalid will be returned in response to these failed tests.
- QC failures for three tests, Mononucleotide Amplification QC, Marker Signal QC and Intensity Difference QC, relate to insufficient data at the marker level and return a marker result of No Call. The software will return a sample MSI result when two or more other mononucleotide-repeat markers are identified as unstable (MSI-H) or four or more other mononucleotide-repeat markers are identified as stable (MSS). When these criteria are not met, a sample result of Invalid is returned because the sample interpretation is ambiguous.

When a sample or control fails one or more QC tests, the failure reason is displayed on the software's Sample Data screen in abbreviated form in the 'QC Messages' column of the results table and in full form in the QC Table section.

Table 8. Automated Data Quality-Control Tests Implemented in the OncoMate® MSI Dx Analysis Software. Invalid results are assigned at the marker and sample levels in response to critical QC failures (i.e., failures that require repeated sample analysis), while No Call marker results are assigned for noncritical QC Failures associated with insufficient information for a given marker. When a control sample fails any QC test, a sample result of Invalid is assigned to all patient samples in the batch. Consult Section 11, Troubleshooting for guidance on resolving QC failures.

QC Test	Description of QC Test Returned by the Software When a Failure is Observed[1]	Sample Types Evaluated[2]	Patient Sample Result for Failed QC Test
Sizing Quality QC (Size Standard 500)	Quality of the Size Standard 500 fragments is assessed based on peak shape, size matching and a size-calling curve.	+, –, N, T	Sample Invalid[3]
Negative Control QC (All markers)	For negative amplification controls, there must be no peaks detected above the analytical threshold.	–	All samples Invalid
Control Identity QC (Pentanucleotide markers)	For the positive amplification control, the alleles present in the pentanucleotide markers must match the expected alleles for the 2800M control DNA within ±1.5 base pairs.	+	All samples Invalid
Off-Scale Peak QC (All Markers)	The intensity (RFU) of peaks in a marker must not exceed the maximum detectable range of the capillary electrophoresis instrument.	+, N, T	Marker Invalid Sample Invalid[3]
Spectral Pull-Up QC (All Markers)	Allele peaks that are aligned by length in separate dye channels are evaluated for spectral pull-up (i.e., signal bleedthrough between dye channels).	+, N, T	Marker Invalid Sample Invalid[3]
Broad Peak QC[4] (All Markers)	The width of peaks must not exceed the acceptable threshold assigned for MSI analysis.	+, N, T	Marker Invalid Sample Invalid[3]
Pentanucleotide Amplification QC (Pentanucleotide markers)	At least one allele above the analytical threshold (175RFU) must be present within each pentanucleotide-repeat marker.	+, N, T	Marker Invalid Sample Invalid[3]
Mononucleotide Amplification QC (Mononucleotide markers)	At least one allele above the analytical threshold (175RFU) must be present within each mononucleotide-repeat marker.	+, N, T	Marker No Call[3,5]
DNA Contamination QC (Pentanucleotide markers)	For each pentanucleotide marker, there can be no more than two alleles present in the normal sample.	+, N	Marker Invalid Sample Invalid[3]
Patient Sample Identity QC (Pentanucleotide markers)	For each pentanucleotide marker, the alleles identified in the normal sample must be present in the tumor sample (within ±1.5 base pair).	T	Marker Invalid Sample Invalid

QC Test	Description of QC Test Returned by the Software When a Failure is Observed[1]	Sample Types Evaluated[2]	Patient Sample Result for Failed QC Test
Marker Signal QC (Mononucleotide markers)	For mononucleotide markers that have been interpreted as stable, allele peak heights in the normal and tumor samples must be ≥700RFU to ensure assay sensitivity.	N, T	Marker No Call[5]
Intensity Difference QC (Mononucleotide markers)	For mononucleotide markers, the peak heights, N/T, must be similar to ensure assay accuracy.	Sample pair	Marker No Call[5]
Unexpected Peak(s) QC (Mononucleotide markers)	For mononucleotide markers, DNA fragments indicating instability must be absent from the normal sample.	Sample pair	Marker Invalid Sample Invalid

[1]The QC messages returned by the software vary when a control sample or a patient sample (normal, tumor, or normal and tumor) fails a QC test.

[2]N, normal sample; T, tumor sample; +, positive control; –, negative control

[3]The MSI result is "Invalid" for all patient samples in a batch when a control sample fails the QC test.

[4]The known broad-peak artifact in NR-21 (approximate range 83–90bp) is unrelated to instability and ignored by the OncoMate® MSI Dx Analysis Software. The software will return a result of "Invalid" when a broad peak other than the known NR-21 artifact is observed.

[5]The software will return an MSI result for samples with No Call marker results when sufficient data are available to make a call (≥2 Unstable markers or ≥4 Stable markers) and no Invalid results are observed. The software assigns an MSI result of Invalid when marker results are ambiguous.

6.4 Summary of Known Amplification Artifacts and Capillary Electrophoresis Anomalies

A known amplification artifact is observed within the NR-21 marker as a single broad peak in the size range of 83–90 base pairs. The OncoMate® MSI Dx Analysis Software will not call this peak as an allele or consider this peak when determining the stability of the NR-21 marker. Although the peak may appear on the electropherogram, it will not affect automated MSI interpretation.

Another known amplification artifact occurs when too much or highly fragmented DNA is used as input to the OncoMate® MSI Dx Analysis System. The baseline signal in the BAT-26 marker (blue channel) may become elevated and jagged. Except for extreme inputs (e.g., ≥4ng 2800M Control DNA), the OncoMate® MSI Dx Analysis Software will not call these peaks as alleles or consider them when determining the stability of the BAT-26 marker. A single broad peak artifact may occasionally be observed in BAT-26 between 95bp and 100bp without a corresponding jagged baseline. If repeated Broad Peak QC failures are observed for BAT-26, verify that the thermal cycler used for amplification meets assay requirements and is calibrated.

The OncoMate® MSI Dx Analysis Software minimizes the effect of known, but random, anomalies that may be observed during capillary electrophoresis. Three such rare anomalies predominate: failed injections, broad peaks and signal spikes.

1. When an injection fails, little or no sample DNA is injected into capillary array. In these cases, a Sizing Quality QC failure will be observed due to the lack (or poor quality) of Size Standard 500 peaks, and the sample result will be Invalid.
2. A peak may be detected during capillary electrophoresis that exhibits a broad (i.e., not sharp) morphology. A broad peak may originate from polymer crystals or other aberrant material migrating through the capillary array. When the analysis software detects a broad peak, the sample result will be Invalid.
3. A signal “spike” may be observed during capillary electrophoresis in the form of a near-zero width peak that spans all color channels. Such spikes are detected by the analysis software and may result in an Off-Scale or Sizing Quality QC failure.

6.5 Data Review of Software Results

DNA recovered from FFPE samples may be highly fragmented, and overestimation of template DNA concentration can lead to low allele signal. Low signal may be compounded for longer markers because longer DNA fragments are typically in lower abundance than shorter fragments in FFPE DNA samples. The peak intensities for the longer markers may not be adequate for data evaluation. Such samples may exhibit Amplification QC, Marker Signal QC or Patient Sample Identity QC failures in the OncoMate® MSI Dx Analysis Software.

Cancerous tissues can display general genomic instability, such as loss of heterozygosity (LOH), independent of microsatellite instability. LOH may lead to severe allelic imbalance in the tumor and result in a pentanucleotide allele that is present in the normal sample but is below the assay analytical threshold (175RFU) in the tumor sample. In such cases, LOH will interfere with sample authentication (i.e., interpretation of identity between matched normal and tumor samples) and lead to an Invalid sample MSI result. Pentanucleotide-repeat markers may also display a different number of alleles in a tumor sample relative to the matched normal sample. The presence of additional alleles in a tumor sample will not prompt a QC failure.

Microsatellite instability may manifest as a spectrum of allelic profiles, from subtle alterations from the normal profile to distinct, novel alleles several bases removed from the normal allele. Due to the individual nature of cancer development and progression, rare tumor profiles may challenge the algorithms used in the analysis software. We recommend a data review of MSI results to identify samples with data profiles your laboratory may want to evaluate further based on current professional standards. For example, the analysis software will score markers that exhibit expansions as stable. Expansions occur rarely in MSI-H tumors (see reference 6).

For tumor samples exhibiting instability at a single locus (1/5 alleles unstable), examine electropherograms for unexpected and known artifacts that may be responsible for the unstable result (see Section 6.4 and assess tumor content of the sample. Pathology estimation of tumor content is subjective. If tumor content is near the recommended assay minimum, consider repeat testing after enriching tumor content for the sample or orthogonal testing to rule out a false-negative test result. Samples with a single unstable marker should be interpreted by healthcare professionals in conjunction with other clinical findings, family history and other laboratory data according to your laboratory's procedures and current professional standards.

Heterozygosity due to human genetic diversity may be observed in the normal sample and is accounted for during sample analysis by the OncoMate® MSI Dx Analysis Software. Heterozygosity is characterized by two allele-peak profiles at a given marker with balanced, or approximately equivalent, peak heights. Heterozygosity in the mononucleotide markers is uncommon and, when present, is usually limited to one or two markers per individual. If multiple markers in the normal sample exhibit peak profiles shifted toward shorter fragment sizes, then this may indicate cross-contamination with tumor DNA and suggests sample handling issues, such as accidental mixing of tumor and normal tissues or DNA. We recommend visual inspection of electropherograms, even when the software returns a valid MSI result.

7. Assay Quality Controls

7.1 Spectral Calibration

During capillary electrophoresis, dye-labeled OncoMate® MSI Dx Analysis System amplification products are separated and detected using the Applied Biosystems® 3500 Dx Genetic Analyzer. Prior to analysis, the Applied Biosystems® 3500 Dx Genetic Analyzer is calibrated with matrix standards so that the fluorescent signals resulting from the set of specific dyes used in the assay can be distinguished. The OncoMate® 5C Matrix Standard consists of DNA fragments labeled with five different fluorescent dyes (fluorescein, JOE, TMR-ET, CXR-ET and WEN) in one tube. The calibration is performed using the 'OncoMate_MSI' dye set, which is installed on the Applied Biosystems® 3500 Dx Genetic Analyzer using the OncoMate® MSI Dx Assay Installer. Once generated, the spectral calibration file is applied automatically during sample detection to account for the spectral overlap among the dyes and to separate the raw fluorescent signals into individual dye signals.

7.2 Matched Normal Sample

Mononucleotide-repeat markers can show heterozygosity or variation in normal allele length between individuals. To control for such variations in normal alleles, the analysis software uses data generated from matched normal DNA as an allelic reference for novel tumor alleles. For this reason, the OncoMate® MSI Dx Analysis Software requires a patient-matched normal sample to be processed in parallel with every tumor sample. Unmatched samples will be excluded from analysis. If analysis of a tumor or normal sample must be repeated for any reason, the paired tumor or normal sample must be rerun as well.

During capillary electrophoresis run setup, information for paired samples (Sample Name and normal/tumor designation) must be entered into the Applied Biosystems® 3500 Dx Genetic Analyzer DCS following prescribed conventions, and patient samples must be identified as 'Samples' (see Section4.9, Step 6 and the *OncoMate® MSI Dx Analysis Software Technical Manual* #TM680 for more information). Improperly identified samples cannot be paired by the OncoMate® MSI Dx Analysis Software and will not be imported or analyzed.

7.3 Positive and Negative Controls

Positive and no-template ("negative") amplification control reactions using 2800M Control DNA and Water, Amplification Grade, respectively, must be analyzed concurrently with patient samples to verify assay performance. At least one 2800M Control DNA amplification reaction and one negative control amplification reaction must be completed for each plate (i.e., batch) of patient samples analyzed using the OncoMate® MSI Dx Analysis Software. The negative control reaction is analyzed to ensure that no unexpected amplification occurred in no-template reactions, which would indicate the presence of DNA contamination and lead to an Invalid assay result. The positive control reaction is analyzed to demonstrate that the amplification chemistry performed as expected. See Table 9 for expected 2800M Control DNA results. No-template controls should not have amplified peaks above the 175RFU calling threshold.

During capillary electrophoresis run setup, the positive and negative amplification controls must be identified as "Positive Control" and "Negative Control" in the Applied Biosystems® 3500 Dx Genetic Analyzer DCS. If amplification controls are not processed with patient samples or misidentified, the patient samples will not be imported or analyzed by the analysis software.

Table 9. Expected Amplification and Analysis Results Using 1ng of 2800M Control DNA.

Marker Name	2800M Alleles (bp)[1,2]
NR-21	101
BAT-26	115
BAT-25	124.5
NR-24	134
MONO-27	152.5
Penta C	178.5, 184
Penta D	195.5, 200.5

[1]Allele sizes were determined using the Applied Biosystems® 3500 Dx Genetic Analyzer with POP-7® polymer and a 50cm capillary.

[2]Instrument-to-instrument and day-to-day variability in the performance of capillary electrophoresis instruments may result in a ±bp difference in the allele sizes for 2800M Control DNA. We observed ±1.5bp difference in the analytical studies when using 1ng of Control 2800M DNA. Ninety-seven percent of alleles were within 1bp.

7.4 Capillary Electrophoresis Size Standards

All analyzed samples and controls must contain Size Standard 500 (added prior to capillary electrophoresis). The Size Standard 500 contains a series of 21 DNA fragments of known lengths (60, 65, 80, 100, 120, 140, 160, 180, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500bp), also referred to as a DNA ladder. Each fragment is labeled with WEN dye and is detected separately (as a fourth color, orange) in the presence of OncoMate® MSI Dx Analysis System amplified products using the Applied Biosystems® 3500 Dx Genetic Analyzer. For each sample or control, amplified DNA fragments are sized with reference to the size standard fragments using the Local Southern method (16). The size standard controls for capillary-to-capillary variations in sizing precision during capillary electrophoresis and allows direct comparison of samples across the capillary electrophoresis run. Only the 60-base to 300-base fragments are analyzed for fragment sizing in the OncoMate® MSI Dx Analysis Software.

7.5 Quality Requirements for Data Interpretation

The OncoMate® MSI Dx Analysis Software evaluates the quality of capillary electrophoresis data to ensure that a valid MSI determination can be made (see Section 6.3, Table 8, for a summary of QC metrics evaluated). Consult the *OncoMate® MSI Dx Analysis Software Technical Manual* #TM680 for more information. Positive and negative amplification controls must be analyzed in the same plate as corresponding patient samples during the capillary electrophoresis separation run. If amplification controls are not processed with patient samples or if they are missing during data import, the patient samples will not be imported or analyzed by the analysis software.

8. Expected Values

The OncoMate® MSI Dx Analysis System determines microsatellite instability status based on results generated for five mononucleotide repeat markers (BAT-25, BAT-26, NR-21, NR-24 and MONO-27). A tumor sample is interpreted as MSI-H when two or more markers are Unstable. A tumor sample is interpreted as MSS when fewer than two markers are interpreted as Stable. A sample may be interpreted as “Invalid” in response to specific QC failures.

9. Performance Characteristics in Endometrial Carcinoma

All analytical studies followed the procedure outlined in the OncoMate® MSI Dx Analysis System Technical Manual unless noted otherwise in the study results section.

9.1 Analytical Performance in Endometrial Carcinoma

For the analytical performance summarized below, an MSS result is considered the positive result and an MSI-H result is considered the negative result, unless noted otherwise in the study results. The OncoMate® MSI Dx Analysis System MSS test result is equivalent to a not-MSI-H result.

9.1.1 Accuracy

Accuracy of the OncoMate® MSI Dx Analysis System was characterized by evaluating agreement of the test with a commercially available immunohistochemistry (IHC) panel. A comparison of MMR and MSI results was performed using 255 endometrial cancer samples obtained from the KEYNOTE-775 clinical trial. See Section 9.2 for additional details from the clinical trial.

Treating the commercially available immunohistochemistry (IHC) results as the reference method, the positive percent agreement (PPA) between MSS and proficient MMR (pMMR) results was 99.0% (205/207), with a 95% confidence interval (95% CI) of 96.5–99.7%. The negative percent agreement (NPA) between MSI-H and deficient MMR (dMMR) results was 91.7% (44/48), with a 95% confidence interval of 80.4–96.7%. The overall percent agreement (OPA) was 97.6% (249/255), with a 95% confidence interval of 95.0–98.9%.

The data demonstrated the OncoMate® MSI Dx Analysis System and an orthogonal IHC method are highly concordant.

Table 10. Accuracy Absolute Frequencies and Agreement Estimates Generated Using the OncoMate® MSI Analysis System and an MMR IHC Panel.

OncoMate® MSI Dx Analysis System Result	MMR IHC Panel Result		Total
	pMMR	dMMR	
MSS (not MSI-H)	205	4	209
MSI-H	2	44	46
Total	207	48	255

	Estimate (n/N)	95% CI	
		Lower Limit	Upper Limit
PPA	99.0% (205/207)	96.5%	99.7%
NPA	91.7% (44/48)	80.4%	96.7%
OPA	97.6% (249/255)	95.0%	98.9%

9.1.2 Reproducibility

The Reproducibility study characterized the precision and reproducibility of the OncoMate® MSI Dx Analysis System when testing MSS and MSI-H FFPE endometrial tumors.

To assess within-lot reproducibility and precision, three endometrial tumors (one MSS and two MSI-H) were analyzed on five nonconsecutive days using the same OncoMate® MSI Dx Analysis System lot at three different study sites. A single operator team at each study site analyzed five replicates for each sample per day using a single CE instrument. To assess between-lot reproducibility and precision, the same three endometrial tumors used for evaluating within-lot reproducibility were analyzed on five nonconsecutive days using three OncoMate® MSI Dx Analysis System lots at a single study site. A single operator analyzed five replicates for each sample per day using a single capillary electrophoresis (CE) instrument.

PPA, NPA and two-sided 95% confidence intervals were calculated by comparing observed MSI results for each sample replicate to the majority MSI result observed across all replicates. Data across individual factors (site, lot and day) and all factors were summarized to evaluate the reproducibility of the OncoMate® MSI Dx Analysis System.

PPA and NPA values for MSS and MSI-H results, respectively, across site, day, lot or run were 100%, demonstrating high reproducibility for each factor tested. Across all study factors, PPA was 100% (125/125, 95% CI of 97.1–100%) and NPA was 100% (250/250, 95% CI of 98.4–100%). These data are shown in Table 11.

Table 11. PPA and NPA Estimates for Observed MSI Result as Compared to Majority MSI Result By Study Site, Lot and Day.

		PPA			NPA		
			95% CI			95% CI	
		Estimate	Lower Limit	Upper Limit	Estimate	Lower Limit	Upper Limit
Site	1 (Lot 1)	100% (25/25)	86.3%	100%	100% (50/50)	92.9%	100%
	2 (Lot 1)	100% (25/25)	86.3%	100%	100% (50/50)	92.9%	100%
	3 (Lot 1)	100% (25/25)	86.3%	100%	100% (50/50)	92.9%	100%
	Overall, Sites 1–3	**100% (75/75)**	**95.2%**	**100%**	**100% (150/150)**	**97.6%**	**100%**
Lot	1 (Site 1)	100% (25/25)	86.3%	100%	100% (50/50)	92.6%	100%
	2 (Site 1)	100% (25/25)	86.3%	100%	100% (50/50)	92.6%	100%
	3 (Site 1)	100% (25/25)	86.3%	100%	100% (50/50)	92.6%	100%
	Overall, Lots 1–3	**100% (75/75)**	**95.2%**	**100%**	**100% (150/150)**	**97.6%**	**100%**

		PPA			NPA		
			95% CI			95% CI	
		Estimate	Lower Limit	Upper Limit	Estimate	Lower Limit	Upper Limit
Day (Lot 1, Sites 1–3)	1	100% (15/15)	78.2%	100%	100% (30/30)	88.4%	100%
	2	100% (15/15)	78.2%	100%	100% (30/30)	88.4%	100%
	3	100% (15/15)	78.2%	100%	100% (30/30)	88.4%	100%
	4	100% (15/15)	78.2%	100%	100% (30/30)	88.4%	100%
	5	100% (15/15)	78.2%	100%	100% (30/30)	88.4%	100%
	Overall, Sites 1–3	**100% (75/75)**	**95.2%**	**100%**	**100% (150/150)**	**97.6%**	**100%**
Day (Site 1, Lots 1–3)	1	100% (15/15)	78.2%	100%	100% (30/30)	88.4%	100%
	2	100% (15/15)	78.2%	100%	100% (30/30)	88.4%	100%
	3	100% (15/15)	78.2%	100%	100% (30/30)	88.4%	100%
	4	100% (15/15)	78.2%	100%	100% (30/30)	88.4%	100%
	5	100% (15/15)	78.2%	100%	100% (30/30)	88.4%	100%
	Overall, Lots 1–3	**100% (75/75)**	**95.2%**	**100%**	**100% (150/150)**	**97.6%**	**100%**
Overall, Across All Factors		**100% (125/125)**	**97.1%**	**100%**	**100% (250/250)**	**98.4%**	**100%**

At the locus-level, overall agreement for MSI-H and MSS endometrial cancer tumor samples were reported, with a PPA of ≥98.8% (lowest 95% CI bound: 96.5%) for both sample types at all biomarkers with the exception of BAT-25. For the BAT-25 locus, overall PPA was 79.2% (95% CI: 73.6, 84.1) for MSI-H endometrial cancer tumor samples and 100% (95% CI: 97.1, 100) for MSS endometrial cancer tumor samples. The variability at the BAT-25 locus was due to a single MSI-H sample having an observed allele size difference between tumor and normal sample close to the cutoff (i.e., 2bp), which resulted in 58.4% (73/125) agreement with the majority result. These results are summarized in Table 12.

Table 12. Percent Agreement of Sample-Level MSI Results and Locus-Level Stability Results Compared to Majority Result.

				NR-21		BAT-25		BAT-26		NR-24		MONO-27	
Sample ID	Majority MSI Result	Percent Tumor Content	Percent Agreement with Majority MSI Result (n/n); 95% CI	Percent Agreement with Majority MSI Result (n/n)	95% CI Lower Limit; 95% CI Upper Limit	Percent Agreement with Majority MSI Result (n/n)	95% CI Lower Limit; 95% CI Upper Limit	Percent Agreement with Majority MSI Result (n/n)	95% CI Lower Limit; 95% CI Upper Limit	Percent Agreement with Majority MSI Result (n/n)	95% CI Lower Limit; 95% CI Upper Limit	Percent Agreement with Majority MSI Result (n/n)	95% CI Lower Limit; 95% CI Upper Limit
1	MSI-H	95%	100% (125/125); 97.1, 100	99.2% (124/125)	95.6%; 100%	100% (125/125)	97.1%; 100%	100% (125/125)	97.1%; 100%	100% (125/125)	97.1%; 100%	100% (125/125)	97.1%; 100%
2	MSI-H	70%	100% (125/125); 97.1, 100	98.4% (123/125)	94.3%; 99.8%	58.4% (73/125)	49.3%; 67.2%	100% (125/125)	97.1%; 100%	100% (125/125)	97.1%; 100%	100% (125/125)	97.1%; 100%
3	MSS	80%	100% (125/125); 97.1, 100	100% (125/125)	97.1%; 100%	100% (125/125)	97.1%; 100%	100% (125/125)	97.1%; 100%	100% (125/125)	97.1%; 100%	100% (125/125)	97.1%; 100%
Overall for MSI-H Samples				98.8% (247/250)	96.5%; 99.8%	79.2% (198/250)	73.6%; 84.1%	100% (250/250)	98.5%; 100%	100% (250/250)	98.5%; 100%	100% (250/250)	98.5%; 100%
Overall for MSS Samples				100% (125/125)	97.1%; 100%	100% (125/125)	97.1%; 100%	100% (125/125)	97.1%; 100%	100% (125/125)	97.1%; 100%	100% (125/125)	97.1%; 100%

Promega

To assess precision and reproducibility at the assay limit of detection for tumor content, six endometrial tumor samples (three MSI-H and three MSS) with 30% tumor content were evaluated. Each sample was analyzed on five nonconsecutive days at each of two study sites using two OncoMate® MSI Dx Analysis System lots. At each site, a single operator analyzed five replicates for each sample per day using a single CE instrument.

Across site, day, lot or run, PPA for MSS results was 100% and NPA for MSI-H results was 100%. Across all study factors, PPA was 100% (150/150, 95% CI of 97.6–100%) and NPA was 100% (150/150, 97.6–100%). See Table 13.

Table 13. PPA and NPA Estimates for Observed MSI Result as Compared to Majority MSI Result for MSI-H and MSS Endometrial Tumor Samples With Tumor Content At 30% By Study Site, Lot and Day.

		PPA				NPA			
				95% CI				95% CI	
		N	Estimate	Lower Limit	Upper Limit	N	Estimate	Lower Limit	Upper Limit
Site	1	75	100%	95.2%	100%	75	100%	95.2%	100%
	2	75	100%	95.2%	100%	75	100%	95.2%	100%
Lot	1	75	100%	95.2%	100%	75	100%	95.2%	100%
	2	75	100%	95.2%	100%	75	100%	95.2%	100%
Day (Site 1)	1 (Lot 1)	15	100%	78.2%	100%	15	100%	78.2%	100%
	2 (Lot 2)	15	100%	78.2%	100%	15	100%	78.2%	100%
	3 (Lot 1)	15	100%	78.2%	100%	15	100%	78.2%	100%
	4 (Lot 2)	15	100%	78.2%	100%	15	100%	78.2%	100%
	5 (Lot 1)	15	100%	78.2%	100%	15	100%	78.2%	100%
	Overall, Site 1, Lot 1	**45**	**100%**	**92.1%**	**100%**	**45**	**100%**	**92.1%**	**100%**
	Overall, Site 1, Lot 2	**30**	**100%**	**88.4%**	**100%**	**30**	**100%**	**88.4%**	**100%**

		PPA				NPA			
				95% CI				95% CI	
		N	Estimate	Lower Limit	Upper Limit	N	Estimate	Lower Limit	Upper Limit
Day (Site 2)	1 (Lot 2)	15	100%	78.2%	100%	15	100%	78.2%	100%
	2 (Lot 1)	15	100%	78.2%	100%	15	100%	78.2%	100%
	3 (Lot 2)	15	100%	78.2%	100%	15	100%	78.2%	100%
	4 (Lot 1)	15	100%	78.2%	100%	15	100%	78.2%	100%
	5 (Lot 2)	15	100%	78.2%	100%	15	100%	78.2%	100%
	Overall, Site 2, Lot 1	**30**	**100%**	**88.4%**	**100%**	**30**	**100%**	**88.4%**	**100%**
	Overall, Site 2, Lot 2	**45**	**100%**	**92.1%**	**100%**	**45**	**100%**	**92.1%**	**100%**
Across All Factors		**150**	**100%**	**97.6%**	**100%**	**150**	**100%**	**97.6%**	**100%**

At the locus-level, overall agreement for MSI-H and MSS endometrial cancer tumor samples were reported, with an overall agreement of 100% (95% CI: 97.6, 100.0) for both sample types at all biomarkers with the exception of NR-24 and MONO-27. For the NR-24 locus, overall agreement was 100% (95% CI: 97.6, 100.0) for MSI-H endometrial cancer tumor samples and 98% (95% CI: 94.3, 99.6) for MSS endometrial cancer tumor samples. For the MONO-27 locus, the overall agreement was 86.0% (95% CI: 79.4, 91.1) for MSI-H endometrial cancer tumor samples and 98.7% (95% CI: 95.3, 99.8) for MSS endometrial cancer tumor samples. The variability at the MONO-27 locus was due to a single MSI-H sample having an observed allele size difference between tumor and normal sample close to the cutoff (i.e., 2bp), which resulted in 58.0% (29/50) agreement with the majority result. These results are summarized in Table 14, below.

Table 14. Percent Agreement of Sample Level MSI Results and Locus Level Stability Results Compared to the Majority Result for Challenging Samples.

			NR-21		BAT-25		BAT-26		NR-24		MONO-27	
Sample ID	Majority MSI Result	Percent Agreement with Majority MSI Result (n/n); 95% CI	Percent Agreement with Majority MSI Result (n/n)	95% CI Lower Limit; 95% CI Upper Limit	Percent Agreement with Majority MSI Result (n/n)	95% CI Lower Limit; 95% CI Upper Limit	Percent Agreement with Majority MSI Result (n/n)	95% CI Lower Limit; 95% CI Upper Limit	Percent Agreement with Majority MSI Result (n/n)	95% CI Lower Limit; 95% CI Upper Limit	Percent Agreement with Majority MSI Result (n/n)	95% CI Lower Limit; 95% CI Upper Limit
1	MSI-H	100% (50/50); 92.9, 100	100% (50/50)	92.9%; 100%	100% (50/50)	92.9%; 100%	100% (50/50)	92.9%; 100%	100% (50/50)	92.9%; 100%	100% (50/50)	92.9%; 100%
2	MSI-H	100% (50/50); 92.9, 100	100% (50/50)	92.9%; 100%	100% (50/50)	92.9%; 100%	100% (50/50)	92.9%; 100%	100% (50/50)	92.9%; 100%	100% (50/50)	92.9%; 100%
3	MSI-H	100% (50/50); 92.9, 100	100% (50/50)	92.9%; 100%	100% (50/50)	92.9%; 100%	100% (50/50)	92.9%; 100%	100% (50/50)	92.9%; 100%	58.0% (29/50)	43.2%; 71.8%
4	MSS	100% (50/50); 92.9, 100	100% (50/50)	92.9%; 100%	100% (50/50)	92.9%; 100%	100% (50/50)	92.9%; 100%	100% (50/50)	92.9%; 100%	100% (50/50)	92.9%; 100%
5	MSS	100% (50/50); 92.9, 100	100% (50/50)	92.9%; 100%	100% (50/50)	92.9%; 100%	100% (50/50)	92.9%; 100%	94.0% (47/50)	83.5%; 100%	96.0% (48/50)	86.3%; 99.5%
6	MSS	100% (50/50); 92.9, 100	100% (50/50)	92.9%; 100%	100% (50/50)	92.9%; 100%	100% (50/50)	92.9%; 100%	100% (50/50)	92.9%; 100%	100% (50/50)	92.9%; 100%
Overall for MSI-H Samples			100% (150/150)	97.6%; 100%	100% (150/150)	97.6%; 100%	100% (150/150)	97.6%; 100%	100% (150/150)	97.6%; 100%	86.0% (129/150)	79.4%; 91.1%
Overall for MSS Samples			100% (150/150)	97.6%; 100%	100% (150/150)	97.6%; 100%	100% (150/150)	97.6%; 100%	98.0% (147/150)	94.3%; 99.6%	98.7% (148/150)	95.3%; 99.8%

Together, these data demonstrated that the OncoMate® MSI Dx Analysis System generates highly reproducible and consistent MSI results across sites, days and product material lots.

9.1.3 Limit of Detection

The limit of detection (LOD) study was designed to estimate the limit of detection of the OncoMate® MSI Dx Analysis System in terms of the lowest tumor content and DNA input amount required to correctly assign an MSI result with 95% percent positivity.

Tumor content LOD was established using one MSI-H sample, as depicted in Table 15 and Table 16, from an endometrial tumor that was analyzed at five different levels of tumor content (10%, 20%, 30%, 40% and the original tumor content) and a constant DNA input amount (1.0ng). This LOD establishment study used two reagent lots, with five runs per lot on nonconsecutive days and at least one capillary electrophoresis (CE) instrument, for a total of 40 replicates per sample at each level of tumor content (four replicates/run•lot × five runs × two lots). The lowest tumor content achieving a percent positivity ≥95% (hit rate ≥0.95) across all factors and samples was 20%. The absolute and relative frequency for each tumor content percentage is summarized in Table 15 and Table 16.

Table 15. Percent Tumor Content Limit of Detection for MSI.

Absolute and Relative MSI-H Frequency by Percent Tumor Content									
10%		20%		30%		40%		Original Tumor Content	
Lot 1	Lot 2	Lot 1	Lot 2	Lot 1	Lot 2	Lot 1	Lot 2	Lot 1	Lot 2
75.0% (15/20)	90.0% (18/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)

Table 16. Percent Tumor Content Limit of Detection per Locus.

Lot Number	Tumor Content	NR-21	BAT-25	BAT-26	NR-24	MONO-27
1	10%	5.0% (1/20)	5.0% (1/20)	65.0% (13/20)	95.0% (19/20)	35.0% (7/20)
	20%	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)
	30%	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)
	40%	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)
	60%	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)
2	10%	10.0% (2/20)	0% (0/20)	75.0% (15/20)	90.0% (18/20)	30.0% (6/20)
	20%	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)
	30%	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)
	40%	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)
	60%	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)

DNA input LOD was established using a dilution series of DNA purified from two endometrial FFPE tumor (one MSI-H and one MSS) and matched normal tissue as depicted in Table 17 and Table 18. Each sample was analyzed at six different DNA input amounts (0.05ng, 0.2ng, 0.5ng, 0.75ng, 1.0ng and 1.25ng per reaction). DNA from MSI-H tumor samples was blended with DNA from the matched normal sample, as described above, to represent the minimum tumor-content assay requirement of 30%. MSS tumor samples were analyzed at the original tumor content. Each tumor sample was analyzed using two OncoMate® MSI Dx Analysis System lots, for a total of 40 replicates per sample at each DNA input amount (four replicates/run•lot × five runs × two lots). Results from this study are summarized in Table 17 and Table 18.

Table 17. DNA Input Amount (ng) Limit of Detection for MSI.

		Absolute and Relative MSI Status Frequency by DNA Input Amount (ng)											
Sample ID	Known MSI Result	0.05ng		0.2ng		0.5ng		0.75ng		1.0ng		1.25ng	
		Lot 1	Lot 2	Lot 1	Lot 2	Lot 1	Lot 2	Lot 1	Lot 2	Lot 1	Lot 2	Lot 1	Lot 2
1	MSS	0% (0/20)	0% (0/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)
2	MSI-H	5.0% (1/20)	15.0% (3/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)

Table 18. DNA Input Amount (ng) Limit of Detection per Locus.

Known MSI Result	DNA Input (ng)	Percent Absolute and Relative Stability Call Frequencies at Locus Level (n/n)									
		NR-21		BAT-25		BAT-26		NR-24		MONO-27	
		Lot 1	Lot 2	Lot 1	Lot 2	Lot 1	Lot 2	Lot 1	Lot 2	Lot 1	Lot 2
MSS	0.05	0% (0/20)	10.0% (2/20)	0% (0/20)	0% (0/20)	0% (0/20)	0% (0/20)	0% (0/20)	0% (0/20)	0% (0/20)	0% (0/20)
	0.2	100% (20/20)	100% (20/20)	85.0% (17/20)	100% (20/20)	100% (20/20)	100% (20/20)	95.0% (19/20)	100% (20/20)	100% (20/20)	95.0% (19/20)
	0.5	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)
	0.75	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)
	1.0	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)
	1.25	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)
MSI-H	0.05	85.0% (17/20)	90.0% (18/20)	60.0% (12/20)	55.0% (11/20)	50.0% (10/20)	55.0% (11/20)	20.0% (4/20)	15.0% (3/20)	35.0% (7/20)	50.0% (10/20)
	0.2	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)
	0.5	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)
	0.75	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)
	1.0	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)
	1.25	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)

Based on the results of the study, the LOD for endometrial cancer specimens is 20% tumor content at 1ng of DNA input and 0.2ng of DNA input at 30% tumor content.

9.1.4 Limit of Blank

A Limit of Blank study was conducted to confirm that MSS tumors consistently returned MSS results when tested using the OncoMate® MSI Dx Analysis System. MSI results were generated in five replicates per sample per run across three days using two amplification kit lots for a total of thirty (30) replications of each MSS sample. Four MSS endometrial cancer samples with 30–80% tumor content were tested.

All samples evaluated returned MSS results (100%; 120/120). This study demonstrated that the OncoMate® MSI Dx Analysis System can correctly generate MSS results with no significant background that contributes to false MSI-H results.

9.1.5 Matrix Equivalence of Extracted Tissue and Blood

The Matrix Equivalence study assessed the equivalence of matched normal FFPE tissue and matched normal blood samples when determining MSI status using the OncoMate® MSI Dx Analysis System.

DNA extractions were performed with three lots each of both the Maxwell® CSC DNA FFPE and Maxwell® CSC Blood DNA Kits using matched endometrial tumor and normal FFPE samples and matched normal blood samples. Two DNA purification runs were performed per lot and per sample. Seven MSI-H and two MSS endometrial tumors were evaluated. The MSI-H samples ranged from 30–90% tumor content, and the MSS samples ranged from 50–90% tumor content. The study generated 54 MSI results using tumor FFPE samples with matched normal FFPE samples, and 54 MSI results using tumor FFPE samples with matched normal blood samples, for a total of 108 MSI results and 54 MSI result comparisons.

For each donor, the MSI result obtained using FFPE sample as the matched normal sample type was compared to the MSI result obtained using blood as the matched normal sample type. Positive percent agreement, negative percent agreement and overall percent agreement estimates as well as two-sided 95% confidence intervals were calculated.

PPA and NPA values were both 100% (12/12 and 42/42, respectively), with lower 95% CI limits of 73.5% and 91.6% respectively; OPA was 100% with a lower 95% CI limit of 93.4%. Results are summarized in Table 19. In conclusion, this study demonstrated that concordant MSI results are obtained using the OncoMate® MSI Dx Analysis System when either blood or FFPE samples are used as the matched normal sample.

Table 19. Absolute and Relative Agreement Between MSI Results Generated Using Tumor FFPE Tissue with Either Matched Normal FFPE Tissue or Matched Normal Blood Sample.

	FFPE Normal Tissue Result		
Blood Normal Result	MSS	MSI-H	Total
MSS	12	0	12
MSI-H	0	42	42
Total	12	42	54

		95% CI	
	Estimate (n/N)	Lower Limit	Upper Limit
PPA	100% (12/12)	73.5%	100%
NPA	100% (42/42)	91.6%	100%
OPA	100% (54/54)	93.4%	100%

9.1.6 DNA Extraction Methods Equivalence

The suitability of various DNA purification methods for use with the OncoMate® MSI Dx Analysis System was demonstrated by comparing results generated from DNA extracts using six commercially available DNA extraction methods, three each for FFPE tissue and blood sample sources, including the Maxwell® CSC DNA FFPE Kit, ReliaPrep™ FFPE gDNA MiniPrep System and Maxwell® CSC Blood DNA Kit.

Each of the DNA extraction methods for both blood and FFPE samples were performed by two operator teams using two replicate extractions per sample, repeated on 3 nonconsecutive days. Extractions were performed on FFPE tumor and matched FFPE samples from one MSS and four MSI-H endometrial tumors. The MSI-H samples contained 30–90% tumor content, and the MSS sample had 80% tumor content. The minimum DNA yield for testing and test result agreement among methods and replicates were summarized.

Across all DNA extraction methods and sample sources, DNA yield for 100% (540/540) of extractions was greater than or equal to the minimum DNA input amount required by the OncoMate® MSI Dx Analysis System (1ng total DNA at a concentration ≥0.17ng/µl).

MSI results generated using FFPE tissue or blood as the matched normal sample were compared across all DNA purification methods to calculate average positive agreement (APA) and average negative agreement (ANA) values. Across all DNA purification methods evaluated, both the APA and ANA were 100% when MSI results were generated using FFPE or blood tissue as the matched normal sample

This study demonstrated that the OncoMate ® MSI Dx Analysis System is suitable for use with a range of DNA extraction methods for blood and FFPE samples. DNA purified using various DNA extraction methods generated comparable MSI results and sufficient DNA yields to detect microsatellite instability using the OncoMate® MSI Dx Analysis System.

9.1.7 DNA Interference

The DNA Interference study evaluated the potential effect of endogenous and exogenous interfering substances associated with FFPE tissue samples, blood, DNA purification reagents and blood collection devices on the performance of the OncoMate® MSI Dx Analysis System. The study specifically characterized effects of hemoglobin (10mg/ml), triglycerides (16.94mM), mucin (1mg/ml), melanin (0.005%), conjugated bilirubin (475µM), unconjugated bilirubin (684µM), EDTA (5.4mg/ml) and necrosis (40–90%) on MSI results.

Sample lysates of tumor FFPE tissue, matched normal FFPE tissue and matched normal blood samples were generated for the extraction chemistries evaluated. Each potential interferent was added to the lysates to the concentration listed above before DNA purification. Three different DNA extraction systems were used to extract DNA from each sample source: blood and FFPE tissue. Extractions were performed using one MSS and two MSI-H endometrial tumors. Mucin and necrosis were tested with matched tumor and normal FFPE tissue only. A separate set of one MSS and two MSI-H endometrial tumors was used for the necrosis samples.

The effect of added interferents on the MSI result was evaluated by comparing the concordance between results generated for samples from the same donor with and without the interferent. Since the necrotic content of a tumor cannot be adjusted, no addition to the lysate was required for the necrotic samples. Instead, the MSI result for the necrosis samples was evaluated by comparing OncoMate® MSI Dx Analysis System results from two independent sample sets of tumors: one sample set with high levels of necrosis (50–90%) and one sample set with low to no necrosis (0–20%).

For hemoglobin, triglycerides, conjugated bilirubin, unconjugated bilirubin, melanin and EDTA, concordant MSI results were obtained for 100% of the sample replicates (18/18 for each potential interferent) when using a matched normal sample from either blood or FFPE tissue and with all three DNA extraction systems. For mucin, concordant results were obtained for 88.9% of all sample replicates (16/18). Finally, 100% of sample replicates (18/18) with high necrosis yielded concordant MSI results to sample replicates with low to no necrosis with each of the three DNA extraction systems used (Table 20 and Table 21)

These data demonstrate that the OncoMate® MSI Dx Analysis System tolerates the presence of a variety of common interferents at biologically relevant concentrations, including hemoglobin, triglycerides, conjugated bilirubin, unconjugated bilirubin, melanin, EDTA, mucin and sample necrosis.

Table 20. Number and Percent Concordant MSI Results by Interferent in Water Diluent.

Interferent (Final Concentration)	Matched Normal Sample Type	MSI Result			Total	
		MSS	MSI-H	Invalid		
		n/6	n/12	n/18	n/18	Percent
Conjugated Bilirubin (475µM)	Blood	6/6	12/12	0/18	18/18	100%
	Tissue	6/6	12/12	0/18	18/18	100%
EDTA (5.4mg/ml)	Blood	6/6	12/12	0/18	18/18	100%
	Tissue	6/6	12/12	0/18	18/18	100%
Hemoglobin (10mg/ml)	Blood	6/6	12/12	0/18	18/18	100%
	Tissue	6/6	12/12	0/18	18/18	100%
Triglycerides (16.94mM)	Blood	6/6	12/12	0/18	18/18	100%
	Tissue	6/6	12/12	0/18	18/18	100%
Mucin[1] (1mg/ml)	Tissue	4/6	12/12	2/18	16/18	88.9%
Necrosis[1] (50–90%)	Tissue	6/6	12/12	0/18	18/18	100%
No Interferent, Water	Blood	6/6	12/12	0/18	18/18	100%
	Tissue	6/6	12/12	0/18	18/18	100%

[1]Necrosis and mucin were not evaluated for blood samples.

Table 21. Number and Percent Concordant MSI Results by Interferent in DMSO Diluent.

Interferent (Final Concentration)	Matched Normal Sample Type	MSI Result			Total	
		MSS	MSI-H	Invalid		
		n/6	n/12	n/18	n/18	Percent
Melanin (0.005%)	Blood	6/6	12/12	0/18	18/18	100%
	Tissue	6/6	12/12	0/18	18/18	100%
Unconjugated Bilirubin (684µM)	Blood	6/6	12/12	0/18	18/18	100%
	Tissue	6/6	12/12	0/18	18/18	100%
No Interferent, DMSO	Blood	6/6	12/12	0/18	18/18	100%
	Tissue	6/6	12/12	0/18	18/18	100%

9.1.8 Normal Range and Cutoff

The OncoMate® MSI Dx Analysis System is intended to measure changes in amplified fragment length. The Normal Range study was conducted to verify the system's capability to resolve amplicons that differ by ≥2 base pairs. Two sets of seven synthetic DNA fragments ("resolution markers") were analyzed during this study. These fragments consist of dye-labeled amplicons of known size that are separated by 1bp within each set, with the two sets designed to bracket the upper (Large) and lower (Small) ends of the amplicon size range of the MSI markers (86–188bp).

Three MSS endometrial tumor sample pairs were amplified in duplicate using the OncoMate® MSI Dx Analysis System. Amplified products were then analyzed by capillary electrophoresis in the presence and absence of resolution markers on each of two nonconsecutive days. Across the three samples, 12 replicates were analyzed in the presence of resolution markers and 12 replicates in the absence of resolution markers. Tumor samples had 40–80% tumor content. The observed size of each resolution marker was summarized.

Resolution fragments were sized precisely, with standard deviations ranging from 0.095–0.175bp. When observed resolution marker size was plotted as a function of known size, linear regression yielded r^2 values ≥0.995. Mean absolute differences calculated for fragments separated by 2bp were also precise, ranging from 1.90–2.35bp. The results are reported in Table 22.

Accordingly, this study demonstrated sufficient fragment size resolution and precision to support reliable detection of ≥2bp deletions by the system.

Table 22. Absolute Differences in Observed Sizes of Resolution Markers.

Resolution Marker Pair	Known Size Difference (bp)	Observed Average Size Difference (bp)
Res_Large 1 and Res_Large 3	2.00	2.12
Res_Large 2 and Res_Large 4	2.00	2.00
Res_Large 3 and Res_Large 5	2.00	1.90
Res_Large 4 and Res_Large 6	2.00	2.02
Res_Large 5 and Res_Large 7	2.00	2.13
Res_Small 1 and Res_Small 3	2.00	2.35
Res_Small 2 and Res_Small 4	2.00	2.22
Res_Small 3 and Res_Small 5	2.00	2.08
Res_Small 4 and Res_Small 6	2.00	2.19
Res_Small 5 and Res_Small 7	2.00	2.34

9.1.9 Extracted DNA Stability

The stability of DNA purified from blood and FFPE tissue and stored at –30°C to –10°C was assessed for use in the OncoMate ® MSI Dx Analysis System by comparing MSI results generated at a baseline time point (Time 0) to multiple time points, including an assessment at 53 weeks.

DNA eluates evaluated during this study were purified from one MSS and three MSI-H endometrial tumors. Each FFPE and blood sample was extracted using three DNA extraction systems in duplicate. In total, 24 MSI results were generated for matched normal blood samples and 24 MSI results were generated for matched normal FFPE tissue samples at each time point.

Across all individuals, sample sources, DNA extraction methods and time points, 100% of the MSI were concordant with Time 0 results for eluates tested using FFPE normal tissue (96/96) and normal blood (96/96). These results demonstrate that the OncoMate® MSI Dx System is compatible with DNA eluates from various DNA extraction methods using FFPE and blood samples when stored for up to 12 months at –30°C to –10°C.

9.1.10 Slide Stability

The stability of FFPE tissue sections mounted on glass slides and stored at +20°C to +25°C for use with the OncoMate® MSI Dx Analysis System was assessed by comparing MSI results generated at a baseline time point (Time 0) to multiple time points, including an assessment at 79 weeks. At each time point, DNA was purified from two slides per sample of tumor and matched normal sections, and each purified DNA sample was amplified in duplicate using a single lot of the OncoMate® MSI Dx Analysis System. The study samples comprised of two MSI-H and two MSS endometrial tumors with tumor content of 50–70%.

Across all samples and time points, 100% of MSI results (64/64) were concordant with MSI results at Time 0, for slides stored at +20°C to +25°C. These results demonstrated the OncoMate® MSI Dx System can successfully determine MSI status of tumors from tissue stored on slides for up to 18 months at +20°C to +25°C.

9.2 Clinical Performance in Endometrial Carcinoma

Clinical Performance of Pembrolizumab (KEYTRUDA) and Lenvatinib (LENVIMA) in the KEYNOTE-775/Study-309

The KEYNOTE-775/Study-309 (NCT03517449) was a multicenter, open-label, randomized, active-controlled trial that compared efficacy and safety of lenvatinib in combination with pembrolizumab versus treatment of physician's choice (TPC) in participants with advanced endometrial cancer who had been previously treated with at least one prior platinum-based chemotherapy regimen in any setting, including in the neoadjuvant and adjuvant settings. Patients (n = 827) were enrolled who had no history of exposure to vascular endothelial growth factor or PD-1-targeting regimens. Patients were stratified by MMR status using central testing with an IHC-based clinical trial assay (CTA), resulting in 697 with pMMR disease and 130 with dMMR disease. The pMMR patients were further broken into stratified groups based on Eastern Cooperative Oncology Group (ECOG) performance status, geographic regions, and history of pelvic radiation. Patients were randomly assigned, in a one-to-one ratio, to receive either lenvatinib (20mg, administered orally once daily) plus pembrolizumab (200mg, administered intravenously every 3 weeks) or chemotherapy of the treating physician's choice (doxorubicin at 60mg per square meter of body-surface area, administered intravenously every 3 weeks, or paclitaxel at 80mg per square meter, administered intravenously weekly [with a cycle of 3 weeks on and 1 week off]). Participants (346) were randomized to the pembrolizumab plus lenvatinib arm, and 351 participants were randomized to the TPC arm. The major efficacy outcome measures were overall survival (OS) and progression-free survival (PFS) as assessed by blinded independent central review (BICR) according to Response Evaluation Criteria in Solid Tumors, version 1.1 (RECIST v1.1), modified to follow a maximum of ten target lesions total and a maximum of five target lesions per organ. Additional efficacy outcome measures included objective response rate (ORR) and duration of response (DoR), as assessed by BICR. The end points were evaluated in patients with pMMR disease and in all patients.

The pMMR population characteristics were: median age of 65 years (range: 30 to 86), 52% age 65 or older; 62% White, 22% Asian, and 3% Black; 60% ECOG PS of 0 and 40% ECOG PS of 1. The histologic subtypes were endometrioid carcinoma (55%), serous (30%), clear cell carcinoma (7%), mixed (4%), and other (3%). All 697 of these patients received prior systemic therapy for endometrial carcinoma: 67% had one, 30% had two, and 3% had three or more prior systemic therapies. Thirty-seven percent of patients received only prior neoadjuvant or adjuvant therapy.

A clinical bridging study from the CTA to the OncoMate® MSI Dx Analysis System was conducted to establish the clinical validity of the test as a companion diagnostic (CDx) to identify endometrial carcinoma patients with MSS (not MSI-H) tumor status for treatment with pembrolizumab and lenvatinib. Of the 827 participants in the original KEYNOTE-775 study, samples from 523 participants were available for testing and met OncoMate® MSI Dx Analysis System requirements.

For the clinical performance with KEYNOTE-775 summarized below and subsequent testing for this intended purpose, a MSS (not MSI-H) OncoMate® MSI Dx Analysis System result is considered the positive result, and an MSI-H OncoMate® MSI Dx Analysis System result is considered the negative result.

Success Rate Analysis

The rate of valid test results (MSS or MSI-H result) with the OncoMate® MSI Dx Analysis System was evaluated using the eligible participant samples from the KEYNOTE-775 clinical bridging study.

Within the study, the valid result success rate (MSS or MSI-H result) was 93.5% (489/523). The OncoMate® MSI Dx Analysis Software identified 34 samples (6.5%, 34/523) exhibiting data quality issues attributable to the samples. Data quality issues included evidence of DNA contamination, mismatched normal and tumor DNA samples, and low peak intensity associated with poor sample quality.

Concordance Analysis

To estimate concordance between the CTA and the OncoMate® MSI Dx Analysis System (CDx), the agreement between the two methods was calculated using the 489 KEYNOTE-775 participant samples which yielded evaluable CTA MMR results and OncoMate® MSI Dx Analysis System results. Positive percent agreement and negative percent agreement were estimated along with 95% confidence intervals, using the CTA as the reference.

The PPA between MSS (not MSI-H) and pMMR results was 98.8% (402/407), with a 95% confidence interval of 97.2–99.5%. The NPA between MSI-H and dMMR results was 91.5% (75/82), with a 95% confidence interval of 83.4–95.8%. Across all samples, the overall percent agreement was 97.5%, (477/489) with a 95% confidence interval of 95.8–98.6%. These results are shown in Table 23.

Table 23. Agreement Analysis Between the OncoMate® MSI Dx Analysis System and the CTA in the CDx-Evaluable Subpopulation of the KEYNOTE-775 Study Participants.

OncoMate® MSI Dx Analysis System Result (CDx)	CTA		Total
	pMMR	dMMR	
MSS (not MSI-H)	402	7	409
MSI-H	5	75	80
Total	407	82	489

	Estimate (n/N)	95% CI	
		Lower Limit	Upper Limit
PPA	98.8% (402/407)	97.2%	99.5%
NPA	91.5% (75/82)	83.4%	95.8%
OPA	97.5% (477/489)	95.8%	98.6%

Clinical Efficacy Results

The major efficacy outcome measures were OS and PFS as assessed by BICR according to RECISTv1.1, modified to follow a maximum of ten target lesions total and a maximum of five target lesions per organ. Additional efficacy outcome measures included ORR and DoR, as assessed by BICR. For the bridging study, efficacy was assessed in participants (n = 409) with MSS (not MSI-H) tumors as defined by OncoMate® MSI Dx Analysis System. Statistical testing was not performed on this population due to insufficient sample size; estimates and confidence intervals were used instead.

Pembrolizumab plus lenvatinib efficacy in the subset of KEYNOTE-775 participants with MSS (not MSI-H) tumor status by the OncoMate ® MSI Dx Analysis System was comparable to the efficacy in all CTA-pMMR participants randomized in the trial. Efficacy results are summarized in Table 24.

Table 24. Clinical Benefit of Pembrolizumab in Combination with Lenvatinib Estimated from KEYNOTE-775.

	pMMR Status by CTA (n = 697)		MSS (not MSI-H) by CDx* (n = 409)		CDx Unevaluable (n = 338)	
Endpoint	Pembrolizumab plus Lenvatinib (n = 346)	TPC Group (n = 351)	Pembrolizumab plus Lenvatinib (n = 218)	TPC Group (n = 191)	Pembrolizumab plus Lenvatinib (n = 155)	TPC Group (n = 183)
OS median in months (95% CI)	17.4 (14.2, 19.9)	12.0 (10.8, 13.3)	18.0 (14.0, 20.5)	11.3 (10.0, 12.8)	17.1 (12.2, 20.0)	13.0 (10.7, 15.2)
OS HR[1] (95% CI)	0.68 (0.56, 0.84)		0.60 (0.46, 0.78)		0.80 (0.59, 1.08)	
PFS median in months (95% CI)	6.6 (5.6, 7.4)	3.8 (3.6, 5.0)	7.2 (5.6, 7.6)	3.7 (3.6, 5.4)	6.1 (5.4, 7.5)	4.1 (3.5, 5.6)
PFS HR[1] (95% CI)	0.60 (0.50, 0.72)		0.59 (0.47, 0.74)		0.63 (0.48, 0.83)	
Percent ORR[2]	30.3 (25.5, 35.5)	15.1 (11.5, 19.3)	32.6 (26.4, 39.2)	15.7 (10.9, 21.7)	26.5 (19.7, 34.1)	15.8 (10.9, 22.0)
Percent ORR[2] difference (95% CI)	15.2 (9.1, 21.4)		16.9 (8.6, 24.9)		10.6 (1.9, 19.5)	
DoR median in months (range)	9.2 (1.6, 23.7+)	5.7 (0.0, 24.2+)	9.2 (1.6, 23.7+)	5.8 (1.8, 24.2+)	10.9 (1.6, 18.8+)	5.2 (0.0, 15.6+)

OS: Overall Survival. PFS: Progression-Free Survival. ORR: Overall Response Rate. DoR: Duration of Response
*MSS (not MSI-H) population consists of n = 402 participants with pMMR status by CTA and MSS (not MSI-H) by the CDx (drug efficacy population) and n = 7 participants with dMMR status by the CTA and MSS (not MSI-H) by the CDx. The n = 7 participants with dMMR status by CTA were not included in the n = 697 drug efficacy population from KEYNOTE-775. The KEYNOTE-775 study enrolled 827 subjects total, with 697 classified as pMMR and 130 as dMMR by the CTA. When evaluating participants using the CDx, the total population was 747 subjects (combining CDx-evaluable and CDx-unevaluable participants). The discrepancy between the pMMR population by CTA (697 subjects) and the total CDx population (747 subjects) occurs because the CDx analysis included dMMR participants when assessing clinical benefit.

[1]Based on the stratified Cox regression model for the CTA-identified pMMR participants and on nonstratified Cox regression model for the other two populations.

[2]Response: Best objective response as confirmed complete response or partial response.

Sensitivity analyses with regard to the missing CDx test results were conducted to evaluate the robustness of the efficacy estimates considering OncoMate® MSI Dx Analysis System unevaluable patients enrolled in the KEYNOTE-775 trial. To conduct a missing data sensitivity analysis, data sets were created through multiple imputation, each containing the observed CDx results and imputed CDx results when missing. Efficacy endpoint estimates and hypothesis tests were conducted on each complete data set and summarized over the multiple imputations (Table 24). Amongst all patients enrolled in the KEYNOTE-775 primary efficacy population, 40.9% (338/827) did not have an OncoMate® MSI Dx Analysis System result. The imputed PFS HR and OS HR was estimated to be 0.6 (95% CI: 0.59, 0.61) and 0.68 (95% CI: 0.56, 0.84), respectively. This is comparable to the PFS HR and OS HR for the CTA-positive population (i.e., pMMR) [0.60 (0.50, 0.72) and 0.68 (0.56, 0.84), respectively]. The imputed ORR was estimated to be 30.1% (95% credible interval: 29.7, 30.5), which is comparable to the ORR for the CTA-positive population [30.3% (95% CI: 25.5, 35.5)]. The imputed median DoR was estimated to be 9.2 months, which is comparable to the median DoR for the CTA-positive population (9.2 months).

Efficacy results from the bridging study support the clinical performance of the OncoMate® MSI Dx Analysis System to identify endometrial carcinoma patients with solid tumors that are MSS (not MSI-H) who might benefit from treatment with pembrolizumab plus lenvatinib combination treatment.

10. Performance Characteristics in Solid Tumors

All analytical studies followed the procedure outlined in the OncoMate® MSI Dx Analysis System Technical Manual unless noted otherwise in the study results section

10.1 Analytical Performance in Solid Tumors

Within the analytical performance summarized below, an MSI-H result is considered the positive result and an MSS result is considered the negative result, unless noted otherwise in the study results.

10.1.1 Accuracy

Accuracy of the OncoMate® MSI Dx Analysis System was characterized by evaluating agreement of the test with a commercially available immunohistochemistry (IHC) assay. A comparison of IHC and MSI results was performed using 611 solid tumor samples from the following tumor or tissue types: endometrial, neuroendocrine tumor, anal, vulvar, mesothelioma, cervical, thyroid, gastric, cholangiocarcinoma, small cell lung, salivary, small intestine adenocarcinoma, sarcoma, ovarian, breast, glioma, renal, bladder, pancreatic, nasopharyngeal, adrenocortical, prostate, colorectal, esophageal, head and neck squamous cell carcinoma and liver.

With the commercially available IHC assay as the reference method, positive percent agreement between MSI-H and dMMR results was 88.7% (102/115), with a 95% confidence interval of 81.6–93.3%. Negative percent agreement between MSS and pMMR results was 97.2% (482/496), with a 95% confidence interval of 95.3–98.3%. Overall percent agreement was 95.6% (584/611), with a 95% confidence interval of 93.6–96.9%. See Table 25.

The data demonstrate the OncoMate® MSI Dx Analysis System and an orthogonal IHC method are highly concordant.

Table 25. Accuracy Absolute Frequencies and Agreement Estimates Generated Using the OncoMate® MSI Analysis System and an MMR IHC Panel.

OncoMate® MSI Dx Analysis System Result	MMR IHC Panel Result		Total
	dMMR	pMMR	
MSI-H	102	14	116
MSS	13	482	495
Total	115	496	611

		95% CI	
	Estimate (n/N)	Lower Limit	Upper Limit
PPA	88.7% (102/115)	81.6%	93.3%
NPA	97.2% (482/496)	95.3%	98.3%
OPA	95.6% (584/611)	93.6%	96.9%

10.1.2 Reproducibility

The Reproducibility study characterized precision and reproducibility of the OncoMate® MSI Dx Analysis System when testing MSS and MSI-H FFPE tumors.

To assess within-lot reproducibility and precision, nine samples were analyzed on five nonconsecutive days using the same OncoMate® MSI Dx Analysis System lot at three different study sites. A single operator team at each study site analyzed five replicates for each sample per day using a single CE instrument. The nine samples comprised six MSI-H FFPE tumors from endometrial, gastric, colorectal and lung tissues and three MSS FFPE tumors from endometrial, gastric and breast tissues

To assess between-lot reproducibility and precision, the same nine samples used for evaluating within-lot reproducibility were analyzed on five nonconsecutive days using three OncoMate® MSI Dx Analysis System lots at a single study site. A single operator analyzed five replicates for each sample per day using a single capillary electrophoresis instrument.

PPA, NPA and two-sided 95% confidence intervals were calculated by comparing observed MSI results for each sample replicate to the majority MSI result observed across all replicates. Data across individual factors (site, lot and day) and all factors were summarized to evaluate reproducibility of the OncoMate® MSI Dx Analysis System.

PPA and NPA values for MSS and MSI-H results, respectively, across site, day, lot or run were 100%, demonstrating high reproducibility for each factor tested. Across all study factors, PPA was 100% (750/750, 95% CI of 99.5–100%) and NPA was 100% (375/375, 95% CI of 99.0–100%). These data are shown in Table 26.

Table 26. PPA and NPA Estimates for Observed MSI Result as Compared to Majority MSI Result By Study Site, Lot and Day.

		PPA			NPA		
			95% CI			95% CI	
		Estimate	Lower Limit	Upper Limit	Estimate	Lower Limit	Upper Limit
Site	1 (Lot 1)	100% (150/150)	97.6%	100%	100% (75/75)	95.2%	100%
	2 (Lot 1)	100% (150/150)	97.6%	100%	100% (75/75)	95.2%	100%
	3 (Lot 1)	100% (150/150)	97.6%	100%	100% (75/75)	95.2%	100%
	Overall, Sites 1–3	**100% (450/450)**	**99.2%**	**100%**	**100% (225/225)**	**98.4%**	**100%**
Lot	1 (Site 1)	100% (150/150)	97.6%	100%	100% (75/75)	95.2%	100%
	2 (Site 1)	100% (150/150)	97.6%	100%	100% (75/75)	95.2%	100%
	3 (Site 1)	100% (150/150)	97.6%	100%	100% (75/75)	95.2%	100%
	Overall, Lots 1–3	**100% (450/450)**	**99.2%**	**100%**	**100% (225/225)**	**98.4%**	**100%**
Day (Lot 1, Sites 1–3)	1	100% (90/90)	96.0%	100%	100% (45/45)	92.1%	100%
	2	100% (90/90)	96.0%	100%	100% (45/45)	92.1%	100%
	3	100% (90/90)	96.0%	100%	100% (45/45)	92.1%	100%
	4	100% (90/90)	96.0%	100%	100% (45/45)	92.1%	100%
	5	100% (90/90)	96.0%	100%	100% (45/45)	92.1%	100%
	Overall, Sites 1–3	**100% (450/450)**	**99.2%**	**100%**	**100% (225/225)**	**98.4%**	**100%**

		PPA			NPA		
			95% CI			95% CI	
		Estimate	Lower Limit	Upper Limit	Estimate	Lower Limit	Upper Limit
Day (Site 1, Lots 1–3)	1	100% (90/90)	96.0%	100%	100% (45/45)	92.1%	100%
	2	100% (90/90)	96.0%	100%	100% (45/45)	92.1%	100%
	3	100% (90/90)	96.0%	100%	100% (45/45)	92.1%	100%
	4	100% (90/90)	96.0%	100%	100% (45/45)	92.1%	100%
	5	100% (90/90)	96.0%	100%	100% (45/45)	92.1%	100%
	Overall, Lots 1–3	**100% (450/450)**	**99.2%**	**100%**	**100% (225/225)**	**98.4%**	**100%**
Overall, Across All Factors		**100% (750/750)**	**99.5%**	**100%**	**100% (375/375)**	**99.0%**	**100%**

To assess precision and reproducibility at the assay limit of detection for tumor content, six endometrial tumor samples (three MSI-H and three MSS) with 30% tumor content were evaluated. Each sample was analyzed on five nonconsecutive days at each of two study sites using two OncoMate® MSI Dx Analysis System lots. At each site, a single operator analyzed five replicates for each sample per day using a single CE instrument.

Across both study sites and both reagent lots, PPA for MSI-H results was 100% and NPA for MSS results was 100%. Across all study factors, PPA was 100% (95% CI of 97.6–100%) and NPA was 100% (97.6–100%). See Table 27.

Table 27. PPA and NPA Estimates for Observed MSI Result as Compared to Majority MSI Result for MSI-H and MSS Endometrial Tumor Samples With Tumor Content At 30% By Study Site, Lot and Day.

		PPA				NPA			
				95% CI				95% CI	
		N	Estimate	Lower Limit	Upper Limit	N	Estimate	Lower Limit	Upper Limit
Site	1	75	100%	95.2%	100%	75	100%	95.2%	100%
	2	75	100%	95.2%	100%	75	100%	95.2%	100%
Lot	1	75	100%	95.2%	100%	75	100%	95.2%	100%
	2	75	100%	95.2%	100%	75	100%	95.2%	100%

		PPA				NPA			
				95% CI				95% CI	
		N	Estimate	Lower Limit	Upper Limit	N	Estimate	Lower Limit	Upper Limit
Day (Site 1)	1 (Lot 1)	15	100%	78.2%	100%	15	100%	78.2%	100%
	2 (Lot 2)	15	100%	78.2%	100%	15	100%	78.2%	100%
	3 (Lot 1)	15	100%	78.2%	100%	15	100%	78.2%	100%
	4 (Lot 2)	15	100%	78.2%	100%	15	100%	78.2%	100%
	5 (Lot 1)	15	100%	78.2%	100%	15	100%	78.2%	100%
	Overall, Site 1, Lot 1	**45**	**100%**	**92.1%**	**100%**	**45**	**100%**	**92.1%**	**100%**
	Overall, Site 1, Lot 2	**30**	**100%**	**88.4%**	**100%**	**30**	**100%**	**88.4%**	**100%**
Day (Site 2)	1 (Lot 2)	15	100%	78.2%	100%	15	100%	78.2%	100%
	2 (Lot 1)	15	100%	78.2%	100%	15	100%	78.2%	100%
	3 (Lot 2)	15	100%	78.2%	100%	15	100%	78.2%	100%
	4 (Lot 1)	15	100%	78.2%	100%	15	100%	78.2%	100%
	5 (Lot 2)	15	100%	78.2%	100%	15	100%	78.2%	100%
	Overall, Site 2, Lot 1	**30**	**100%**	**88.4%**	**100%**	**30**	**100%**	**88.4%**	**100%**
	Overall, Site 2, Lot 2	**45**	**100%**	**92.1%**	**100%**	**45**	**100%**	**92.1%**	**100%**
Across All Factors		**150**	**100%**	**97.6%**	**100%**	**150**	**100%**	**97.6%**	**100%**

Together, these data demonstrated that the OncoMate® MSI Dx Analysis System generates highly reproducible and consistent MSI results between and across sites, CE instruments, days and product material lots.

10.1.3 Limit of Detection

The LOD study determined the minimum tumor content and DNA input amount required for the OncoMate® MSI Dx Analysis System to correctly assign an MSI result with 95% positivity. The LOD was characterized using samples from a variety of solid tumor tissues.

To determine the LOD for tumor content, two tumor samples were evaluated on five nonconsecutive days using two lots of the OncoMate® MSI Dx Analysis System with four replicates per sample per day. Forty replicates of each sample were evaluated at five levels of tumor content, 10%, 20%, 30%, 40% and the original tumor content, using a DNA input amount of 1.0ng. The absolute and relative frequencies of positive results were summarized. The lowest tumor content percentage with a positivity hit rate ≥0.95 at the sample level for all samples for the worse-performing reagent lot when tested at 1.0ng DNA input was identified as the LOD.

The absolute and relative frequencies for each tumor content percentage are summarized in Table 28. The lowest tumor content achieving a percent positivity ≥95% (hit rate ≥0.95) across all factors and samples was 20%.

Table 28. Absolute and Relative MSI-H Result Frequency by Percent Tumor Content.

Sample Number	Sample or Tissue Type	Absolute and Relative MSI-H Frequency by Percent Tumor Content									
		10%		20%		30%		40%		Original Tumor Content	
		Lot 1	Lot 2	Lot 1	Lot 2	Lot 1	Lot 2	Lot 1	Lot 2	Lot 1	Lot 2
1	Colorectal	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)
2	Endometrial	75.0% (15/20)	90.0% (18/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)

To verify the LOD for sample tumor content, ten MSI-H tumor samples from eight tissues, which included prostate, melanoma, endometrial, liver, gastric, ovarian, colorectal and small bowel, were tested on five nonconsecutive days with four replicates per sample per day. Samples were evaluated at 30% tumor content using a 1.0ng DNA input. The five gastrointestinal tumor samples, which were gastric, colorectal and small bowel tissues, also were evaluated at 20% tumor content and 1.0ng DNA. The observed absolute and relative frequencies of MSI-H results and empirical hit rates were summarized.

All ten tumor samples yielded a positivity hit rate of 1, meaning 100% correct MSI-H results were achieved for the 200 total replicates examined at 30% tumor content and 1.0ng DNA input. At 20% tumor content, all gastrointestinal tumor samples also yielded 100% correct MSI-H results for the 100 total replicates examined. These data are shown in Table 29.

Table 29. MSI Results for Tumor-Content Limit of Detection Verification By Tissue Type and Tumor Content.

	Tissue Type	Sample ID	Known MSI Result	Tumor Content	Observed MSI Result		Hit Rate
					MSI-H	MSS	
Nongastrointestinal	Prostate	S00949	MSI-H	30%	100% (20/20)	0% (0/20)	1
	Skin	S00940	MSI-H	30%	100% (20/20)	0% (0/20)	1
	Biliary-cholangio (liver)	S00732	MSI-H	30%	100% (20/20)	0% (0/20)	1
	Ovarian	S00948	MSI-H	30%	100% (20/20)	0% (0/20)	1
	Endometrial	S00476	MSI-H	30%	100% (20/20)	0% (0/20)	1
Gastrointestinal; 20% tumor content	Small Bowel	S00951	MSI-H	20%	100% (20/20)	0% (0/20)	1
		S00477	MSI-H	20%	100% (20/20)	0% (0/20)	1
	Gastric	S00960	MSI-H	20%	100% (20/20)	0% (0/20)	1
		S00914	MSI-H	20%	100% (20/20)	0% (0/20)	1
	Colorectal	S00520	MSI-H	20%	100% (20/20)	0% (0/20)	1
Gastrointestinal; 30% tumor content	Small Bowel	S00951	MSI-H	30%	100% (20/20)	0% (0/20)	1
		S00477	MSI-H	30%	100% (20/20)	0% (0/20)	1
	Gastric	S00960	MSI-H	30%	100% (20/20)	0% (0/20)	1
		S00914	MSI-H	30%	100% (20/20)	0% (0/20)	1
	Colorectal	S00520	MSI-H	30%	100% (20/20)	0% (0/20)	1
Total 30% Tumor Content, Nongastrointestinal Tumors					100% (100/100)	0% (0/100)	1
Total 20% Tumor Content, Gastrointestinal Tumors					100% (100/100)	0% (0/100)	1
Total 30% Tumor Content, Gastrointestinal Tumors					100% (100/100)	0% (0/100)	1
Total					100% (300/300)	0% (0/300)	1

To determine the LOD for DNA input amount, DNA was purified from two MSI-H and two MSS from colorectal and endometrial tumors. Each sample was analyzed at six different DNA input amounts ranging from 0.05ng to 1.25ng per reaction across five nonconsecutive days using two OncoMate® MSI Dx Analysis System lots. The MSI-H endometrial sample was assayed at 30% tumor content while the MSI-H colorectal sample was assayed at 20% and 30% tumor content. Both MSS samples were assayed at their original tumor contents. Forty replicates were generated per sample for each tumor content and DNA input amount. The lowest DNA input amount with a positivity hit rate ≥0.95 at the sample level for all samples when tested at the LOD tumor content was identified as the DNA-input LOD. Across the samples and factors evaluated, the DNA-input LOD varied from 0.2ng to 0.76ng. Therefore, the lowest DNA input amount that achieved a positivity hit rate ≥0.95 for the worse-performing sample and lot was 0.76ng.

To verify the LOD for sample DNA input amount, 16 FFPE tumor samples (the same ten MSI-H tumor samples used to verify the tumor-content LOD plus an additional six MSS tumor samples) were tested on five nonconsecutive days with four replicates per sample within each run using a single OncoMate® MSI Dx Analysis System lot. Twenty replicates for each of ten MSI-H tumor samples were evaluated at 30% tumor content using a 0.76ng DNA input, and 20 replicates for each of six MSS tumor samples were evaluated at their original tumor content using a 0.76ng DNA input. The MSI-H tissues evaluated comprised prostate, melanoma, endometrial, liver, gastric, ovarian, colorectal and small bowel tumors, and the MSS tissues evaluated comprised endometrial, melanoma and renal tumors. The five MSI-H gastrointestinal tumor samples were also evaluated with 20% tumor content and a 0.76ng DNA input. The observed MSI results were tabulated, and empirical hit rates were summarized.

All ten MSI-H tumor samples yielded a positivity hit rate of 1, meaning 100% correct MSI-H results for the 200 total replicates examined at 30% tumor content and 0.76ng DNA input. At 20% tumor content and 0.76ng DNA input, all MSI-H gastrointestinal tumor samples also yielded 100% correct MSI-H results for the 100 total replicates examined. At their original tumor contents, the six MSS tumor samples yielded 100% correct MSS results for the 120 total replicates examined using 0.76ng DNA input. These data are shown in Table 30.

Together these data support a limit of detection with 95% positivity for the OncoMate® MSI Dx Analysis System of 30% tumor content with 0.76ng DNA input for nongastrointestinal tumor samples and 20% tumor content with 0.76ng DNA input for gastrointestinal tumor samples.

Table 30. MSI Results for DNA-Input Limit of Detection Verification By Tissue Type and Sample. All DNA samples were evaluated at 0.76ng of DNA per reaction.

	Tissue Type	Sample ID	Known MSI Result	Tumor Content	Observed MSI Result		Hit Rate
					MSI-H	MSS[1]	
Nongastrointestinal	Prostate	S00949	MSI-H	30%	100% (20/20)	0% (0/20)	1
	Skin	S00940	MSI-H	30%	100% (20/20)	0% (0/20)	1
	Biliary-cholangio (liver)	S00732	MSI-H	30%	100% (20/20)	0% (0/20)	1
	Ovarian	S00948	MSI-H	30%	100% (20/20)	0% (0/20)	1
	Endometrial	S00476	MSI-H	30%	100% (20/20)	0% (0/20)	1
		S00019	MSS	70%	0% (0/20)	100% (20/20)	1[1]
		S00021	MSS	90%	0% (0/20)	100% (20/20)	1[1]
	Melanoma	S00918	MSS	80%	0% (0/20)	100% (20/20)	1[1]
		S00920	MSS	90%	0% (0/20)	100% (20/20)	1[1]
	Kidney	S00613	MSS	98%	0% (0/20)	100% (20/20)	1[1]
		S00614	MSS	98%	0% (0/20)	100% (20/20)	1[1]
Gastrointestinal; 20% tumor content	Small Bowel	S00477	MSI-H	20%	100% (20/20)	0% (0/20)	1
		S00951	MSI-H	20%	100% (20/20)	0% (0/20)	1
	Gastric	S00914	MSI-H	20%	100% (20/20)	0% (0/20)	1
		S00960	MSI-H	20%	100% (20/20)	0% (0/20)	1
	Colorectal	S00520	MSI-H	20%	100% (20/20)	0% (0/20)	1
Gastrointestinal; 30% tumor content	Small Bowel	S00477	MSI-H	30%	100% (20/20)	0% (0/20)	1
		S00951	MSI-H	30%	100% (20/20)	0% (0/20)	1
	Gastric	S00914	MSI-H	30%	100% (20/20)	0% (0/20)	1
		S00960	MSI-H	30%	100% (20/20)	0% (0/20)	1
	Colorectal	S00520	MSI-H	30%	100% (20/20)	0% (0/20)	1

[1]Hit rates for MSS tumor samples are not informative due to the absence of microsatellite instability.

10.1.4 Limit of Blank

A Limit of Blank study was conducted to confirm that MSS tumors consistently returned MSS results when tested using the OncoMate® MSI Dx Analysis System.

MSI results were generated for five replicates per sample per day across three days using two amplification kit lots for a total of thirty (30) replications of each MSS sample. Four MSS endometrial cancer samples with 30–80% tumor content were tested.

All tests resulted in MSS results (100%; 120/120). No MSS samples were assigned an MSI-H result during this study. This study demonstrated that the OncoMate® MSI Dx Analysis System provides highly reproducible, negative MSS results with no significant background that contributes to false MSI-H results.

10.1.5 Matrix Equivalence of Extracted Tissue and Blood

The Matrix Equivalence study assessed equivalence of matched normal FFPE tissue and matched normal blood samples when determining MSI status using the OncoMate® MSI Dx Analysis System.

DNA extractions were performed with three lots each of both the Maxwell® CSC DNA FFPE and Maxwell® CSC Blood DNA Kits using matched tumor and normal FFPE samples and matched normal blood samples. Two DNA purification runs were performed per lot and per sample. Eighteen (MSI-H and 20 MSS samples were evaluated, derived from colorectal, endometrial, adrenal, pancreatic, gastric, melanoma, breast, lung, prostate, bladder, renal, head and neck tumor, and cervical tumors. The MSI-H samples ranged from 30% to 90% tumor content, and the MSS samples ranged from 30% to 95% tumor content. The study generated 228 MSI results using tumor FFPE samples with matched normal FFPE samples and 228 MSI results using tumor FFPE samples with matched normal blood samples, for a total of 456 MSI results and 228 MSI result comparisons.

For each donor, the MSI result obtained using FFPE sample as the matched normal sample type was compared to the MSI result obtained using blood as the matched normal sample type. Positive percent agreement (PPA), negative percent agreement (NPA) and overall percent agreement (OPA) estimates as well as two-sided 95% confidence intervals (95% CI) were calculated.

PPA and NPA values were both 100% (108/108, 120/120 respectively), with lower 95% CI limits of 96.6% and 97.0% respectively, and OPA was 100% with a lower 95% CI limit of 98.4%.

Based on the study results, concordant MSI results are obtained using the OncoMate® MSI Dx Analysis System when either blood or FFPE samples are used as the matched normal sample across a variety of cancer types.

10.1.6 DNA Extraction Methods Equivalence

The suitability of various DNA purification methods for use with the OncoMate® MSI Dx Analysis System was demonstrated by comparing results generated from DNA extracts derived from multiple tissue sources using six commercially available DNA extraction methods, three each for FFPE tissue and blood sample sources, including the Maxwell® CSC DNA FFPE Kit, ReliaPrep™ FFPE gDNA MiniPrep System and Maxwell® CSC Blood DNA Kit.

Each of the DNA extraction methods for both blood and FFPE samples was performed by two operator teams using two replicate extractions per sample, repeated on three nonconsecutive days. DNA was purified from FFPE tumor and matched FFPE samples for seven MSI-H and 12 MSS samples from 12 tissues (colorectal, endometrial, pancreatic, gastric, melanoma, renal, liver, thyroid, lung, prostate, bladder and small bowel). Matched normal blood samples were available for 10 of the 12 MSS tumor samples. The MSI-H samples contained 30–90% tumor content, and the MSS samples had 30–95% tumor content. The minimum DNA yield for testing and test result agreement among methods and replicates were summarized.

Across all DNA extraction methods and sample sources, DNA yield for 99.7% (1974/1980) of extractions was greater than or equal to the minimum DNA input amount required by the OncoMate® MSI Dx Analysis System (1ng total DNA at a concentration ≥0.17ng/µl).

MSI results generated using FFPE tissue or blood as the matched normal sample were compared across all DNA purification methods to calculate average positive agreement (APA) and average negative agreement (ANA) values. Across all DNA purification methods evaluated, both the APA and ANA were 100% when MSI results were generated using FFPE or blood tissue as the matched normal sample (Table 31).

Table 31. Average Agreement Values for MSI Results Generated Using Tumor FFPE Tissue with Matched Normal FFPE Tissue or Matched Normal Blood Samples After DNA extraction with Three Separate DNA Extraction Systems.

Measure of Agreement	MSI Result Using FFPE Tissue Normal			MSI Result Using Blood Tissue Normal		
	Percent	95% CI		Percent	95% CI	
		Lower Limit	Upper Limit		Lower Limit	Upper Limit
Average Positive Agreement	100% (252/252)	98.55%	100%	100% (252/252)	98.55%	100%
Average Negative Agreement	100% (432/432)	99.15%	100%	100% (360/360)	98.98%	100%

This study demonstrated that the OncoMate ® MSI Dx Analysis System is suitable for use with a variety of DNA extraction methods for blood and FFPE samples from multiple organ sources. DNA purified using various DNA extraction methods generated comparable MSI results and sufficient DNA yields to detect microsatellite instability using the OncoMate® MSI Dx Analysis System.

10.1.7 DNA Interference

The DNA Interference study evaluated the potential effect of endogenous and exogenous interfering substances associated with FFPE tissue samples, blood, DNA purification reagents and blood collection devices on OncoMate® MSI Dx Analysis System performance. The study specifically characterized the effects of hemoglobin (10mg/ml), triglycerides (16.94mM), mucin (1mg/ml), melanin (0.005%), conjugated bilirubin (475µM), unconjugated bilirubin (684µM), EDTA (5.4mg/ml) and necrosis (40–90%) on MSI results. Previously generated data characterizing the effects of proteinase K, ethanol and guanidine hydrochloride were re-analyzed using the OncoMate® MSI Dx Analysis Software.

Lysates of tumor FFPE tissue, matched normal FFPE tissue and matched normal blood samples were generated for the extraction chemistries evaluated. Each potential interferent was added to the lysates to the concentration listed above before DNA purification. Three different DNA extraction systems were used to extract DNA from each sample source: blood and FFPE tissue. Extractions were performed on a total of four MSI-H and four MSS tumors across a variety of representative tumor types from colorectal, gastric, endometrial and kidney. Mucin and necrosis were tested with matched tumor and normal FFPE tissue only.

The effect of added interferents on the MSI result was evaluated by comparing concordance between results generated for samples from the same donor with and without the interferent. Since the necrotic content of a tumor cannot be adjusted, no addition to the lysate was required for the necrosis samples. Instead, the MSI results for the necrosis samples were evaluated by comparing OncoMate® MSI Dx Analysis System results from two independent sample sets of colorectal, endometrial, gastric and kidney tumors: one sample set with high levels of necrosis (40–90%) and one sample set with low to no necrosis (0–20%).

For hemoglobin, triglycerides, conjugated bilirubin, unconjugated bilirubin, melanin and EDTA, concordant MSI results were obtained for 100% of the sample replicates (48/48 for each potential interferent) when using a matched normal sample from either blood or FFPE tissue and with all three DNA extraction systems. For mucin, concordant results were obtained for 95.8% of all sample replicates (46/48). Finally, 100% of sample replicates (48/48) with high necrosis (40–90%) yielded concordant MSI results to sample replicates with low to no necrosis (0–20%) with each of the three DNA extraction systems used (Table 32 and Table 33). Concordant MSI results (120/120) were observed between the original analyses and the results generated after re-analysis of the original data with the OncoMate® MSI Dx Analysis Software for proteinase K, ethanol, guanidine hydrochloride and other interferents. See Section 23.6 for the original study results.

These data demonstrate that the OncoMate® MSI Dx Analysis System tolerates the presence of a variety of common interferents at biologically relevant concentrations, including hemoglobin, triglycerides, conjugated bilirubin, unconjugated bilirubin, melanin, EDTA, mucin and necrosis as well as proteinase K, ethanol and guanidine hydrochloride. Thus, the OncoMate® MSI Dx Analysis System can generate an MSI result in the presence of these 11 interferents.

Table 32. Number and Percent Concordant MSI Results by Interferent in Water Diluent.

Interferent (Final Concentration)	Matched Normal Sample Type	MSI Result			Total	
		MSS	MSI-H	Invalid		
		n/24	n/24	n/48	n/48	Percent Correct
Conjugated Bilirubin (475µM)	Blood	24/24	24/24	0/48	48/48	100%
	Tissue	24/24	24/24	0/48	48/48	100%
EDTA (5.4mg/ml)	Blood	24/24	24/24	0/48	48/48	100%
	Tissue	24/24	24/24	0/48	48/48	100%
Hemoglobin (10mg/ml)	Blood	24/24	24/24	0/48	48/48	100%
	Tissue	24/24	24/24	0/48	48/48	100%
Triglycerides (16.94mM)	Blood	24/24	24/24	0/48	48/48	100%
	Tissue	24/24	24/24	0/48	48/48	100%
Mucin[1] (1mg/ml)	Tissue	22/24	24/24	2/48	46/48	95.8%
Necrosis[1] (40–90%)	Tissue	24/24	24/24	0/48	48/48	100%
No Interferent, Water	Blood	24/24	24/24	0/48	48/48	100%
	Tissue	24/24	24/24	0/48	48/48	100%

[1]Necrosis and mucin were not evaluated for blood samples.

Table 33. Number and Percent Concordant MSI Results by Interferent in DMSO Diluent.

Interferent (Final Concentration)	Matched Normal Sample Type	MSI Result			Total	
		MSS	MSI-H	Invalid		
		n/24	n/24	n/48	n/48	Percent Correct
Melanin (0.005%)	Blood	24/24	24/24	0/48	48/48	100%
	Tissue	24/24	24/24	0/48	48/48	100%
Unconjugated Bilirubin (684µM)	Blood	24/24	24/24	0/48	48/48	100%
	Tissue	24/24	24/24	0/48	48/48	100%
No Interferent, DMSO	Blood	24/24	24/24	0/48	48/48	100%
	Tissue	24/24	24/24	0/48	48/48	100%

10.1.8 Normal Range and Cutoff

The OncoMate® MSI Dx Analysis System measures changes in amplified fragment length. The Normal Range study was conducted to verify the system's capability to resolve amplicons that differ by ≥2 base pairs. Two sets of seven synthetic DNA fragments ("resolution markers") were analyzed during this study. These fragments consist of dye-labeled amplicons of known size that are separated by 1bp within each set, with the two sets designed to bracket the upper (Large) and lower (Small) ends of the amplicon size range of the MSI markers (86–188bp).

Nine MSS tumor sample pairs, divided equally among endometrial, gastric and breast tumors, were amplified in duplicate using the OncoMate® MSI Dx Analysis System. Amplified products were then analyzed by capillary electrophoresis in the presence and absence of resolution markers on each of two nonconsecutive days. Across the nine samples, 36 replicates were analyzed in the presence of resolution markers and 36 replicates in the absence of resolution markers.

When two sets of resolution markers were analyzed by CE and actual sizes were characterized, the precision of DNA fragment size determination was sufficient to detect single-base-pair size differences across the amplicon size range (Table 34) Based on the study results, the OncoMate® MSI Dx Analysis System can resolve DNA fragments that differ by a 2-base deletion, the assay threshold for identifying marker instability.

Table 34. Absolute Differences in Observed Sizes of Resolution Markers.

Resolution Marker Pair	Known Size Difference (bp)	Observed Average Size Difference (bp)
Res_Large 1 and Res_Large 3	2.00bp	2.11
Res_Large 2 and Res_Large 4	2.00bp	2.01
Res_Large 3 and Res_Large 5	2.00bp	1.91
Res_Large 4 and Res_Large 6	2.00bp	2.02
Res_Large 5 and Res_Large 7	2.00bp	2.12
Res_Small 1 and Res_Small 3	2.00bp	2.37
Res_Small 2 and Res_Small 4	2.00bp	2.21
Res_Small 3 and Res_Small 5	2.00bp	2.06
Res_Small 4 and Res_Small 6	2.00bp	2.21
Res_Small 5 and Res_Small 7	2.00bp	2.34

10.1.9 Extracted DNA Stability

The stability of DNA purified from blood and FFPE tissue and stored at –30°C to –10°C was assessed for use in the OncoMate ® MSI Dx Analysis System by comparing MSI results generated at a baseline time point (Time 0) to multiple time points, including an assessment at 79 weeks.

DNA eluates evaluated during this study were purified from specimens associated with 6 MSI-H tumors and 12 MSS tumors. Matched normal FFPE samples were available for all tumors while matched normal blood samples were available for 16 of the 18 tumors evaluated (10 of the 12 MSS tumors). Each FFPE and blood sample was extracted using three DNA extraction systems in duplicate. In total, 96 MSI results were generated for matched normal blood samples and 108 MSI results were generated for matched normal FFPE tissue samples at each time point.

Across all individuals, sample sources and DNA extraction methods, 100% of MSI results at each time point, including the 105-week assessment, were concordant with Time 0 results. Therefore, across the five time points evaluated, 432 of 432 eluates tested using FFPE normal tissue and 384 of 384 eluates tested using blood normal tissue were concordant with Time 0 results. These results demonstrate that the OncoMate® MSI Dx Analysis System is compatible with DNA purified from FFPE and blood samples using various DNA extraction methods when stored for up to 18 months at –30°C to –10°C.

10.1.10 Slide Stability

The stability of FFPE tissue sections mounted on glass slides and stored at +20°C to +25°C for use with the OncoMate® MSI Dx Analysis System was assessed by comparing MSI results generated at a baseline time point (Time 0) to multiple time points, including an assessment at 105 weeks. At each time point, DNA was purified from two slides per sample of tumor and matched normal sections, and each purified DNA was amplified in duplicate using a single lot of the OncoMate® MSI Dx Analysis System. The study samples comprised four colorectal cancer sample pairs (two MSI-H and two MSS) and four endometrial cancer sample pairs (two MSI-H and two MSS), ranging from 30% to 70% tumor content.

After 105 weeks of slide storage at +20°C to +25°C, 100% of MSI results (160/160) were concordant with MSI results at Time 0, across all assessed time points. These results demonstrated the OncoMate® MSI Dx Analysis System can successfully determine MSI status of tumors from tissue stored on slides for up to 24 months.

10.2 Clinical Performance in Solid Tumors

A clinical bridging study was conducted to establish the validity of the OncoMate® MSI Dx Analysis System as a companion diagnostic (CDx) to identify patients with MSI-H solid tumors for treatment with pembrolizumab. Available banked specimens from KEYNOTE-158 and KEYNOTE-164 trials were retrospectively tested with the OncoMate® MSI Dx Analysis System. Clinical study results were then bridged to the OncoMate® MSI Dx Analysis System to estimate clinical efficacy and device concordance with the clinical trial assays (CTA). This bridging study included testing of tumor bank samples to establish concordance with a simulated CTA for negative samples.

10.2.1 Background: KEYNOTE-158 and KEYNOTE-164 Clinical Trials

KEYNOTE-158 is an ongoing multicenter, global, open-label trial of pembrolizumab in patients with multiple types of advanced (unresectable and/or metastatic) cancers who have failed prior therapy. All participants enrolled in this study had a histologically or cytologically documented, advanced solid tumor that was incurable and for which prior standard first-line treatment had failed. Participants had progressed on or were intolerant to therapies that are known to provide clinical benefit. KEYNOTE-164 is a completed single-arm, multisite, multicohort, Phase 2 study designed to evaluate the efficacy of pembrolizumab in previously treated patients with unresectable or metastatic MSI-H/dMMR CRC tumors. Local IHC or PCR assays were primarily used to enroll KEYNOTE-158 Cohort K and KEYNOTE-164 participants. All participants received pembrolizumab 200mg once every three weeks.

Primary objectives of KEYNOTE-158 and KEYNOTE-164 were to evaluate ORR per RECIST 1.1 (assessed by central imaging) to pembrolizumab. Secondary objectives included assessment of DOR, PFS and OS in the pembrolizumab-treated participants.

10.2.2 OncoMate® MSI Dx Analysis System Clinical Bridging Study Results

Samples from 404 participants from the KEYNOTE-158 Cohorts A to K (CDx Tested n= 149, 220) and KEYNOTE-164 (n=35) studies were available for testing and met OncoMate® MSI Dx Analysis System requirements. An additional 278 tumor bank specimens were tested to supplement the negative cohorts for estimating NPA. OncoMate® MSI Dx Analysis System data for all evaluable KEYNOTE and tumor bank samples with corresponding CTA results (n = 646) were used to estimate concordance between the CTA and the OncoMate® MSI Dx Analysis System. Evaluable tumors from KEYNOTE-158 Cohort K and KEYNOTE-164 participants (n = 179) collectively represent the CTA-positive efficacy population used in the bridging study to establish the clinical performance of the OncoMate® MSI Dx Analysis System. See Table 35 for sample accountability information.

Table 35. Sample Accountability for Clinical Bridging Study.

Study	N	CDx Tested	CDx-Evaluable	CDx-Positive	CDx-Negative
KN158 Cohort K	355	149	145	87	58
KN158 Cohorts A to J	1066	220	211	0	211
KN164	123	35	34	30	4
Tumor Bank	423	278	257	20	237
Total	**1967**	**682**	**647***	**137**	**510**

* One CDx-evaluable sample from the tumor bank cohort did not have a corresponding CTA result. Therefore, concordance was evaluated for 646 tumors.

Success Rate Analysis

The rate of valid test results (MSS or MSI-H result) with the OncoMate® MSI Dx Analysis System was determined by comparing the tested versus the evaluable participant samples from the clinical bridging study. Across the specimens tested, 94.9% (647/682) returned a valid MSS or MSI-H result. The OncoMate® MSI Dx Analysis Software identified 35 samples (5.1%, 35/682) with data quality issues attributable to the samples. Data quality issues included evidence of DNA contamination, mismatched normal and tumor DNA samples and low peak intensity associated with poor sample quality.

Concordance Analysis

To estimate concordance between the CTA and OncoMate® MSI Dx Analysis System, agreement between these methods was calculated using the 646 samples that yielded evaluable CTA (local IHC or PCR) and OncoMate® MSI Dx Analysis System results. Positive percent agreement (PPA) and negative percent agreement (NPA) were estimated along with 95% confidence intervals, using the CTA as the reference.

Across all tumor types, the PPA between MSI-H and dMMR results was 68.8% (137/199), with a 95% confidence interval of 62.1–74.9%. The NPA between MSS and pMMR results was 100.0% (447/447), with a 95% confidence interval of 99.1–100.0%. The overall percent agreement (OPA) was 90.4%, (584/646) with a 95% confidence interval of 87.9–92.4%. These results are shown in Table 36.

Table 36. Agreement Between CTA and Promega CDx, with CTA as Reference.

	CTA-Positive	CTA-Negative	Total
Promega CDx-Positive[1]	137	0	137
Promega CDx-Negative[2]	62	447	509
Total	199	447	646
PPA (95% CI)	**68.8 (62.1, 74.9)**		
NPA (95% CI)	**100.0 (99.1, 100.0)**		
OPA (95% CI)	**90.4 (87.9, 92.4)**		

[1]CTA-Positive includes samples from KEYNOTE-158 Cohort K, KEYNOTE-164 and tumor bank.

[2]CTA-Negative includes samples from KEYNOTE-158 Cohorts A–J and tumor bank.

Clinical Efficacy Results

The clinical validity of the OncoMate® MSI Dx Analysis System (CDx) to detect MSI-H status in patients with solid tumors was based on estimation of clinical efficacy in the CDx-positive, CTA-positive population. The major efficacy outcome measure was ORR per RECIST 1.1 as assessed by blinded independent central review (BICR) and the secondary efficacy outcome measure was DoR. ORR and DoR values for the CTA positive, CDx positive/CTA positive, CDx negative/CTA positive and CDx missing/CTA positive KEYNOTE study participants are presented in Table 37. Statistical testing was not performed on this population due to insufficient sample size; estimates and confidence intervals were used instead.

Treatment with pembrolizumab provided clinically meaningful benefit in the OncoMate® MSI Dx Analysis System MSI-H efficacy subpopulation, which consists of patients with advanced MSI-H cancers that have progressed on prior standard of care. The ORR in this subpopulation was 45.3% (95% CI: 36.1, 54.8), which is similar to both the ORR that formed the basis for pembrolizumab's authorization in this indication, where ORR was 33.8% (95% CI: 29.7, 38.1), and to the ORR determined for all CTA-Positive individuals, where ORR was 33.5% (95% CI: 29.3, 37.9). Conversely, only 3.2% (95% CI: 0.4, 11.2) of CDx-negative/CTA-positive participants responded to treatment with pembrolizumab.

Table 37. ORR and Duration of Response Estimates by OncoMate® MSI Dx Analysis System Status in KEYNOTE-158 and KEYNOTE-164 Participants Identified as MSI-H or dMMR by IHC- and PCR-based Clinical Trial Assays.

Clinical Outcome	All CTA-Positive (Total) (N=478)	CDx-Positive (MSI-H) and CTA-Positive (N=117)	CDx-Negative (MSS) and CTA-Positive (N=62)	CDx-Missing and CTA-Positive (N=299)
ORR (95% CI[a])	33.5% (29.3, 37.9)	45.3% (36.1, 54.8)	3.2% (0.4, 11.2)	35.1% (29.7, 40.8)
Complete Response	48 (10.0%)	14 (12.0%)	0 (0.0%)	34 (11.4%)
Partial Response	112 (23.4%)	39 (33.3%)	2 (3.2%)	71 (23.7%)
Duration of Response[b]	**N=160**	**N=53**	**N=2**	**N=105**
Median[c] in months (range)	63.2 (1.9+ – 63.2)	NR (3.6 – 61.0+)	NR (18.9 – 28.1+)	63.2 (1.9+ – 63.2)
n (%[c]) with duration ≥6 months	149 (94.9%)	49 (92.5%)	2 (100%)	98 (96.1%)
n (%[c]) with duration ≥12 months	123 (90.3%)	39 (88.4%)	2 (100%)	82 (91.1%)

[a] Based on binomial exact confidence interval method.

[b] Includes participants with confirmed complete response or partial response.

[c] From product-limit (Kaplan-Meier) method for censored data.

"+" indicates there is no progressive disease by the time of last disease assessment.

NR = Not Reached.

Database Cutoff Date: KEYNOTE-164: 2021-02-19, KEYNOTE-158: 2022-01-12

Sensitivity analysis was performed based on multiple imputation to assess the impact of missing OncoMate® MSI Dx Analysis System results from unavailable samples on the clinical efficacy. The analyses of the observed results and the sensitivity imputation show very similar efficacy estimates, supporting the robustness of the observed, primary analysis.

Response to KEYTRUDA for the CTA-positive, OncoMate® CDx-positive/CTA-positive, OncoMate® CDx-negative/CTA-positive and OncoMate® CDx-missing/CTA-positive efficacy subpopulations was analyzed by tumor type. Response rates by tumor type are provided in Table 38.

Efficacy results from the bridging study support the clinical performance of the OncoMate® MSI Dx Analysis System to identify patients with solid tumors that are MSI-H who might benefit from treatment with pembrolizumab.

Table 38. ORR Estimates per tumor Type by OncoMate® MSI Dx Analysis System Status in KEYNOTE-158 and KEYNOTE-164 Participants Identified as MSI-H or dMMR by IHC- and PCR-based Clinical Trial Assays.

Efficacy Subpopulation	All CTA-Positive (N=478)	CDx-Positive (MSI-H) and CTA-Positive (N=117)	CDx-Negative (MSS) and CTA-Positive (N=62)	CDx-Missing and CTA-Positive (N=299)
Tumor Type	**Responder (n) / Subpopulation (N), ORR% (95% CI)**			
Breast	1/13, 7.7% (0.2, 36.0)	1/2, 50.0% (1.3, 98.7)	0/4, 0.0% (0, 60.2)	0/7, 0.0% (0, 41.0)
Cholangiocarcinoma	9/22, 40.9% (20.7, 63.6)	3/4, 75.0% (19.4, 99.4)	0/2, 0.0% (0, 84.2)	6/16, 37.5% (15.2, 64.6)
CRC	42/123, 34.1% (25.8, 43.2)	14/30, 46.7% (28.3, 65.7)	0/4, 0.0% (0, 60.2)	28/89, 31.5% (22.0, 42.2)
Endometrial	42/83, 50.6% (39.4, 61.8)	16/33, 48.5% (30.8, 66.5)	0/3, 0.0% (0, 70.8)	26/47, 55.3% (40.1, 69.8)
Gastric	20/51, 39.2% (25.8, 53.9)	9/23, 39.1% (19.7, 61.5)	0/3, 0.0% (0, 70.8)	11/25, 44.0% (24.4, 65.1)
Glioma	1/21, 4.8% (0.1, 23.8)	0/3, 0.0% (0, 70.8)	1/6, 16.7% (0.4, 64.1)	0/12, 0.0% (0.0, 26.5)
NET	1/11, 9.1% (0.2, 41.3)	0/1, 0.0% (0, 97.5)	0/5, 0.0% (0, 52.2)	1/5, 20% (0.5, 71.6)
Ovarian	8/25, 32.0% (14.9, 53.5)	2/3, 66.7% (9.4, 99.2)	0/5, 0.0% (0, 52.2)	6/17, 35.3% (14.2, 61.7)
Pancreas	4/22, 18.2% (5.2, 40.3)	1/1, 100% (2.5, 100)	0/3, 0.0% (0, 70.8)	3/18, 16.7% (3.6, 41.4)
Sarcoma	3/14, 21.4% (4.7, 50.8)	N/A	0/8, 0.0% (0, 36.9)	3/6, 50.0% (11.8, 88.2)
Small intestine	16/27, 59.3% (38.8, 77.6)	5/7, 71.4% (29.0, 96.3)	0/1, 0.0% (0, 97.5)	11/19, 57.9% (33.5, 79.7)
Other*	13/66, 19.7% (10.9, 31.3)	2/10, 20.0% (2.5, 55.6)	1/18, 5.6% (0.1, 27.3)	10/38, 26.3% (13.4, 43.1)

* Other category includes those tumors that were recorded as "other" and all remaining tumor types with less than 10 samples in the CTA-positive (efficacy) population.

Database Cutoff Date: KEYNOTE 164: 2021-02-19, KEYNOTE 158: 2022-01-12

11. Troubleshooting

Troubleshooting guidance is organized according to QC failures reported in the OncoMate® MSI Dx Analysis software and corrective actions proposed to address these failures. (See Table 8, above, for a summary of QC tests performed by the software.) The Troubleshooting Sequence column provides the typical troubleshooting steps required to address the observed QC failures. For some QC failures, experienced users may be able to identify the root cause of the problem and skip some steps provided in the general troubleshooting sequence. For some samples, multiple rounds of troubleshooting may be required to obtain a valid test result. Any time a sample undergoes troubleshooting, the matched normal or tumor sample and the Positive and Negative Amplification Controls must also be reanalyzed (see Section 7, Assay Quality Controls). When a Positive or Negative Control fails any QC test, the entire batch of samples must also undergo troubleshooting and reanalysis. Note that an "ID Failed" result for pentanucleotide markers will be reported by the software for any QC failure affecting the software's ability to confidently assess sample identity (e.g., DNA Contamination QC Test Failure). Refer to Table 8 for details on specific QC failures that affect Pentanucleotide markers.



See the *Applied Biosystems® 3500 Dx Genetic Analyzer and 3500xL Dx Genetic Analyzer IVD User Guide* for more information about resolving instrument-related problems and correcting mistakes that occur when entering sample information into the instrument's data collection software (DCS). For questions not addressed in this section, consult the other technical manuals associated with the OncoMate® MSI Dx Analysis System (listed in Section 1.2).

If the General Troubleshooting Sequence does not resolve a QC failure, then contact Promega Technical Services for additional guidance. The quality of some samples may be too poor to obtain a valid test result.

Contact information available at: **www.promega.com**; email: **genetic@promega.com**.

Quality Failures and Observations	Possible Causes	Troubleshooting Sequence
Import Failure reported by the OncoMate® MSI Dx Analysis Software pop-up window Samples that were analyzed during the capillary electrophoresis run were not imported into the OncoMate® MSI Dx Analysis Software.	Sample or control information was not entered correctly (i.e., following required conventions) when setting up the run in the Applied Biosystems® 3500 Dx Data Collection Software (DCS). Multiple sample pairs with the same sample name were present in the same batch (e.g., due to re-injection or analysis of identically named replicates). The required controls were not included with the imported batch.	1. Verify that each sample pair has a unique sample name and that amplification controls and patient samples were identified correctly in the Sample Name, Sample Type and UDF1 fields in the Applied Biosystems® 3500 Dx Genetic Analyzer DCS. Refer to Step 6 of Section 4.9 for more information. Open the .fsa files using the 3500 Dx DCS to correct any errors that are discovered using the Rename option, and then save the updated .fsa files. Repeat data analysis, archiving and re-importing the batch, if needed. 2. If the failure wasn't resolved in Step 2, verify that positive and negative controls from the same instrument run and amplification are included in the batch. Repeat any workflow steps required to ensure that positive and negative controls are included in the PCR and capillary electrophoresis runs and are present for import with the batch.

Quality Failures and Observations	Possible Causes	Troubleshooting Sequence
Sizing Quality QC Test Failure Invalid sample result obtained. Size Standard 500 fragments showed low resolution, fewer fragments than expected, unexpected peaks, low peak intensity or excessive peak intensity.	Poor quality capillary electrophoresis injection (Size Standard peaks are missing, have unexpected shape, or demonstrate decreasing peak height with increasing fragment length). One or more bleedthrough peaks from MSI marker peaks were present in the Size Standard 500 dye channel, preventing accurate identification of size standard peaks. Artifacts were present, preventing accurate identification of the Size Standard 500 peaks (e.g., contaminants or crystal deposits were present in the polymer, resulting in capillary electrophoresis artifacts). Incorrect amount of Size Standard 500 in loading cocktail resulted in Size Standard 500 peaks that were too intense (>10,000RFU) or below the system analytical threshold (<175RFU). The typical range for Size Standard 500 fragment peak intensities is approximately 300 to 1,500 RFU.	1. Identify the root cause of the Sizing Quality failure by reviewing the size standard electropherogram for the failing sample in the OncoMate® MSI Dx Analysis Software. Size standard data are viewed by expanding the Size Standard area of the Results View. Reviewing the sample electropherogram using the Applied Biosystems® 3500 Dx Genetic Analyzer Data Collection Software (DCS) may aid in identifying the root cause for Sizing Quality failures. 2. If the Sizing Quality failure can be attributed to bleedthrough from an off-scale marker peak, resolve the off-scale issue first using the Off-Scale QC Test failure troubleshooting section (see the section below). Otherwise, proceed to Step 3. 3. Repeat capillary electrophoresis and data analysis for the affected sample pair(s) and include positive and negative controls. Ensure that the capillary electrophoresis loading cocktail is prepared correctly and that the heat denaturation steps are accurately followed. 4. If the failure was not resolved in Step 3, then repeat PCR amplification of the affected sample pair(s) and include freshly prepared positive and negative controls. **Note:** Observing multiple Sizing Quality failures in a given batch, or an increase in the frequency of observations across batches, may indicate a broader instrumentation issue. See the *Applied Biosystems® 3500 Dx Genetic Analyzer and 3500xL Dx Genetic Analyzer IVD User Guide* for more information about resolving instrument-related problems leading to artifact peaks or reduced peak resolution. If issues are not resolved through routine instrument maintenance, contact Thermo Fisher Scientific Technical Support.

Quality Failures and Observations	Possible Causes	Troubleshooting Sequence
Off Scale QC Test Failure Invalid sample result obtained. Fluorescent signal for marker peaks exceeds the dynamic range of the Applied Biosystems® 3500 Dx Genetic Analyzer.	Too much sample DNA was added to the PCR reaction (one or more markers have off-scale peaks and peak heights are generally high for markers that are not off scale). FFPE sample DNA was degraded or cross linked (observed preferential amplification of small bp amplicons). If an off-scale peak is observed in the positive control, the 2800M Control DNA stock solution or dilution was not vortexed sufficiently, or an error was made when diluting the 2800M Control DNA.	1. If an Off-Scale QC Test failure was observed for a patient sample, proceed to Step 2. If an Off-Scale QC Test failure is observed for the positive control 2800M Control DNA, repeat PCR amplification of the entire batch with a freshly prepared positive control. Ensure that the 2800M Control DNA stock and dilutions are prepared and mixed accurately. 2. Dilute amplification product of the off-scale sample 1:8 in loading cocktail (e.g., 1µl of amplification product plus 7µl of loading cocktail) and repeat capillary electrophoresis analysis using 1µl of the diluted amplification product. Reinject the undiluted paired sample and the positive and negative controls during off-scale sample troubleshooting. 3. If the failure was not resolved in Step 2, then repeat PCR of the off-scale sample with less DNA. Starting with 0.5ng of DNA is recommended but a lower DNA input and additional rounds of PCR troubleshooting may be necessary. Be sure to also repeat PCR for the paired sample (at the original DNA input if suitable signal was present) and for freshly prepared positive and negative amplification controls. 4. If the failure was not resolved in Step 3, then extract DNA from a different slide or section and repeat the sample analysis workflow from PCR through data analysis. **Note:** Frequent Off-Scale QC Test failures may indicate a problem with accuracy of quantitation or dilution. Off-Scale Peaks observed with degraded samples may indicate that sample quality is insufficient for this test. If excessive differences in peak height are routinely observed between smaller (e.g., NR-21) and larger (e.g., Penta C) markers, preparation conditions for FFPE samples and/or storage conditions for FFPE DNA extracts may require adjustment to ensure test compatibility.

Quality Failures and Observations	Possible Causes	Troubleshooting Sequence
Spectral Pull-Up QC Test Failure Invalid sample result obtained. Bleedthrough peaks detected in one or more dye channels.	Poor quality or incorrect spectral calibration. Excessive peak heights observed for a marker in a neighboring dye channel, resulting in bleedthrough.	1. If the Spectral Pull-Up QC Test failure is observed with an Off-Scale Peak QC Test failure, troubleshoot the Off-Scale QC Test failure first. 2. If no associated off-scale peaks are present, repeat spatial and spectral calibration of the instrument. 3. Following the new calibrations, repeat capillary electrophoresis and data analysis for the affected sample pair(s) and include positive and negative controls. **Note:** Be sure to repeat the spectral calibration anytime the instrument is serviced by the manufacturer. Frequent Spectral Pull Up QC Test failures may indicate that additional capillary electrophoresis or assay troubleshooting is required.

Quality Failures and Observations	Possible Causes	Troubleshooting Sequence
Broad Peak QC Test Failure Invalid sample result obtained. Width of marker peak(s) exceed the valued allowed for MSI analysis.	PCR-related artifacts (e.g., due to elevated baseline in BAT-26 with too much DNA input). Capillary electrophoresis-related artifacts (e.g., due to instrument consumables or maintenance issues). Polymer was not at room temperature before installation; the capillary electrophoresis instrument was not adequately preheated.	1. Ensure that a) all manufacturer-recommended capillary electrophoresis instrument maintenance and washes have been performed, b) the array and consumables are within the manufacturer recommended usage, and c) the instrument is preheated following the manufactures' recommendations before starting the run. Then, repeat capillary electrophoresis for the affected sample pair(s) and include positive and negative controls. 2. If failure was not resolved in Step 1, repeat PCR amplification of the affected sample pair(s), including freshly prepared positive and negative controls, and repeat the sample analysis workflow through data analysis.* 3. If failure was not resolved in Step 2, then extract DNA from a different slide or section and repeat the sample analysis workflow through DNA analysis. *When FFPE DNA is highly fragmented or DNA input is excessive, the BAT-26 marker baseline signal may be affected and broad and/or unexpected peaks may be observed. See Section 6.4 for more information on how to identify this and other known artifacts. If this type of artifact is observed, repeat amplification of the affected sample with less DNA. We recommend starting with 0.5ng of DNA, but a lower DNA input and additional rounds of PCR troubleshooting may be necessary.

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Quality Failures and Observations	Possible Causes	Troubleshooting Sequence
Unexpected Peak(s) QC Test Failure Invalid sample results obtained. For mononucleotide-repeat markers, DNA fragments or assay artifacts indicating instability were detected in the normal sample.	Capillary electrophoresis-related artifacts (e.g., due to instrument consumables or maintenance issues). PCR-related artifacts (e.g., due to elevated baseline in BAT-26 with too much DNA input). The normal patient sample was contaminated with another template DNA or amplified product. A patient's tumor sample exhibiting one or more unstable markers was mistakenly identified as the normal sample during sample handling or capillary electrophoresis plate set up. The normal sample peak intensity is much higher than the tumor sample peak intensity.	1. Verify that amplification controls and patient samples were identified correctly and entered in the expected order in the Sample Name, Sample Type and UDF1 fields of the Applied Biosystems® 3500 Dx Genetic Analyzer DCS. Open the .fsa files using the 3500 Dx DCS to correct any errors that are discovered using the Rename option, and then save the updated .fsa files. Repeat data analysis, archiving and re-importing the batch, if needed. 2. If no errors in sample identification were detected in Step 1, repeat capillary electrophoresis for the affected sample pair(s) and include positive and negative controls. 3. If the failure was not resolved in Step 2, then repeat PCR amplification of the affected sample pair(s), including freshly prepared positive and negative controls, and repeat the sample analysis workflow through data analysis. If the normal sample peak intensity is much higher than the tumor sample peak intensity, decrease the normal DNA input or increase the tumor DNA input used for PCR. Adjusting the DNA input for both the normal and tumor sample may be necessary.* 4. If the failure was not resolved in Step 3, then extract DNA from a different slide or section and repeat the sample analysis workflow from PCR through data analysis. *When FFPE DNA is highly fragmented or DNA input is excessive, the baseline signal in the BAT-26 marker may be affected and broad and/or unexpected peaks may be observed. See Section 6.4 for more information on how to identify this and other known artifacts. If this type of artifact is observed, it may be necessary to repeat amplification of the affected sample with less DNA. We recommend starting with 0.5ng of DNA, but a lower DNA input and additional rounds of PCR troubleshooting may be necessary.

Quality Failures and Observations	Possible Causes	Troubleshooting Sequence
Pentanucleotide Amplification QC Test Failure Invalid sample and "ID Failed" marker results observed. No marker peaks detected above the 175RFU analytical threshold. **or** **Mononucleotide Amplification QC Test Failure** No Call marker result(s) observed. No marker peaks detected above the 175RFU analytical threshold. **or** **Marker Signal QC Test Failure** No Call marker result(s) observed. For mononucleotide markers that have been tentatively interpreted as stable, marker peak height(s) are <700RFU.	Not enough DNA in amplification reaction (e.g., degraded sample, incorrect quantitation, dilution, poor sample mixing, user error pipetting or poorly seated pipette tip). 2800M Control DNA stock solution or dilution was not vortexed sufficiently, or an error was made when diluting the 2800M Control DNA.* The presence of PCR inhibitors in the DNA sample (e.g., high salt or altered pH). Amplified product was not added to injection plate (no peaks are visible for any marker; size standard peak heights are more than approximately 2,000RFU). Amplification failure (e.g., thermal cycler problem, user error during PCR amplification mix set up)* *2800M Control DNA positive amplification control also will likely be affected if these are the cause.	1. Verify that HiDi™ formamide was used and stored properly, patient sample DNA was diluted in amplification-grade water, thermal cycler equipment meets the requirements in Section 3, and thermal cycling conditions are correct. Make any required corrections and proceed to Step 2. 2. If Marker Amplification QC failure resulted from not adding amplified product to the capillary electrophoresis loading cocktail (no peaks are visible for any marker and size standard peak heights are greater than approximately 2,000RFU), then repeat capillary electrophoresis and data analysis for the affected sample pair(s) and include a positive and negative control. When amplified product was not added to the intended well of the CE plate, scrutinize batch results to verify that the sample was not accidentally added to the incorrect well. Dispensing amplified product into the wrong well during capillary electrophoresis setup may lead to results that are incorrect or that return a DNA Contamination QC Test Failure. If you suspect that a pipetting error occurred, assemble a new CE plate with the affected samples and positive and negative controls and repeat CE analysis. Otherwise, proceed to Step 3. 3. If Marker Amplification QC failure is observed for the 2800M positive control, repeat PCR amplification, capillary electrophoresis, and data analysis of the entire batch with a freshly prepared positive and negative control. Otherwise, proceed to Step 4. (continued below)

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Quality Failures and Observations	Possible Causes	Troubleshooting Sequence
Pentanucleotide Amplification QC Test Failure **or** **Mononucleotide Amplification QC Test Failure** **or** **Marker Signal QC Test Failure** **(continued)**		4. If the failure was not resolved in Step 3, then repeat PCR amplification of the low-peak-height sample(s) with more DNA input (e.g., 2ng). Also repeat PCR for the paired sample (at the original DNA input if suitable signal was present) and freshly prepared positive and negative amplification controls. Repeat capillary electrophoresis and data analysis. For some samples, a higher DNA input and additional rounds of PCR troubleshooting may be necessary. 5. If the failure was not resolved in Step 4, then extract DNA from a different slide or section and repeat the sample analysis workflow from PCR through data analysis. **Note:** Frequent failures of this type may indicate a sample quality or quantitation issue. Some samples may not have sufficient DNA quality for analysis with the OncoMate® MSI Dx Analysis System. If excessive differences in peak height between smaller (e.g., NR-21) and larger (e.g., Penta C) markers or low peak heights are routinely observed, preparation conditions for FFPE samples and/or storage conditions for FFPE DNA extracts may require adjustment to ensure test compatibility. Matched normal and tumor FFPE samples typically have similar peak intensities. If very low peak intensity is observed for one member of the pair but not the other, this may indicate a user error during PCR or CE setup.

Quality Failures and Observations	Possible Causes	Troubleshooting Sequence
Intensity Difference QC Test Failure No Call marker result(s) observed. For mononucleotide markers, the normal and tumor signal intensities were not similar enough to ensure assay accuracy.	A combination of root causes may lead to divergent peak height between the normal and tumor samples for a given marker. For example, a highly fragmented or poor-quality sample was paired with a high-quality sample (e.g., an FFPE tumor sample with poor quality is paired with a blood normal sample). For more information, see possible causes and notes for Amplification QC and Marker Signal QC Test Failures and Off Scale QC Test Failure.	1. Observe the electropherograms for the normal and tumor samples with the "Normalized" option deselected. If the relative peak intensity between the sample pair is less than approximately 20×, proceed to Step 2. Otherwise, proceed to Step 3. 2. Dilute amplification product for the sample with higher peak intensity 1:8 in loading cocktail (e.g., 1µl of amplification product plus 7µl of loading cocktail) and repeat capillary electrophoresis analysis using 1µl of the diluted amplification product. Reinject the undiluted paired sample and the positive and negative controls during troubleshooting. If this step does not to resolve the Intensity Difference QC failure, proceed to Step 3. 3. Repeat PCR amplification of the sample pair, adjusting the DNA input for one or both paired patient samples to decrease the differences in peak height observed for the affected marker(s). Include freshly prepared positive and negative controls during repeated PCR. Repeat capillary electrophoresis and data analysis. If initial troubleshooting is unsuccessful, additional rounds of PCR with modified DNA inputs may be necessary to resolve the QC failure. **Note:** Peak intensity (RFU) can be viewed by unchecking the "Normalized" box when viewing sample electropherogram data in the OncoMate® MSI Dx Analysis Software. Some samples may not have sufficient DNA quality for analysis with the OncoMate® MSI Dx Analysis System. Frequent failures due to low peak heights may indicate a sample quality or quantitation issue.

Quality Failures and Observations	Possible Causes	Troubleshooting Sequence
Patient Sample Identity QC Test Failure Invalid Sample and "ID Failed" Marker result observed. For each pentanucleotide marker, the alleles identified in the normal sample must be present in the tumor sample.	Normal and tumor DNA samples from different individuals were analyzed as a sample pair. Loss of heterozygosity (LOH) in the tumor sample has caused one of the pentanucleotide alleles to be absent or filtered as stutter by the software. The normal and tumor samples for the patient were inadvertently switched during sample analysis or incorrectly identified during the capillary electrophoresis plate set up. For a homozygous pentanucleotide marker, an artifact or contaminant was recognized by the software as a pentanucleotide allele in the normal sample.	1. Verify that amplification controls and patient samples were identified correctly and entered in the expected order in the Sample Name, Sample Type and UDF1 fields of the Applied Biosystems® 3500 Dx Genetic Analyzer DCS. Open the .fsa files using the 3500 Dx DCS, correct any errors that are discovered using the Rename option, and save the updated .fsa files. Repeat data analysis, archiving and re-importing the batch, if needed. 2. If no errors in sample identification were discovered in Step 1, repeat PCR amplification of the affected sample pair(s).* Include freshly prepared positive and negative controls. Repeat capillary electrophoresis and data analysis. *If the identity failure resulted from low peak height(s) (e.g., due to LOH in the tumor sample), add more DNA (e.g., 2ng) to the amplification reaction for the low-peak-height sample(s). Additional rounds of amplification with higher DNA inputs may be necessary to resolve the QC failure. 3. If the failure was not resolved in Step 2, then extract DNA from a different slide or section and repeat the sample analysis workflow from PCR through data analysis. **Note:** For some samples exhibiting LOH, confirming identity may not be possible and sample testing using an orthogonal method may be necessary.

Quality Failures and Observations	Possible Causes	Troubleshooting Sequence
DNA Contamination QC Test Failure Invalid Sample and "ID Failed" marker result observed. For a pentanucleotide marker, three or more alleles were detected in the normal sample or positive control.	A tumor DNA sample exhibiting pentanucleotide marker instability was assigned as a normal sample. An artifact was recognized by the software as a pentanucleotide allele in the normal sample or Positive Control. The normal patient sample or positive control was contaminated with another template DNA or amplified product. Septa in capillary electrophoresis instrument were contaminated by material from a previous injection.	1. Verify that amplification controls and patient samples were identified correctly and entered in the expected order in the Sample Name, Sample Type and UDF1 fields of the Applied Biosystems® 3500 Dx Genetic Analyzer DCS. Open the .fsa files using the 3500 Dx DCS, correct any errors that are discovered using the Rename option and save the updated .fsa files. Repeat data analysis, archiving and re-importing the batch, if needed. 2. If no errors in sample identification were discovered in Step 1, repeat capillary electrophoresis for affected sample pair(s) and include positive and negative controls. Replacing the instrument consumables, including septa, may be necessary. 3. If the failure was not resolved in Step 2, repeat PCR amplification of the affected sample pair(s) and include freshly prepared positive and negative controls. Repeat capillary electrophoresis and data analysis. 4. If the failure was not resolved in Step 3, then extract DNA from a different slide or section and repeat the sample analysis workflow from PCR through data analysis.
Control Identity QC Test Failure Invalid sample result obtained for all samples in batch. For the positive amplification control, the alleles present in the pentanucleotide markers did not match the expected alleles for the 2800M Control DNA.	A DNA sample other than 2800M Control DNA was amplified or assigned as the positive amplification control.	**Note:** Use aerosol-resistant pipette tips and change gloves regularly. 1. Verify that amplification controls and patient samples were identified correctly and entered in the expected order in the Sample Name and Sample Type fields of the Applied Biosystems® 3500 Dx Genetic Analyzer DCS. Open the .fsa files using the 3500 Dx DCS, correct any errors that are discovered using the Rename option, and save the updated .fsa files. Repeat data analysis, archiving and re-importing the batch, if needed. 2. If no errors in sample identification were discovered in Step 1, repeat PCR amplification for all controls and samples in the batch. Ensure that 2800M Control DNA is used as the positive control.

Quality Failures and Observations	Possible Causes	Troubleshooting Sequence
Negative Control QC Test Failure Invalid sample result obtained for all samples in batch. One or more peaks were detected above the 175RFU analytical threshold in the negative amplification control reaction.	A sample other than no-template control was amplified or assigned as the negative amplification control. The negative control PCR amplification was contaminated with DNA. The negative control capillary electrophoresis reaction was contaminated with amplified product. A capillary electrophoresis artifact was identified as a peak in the negative control.	1. Verify that amplification controls and patient samples were identified correctly and entered in the expected order in the Sample Name, Sample Type and UDF1 fields of the Applied Biosystems® 3500 Dx Genetic Analyzer DCS. Open the .fsa files using the 3500 Dx DCS, correct any errors that are discovered using the Rename option and save the updated .fsa files. Repeat data analysis, archiving and re-importing the batch, if needed. 2. If no errors in sample identification were discovered in Step 1, repeat capillary electrophoresis for the entire batch. 3. If failure was not resolved in Step 2, repeat PCR amplification for all controls and samples in the batch. Ensure that nuclease free water or equivalent is used as the negative control. If any reagents or consumables are suspected to be contaminated, replace them prior to repeating PCR.

12. References

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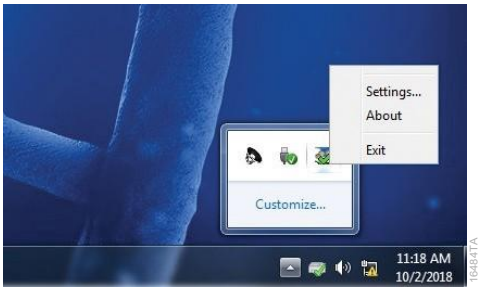
13. Additional Information

For technical assistance, call Promega Technical Services at: 1-800-356-9526 (toll-free) or 608-274-4330 or email: **genetic@promega.com**

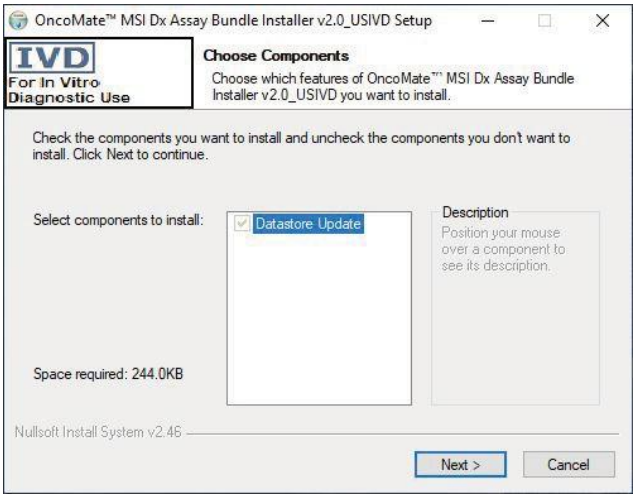
14. Appendix

14.1 OncoMate® MSI Dx Assay Installation

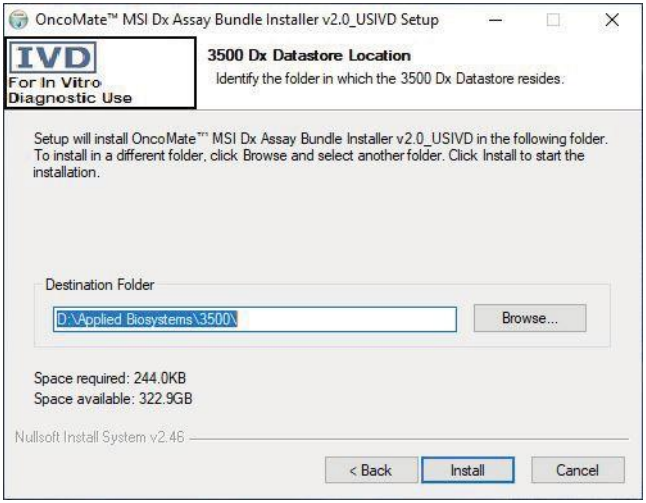
1. Copy the OncoMate_MSI_Dx_Assay_v2.0_IUO_Installer.exe file to a folder on the target computer system or a memory stick.
2. Exit the Applied Biosystems® 3500 Series Data Collection Software.
3. Exit the services started by the data collection software by opening the system tray, right clicking the 3500 Server Monitor (the icon with a bright-green check mark) and selecting **Exit** from the pop-up menu. This process will take approximately 30 seconds to complete.



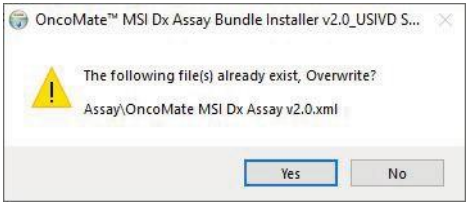
4. Double-click the OncoMate MSI Dx Assay Bundle Installer v2.0_IUO.exe file to begin assay installation.
5. When the following window appears, select **Next >**:



6. The destination folder for the Assay will be displayed. Select **Install**:

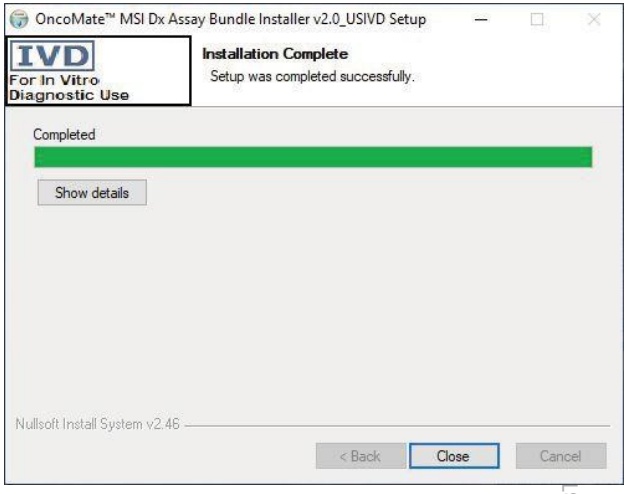


Note: If the Assay was previously installed, the following window will be displayed. Select **Yes** to reinstall the assay:



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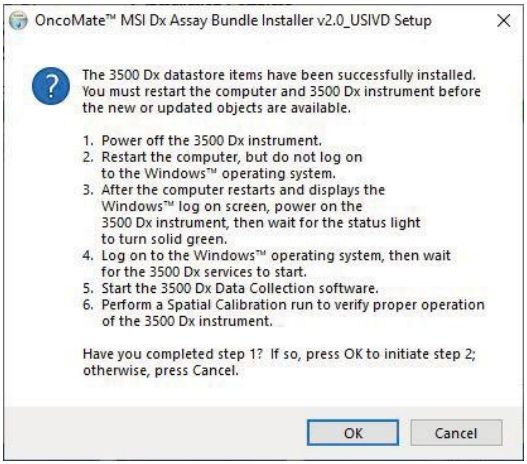
8. When the installation is completed, the following window will be displayed. Select **Close**:



19748T

9. A full system reboot is now required, and a window describing the reboot process will appear. Select **OK** and follow the system reboot instructions, or choose **Cancel** and you will be reminded to restart the system later.

   Note: After selecting **OK**, the system computer will automatically restart.



The Assay installation is logged in the file:

D:\Applied Biosystems\3500\OncoMate® MSI Dx Assay Bundle Installer v2.0_IUO.log

Promega

OncoMate® MSI Dx Analysis System (Part 2)

Part 2 (Sections 15–27) is for use as an aid to identify candidates for Lynch syndrome testing.

Promega

15. Introduction

15.1 About This Guide

This guide describes the OncoMate® MSI Dx Analysis System and is the primary source for information about its intended use, components, limitations, protocol, troubleshooting and more. The assay workflow comprises several components, which are used together to analyze microsatellite instability in formalin-fixed, paraffin-embedded (FFPE) colorectal cancer (CRC) tissue samples: DNA isolated from normal and tumor FFPE tissue samples using the Maxwell® CSC Instrument (Cat.# AS6000) and Maxwell® CSC DNA FFPE Kit (Cat.# AS1350); a dye-based DNA quantification system; the OncoMate® 5C Matrix Standard (Cat.# MD4850); the OncoMate® MSI Dx Analysis System (Cat.# MD2140) for amplification; the Applied Biosystems® 3500 Dx Genetic Analyzer (Thermo Fisher Scientific Cat.# A46344); and the OncoMate® MSI Dx Interpretive Software (Cat.# MD4140).

While this guide provides an overview of the entire assay workflow, emphasis is given to the keystone component of the assay: the OncoMate® MSI Dx Analysis System amplification kit. Section 18, Assay Protocol, includes the procedures required for DNA extraction and quantification, and step-by-step instructions for amplifying sample DNA using the OncoMate® MSI Dx Analysis System. Detailed instructions are also provided in Section 18 to analyze amplified products by capillary electrophoresis and in Sections 19 and 20 to analyze and interpret capillary electrophoresis data using the OncoMate® MSI Dx Interpretive Software. Information on specific functions and capabilities of the OncoMate® MSI Dx Interpretive Software are found in the *OncoMate® MSI Dx Interpretive Software Reference Manual* #TM554.

The following technical manuals contain instructions for the use of associated assay components:

- *Maxwell® CSC Instrument Operating Manual* #TM457
- *Maxwell® CSC DNA FFPE Kit Technical Manual* #TM395
- *OncoMate® MSI Dx Interpretive Software Reference Manual* #TM554
- *OncoMate® 5C Matrix Standard Technical Manual* #TM542
- *Applied Biosystems® 3500 Dx Genetic Analyzer and 3500xL Dx Genetic Analyzer IVD User Guide* (Part #100070881)
- Technical literature for the user-selected, fluorescence-based double-stranded DNA quantification system

15.2 Product Name

OncoMate® MSI Dx Analysis System

Cat.# MD2140, 100 reactions Σ_{100}

Common Name
Fluorescent, multiplex PCR reagents

15.3 Abbreviations

bp, base pair

CE, capillary electrophoresis

CRC, colorectal cancer

DCS, data collection software for the Applied Biosystems® 3500 Dx Genetic Analyzer

dsDNA, double-stranded DNA

FFPE, formalin-fixed, paraffin-embedded

HNPCC, hereditary nonpolyposis colorectal cancer

MMR, mismatch repair

MSI, microsatellite instability

MSI-H, microsatellite instability high

MSS, microsatellite stable

PCR, polymerase chain reaction

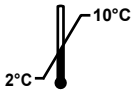
RFU, relative fluorescence unit

QC, quality control

UDF1, user-defined field 1

NA, not applicable

15.4 Key to Symbols Used

Symbol	Explanation	Symbol	Explanation
	In Vitro Diagnostic Medical Device		Protect from light
	Store at 2°C to 10°C		Manufacturer
	Caution		Irritant
	Use by		Contains sufficient for <n> tests
	Do not reuse		Warning. Biohazard
	Consult instructions for use		Catalog number
	Lot number		Serial number

15.5 Intended Use

The OncoMate® MSI Dx Analysis System is a qualitative multiplex polymerase chain reaction (PCR) test intended to detect the deletion of mononucleotides in five microsatellite loci (BAT-25, BAT-26, NR-21, NR-24 and MONO-27) using matched tumor and normal DNA obtained from formalin fixed, paraffin-embedded (FFPE) colorectal tissue sections. The OncoMate® MSI Dx Analysis System is for use with the Applied Biosystems® 3500 Dx Genetic Analyzer and OncoMate® MSI Dx Interpretive Software.

The OncoMate® MSI Dx Analysis System is indicated in patients diagnosed with colorectal cancer (CRC) to detect microsatellite instability (MSI) as an aid in the identification of probable Lynch syndrome to help identify patients that would benefit from additional genetic testing to diagnose Lynch syndrome.

Results from the OncoMate® MSI Dx Analysis System should be interpreted by healthcare professionals in conjunction with other clinical findings, family history and other laboratory data.

The clinical performance of this device to guide treatment decision for MSI-high patients has not been established.

15.6 Summary and Explanation

Lynch syndrome, also called hereditary nonpolyposis colorectal cancer (HNPCC), is an inherited disorder that increases the risk of many types of cancer, particularly cancers of the colon and rectum (1,2). Lynch syndrome accounts for approximately 3% of colorectal cancers (CRC) and is caused by autosomal-dominant germline mutations in DNA mismatch repair (MMR) genes (1,3,4). These mutations impair cellular MMR function, such that mutations introduced during normal cellular DNA replication are not properly repaired. The accumulation of mutations may lead to cellular dysfunction and, eventually, cancer (5–7). Identification of individuals with Lynch syndrome offers an understanding of future cancer susceptibility and an opportunity for increased cancer surveillance. Family members of that individual also may undergo increased medical surveillance or testing for Lynch syndrome (1,8). DNA sequencing to identify pathological mutations in the MMR genes is the definitive diagnostic test for Lynch syndrome (2,5).

Microsatellite instability testing cannot be used to diagnose Lynch syndrome. Instead, MSI testing is a rapid and cost-effective method to identify MMR deficiency in CRC tumor cells (2,4,8,9). Microsatellites are short, DNA-repeat regions that are naturally prone to DNA replication errors that alter (typically shorten) their length. MSI is observed when MMR function is compromised, and errors made during DNA replication are not repaired. As a result, the length of microsatellite alleles may differ in MMR-deficient versus normal tissue samples (5,9–11). CRC patients identified as MSI high (MSI-H) by MSI testing may have Lynch syndrome and are therefore candidates for DNA sequencing to determine whether they have germline mutations in MMR genes (2–4,8). Many professional groups and institutions, including the National Comprehensive Cancer Network (NCCN), endorse universal MMR or MSI testing in all patients with a personal history of colon or rectal cancer to determine which patients should have genetic testing for Lynch syndrome (2,8,12–15).

The OncoMate® MSI Dx Analysis System encompasses a complete workflow for MSI determination, from DNA extraction to data analysis (Figure 19). First, DNA is extracted from FFPE colorectal tissue samples (normal and tumor from the same patient) using the Maxwell® CSC DNA FFPE Kit and Maxwell® CSC Instrument. Double-stranded DNA (dsDNA) is then quantified using a fluorescence-based dsDNA quantification system of your choice. Next, amplification products are generated through multiplex PCR amplification of DNA microsatellite markers using the OncoMate® MSI Dx Analysis System amplification kit. The PCR products are then mixed with Hi-Di™ Formamide and Size Standard 500 and heat-denatured. The resulting single-stranded DNA fragments are separated by size and detected via fluorescence using an Applied Biosystems® 3500 Dx Genetic Analyzer. Following capillary electrophoresis (CE), allele sizes from the CRC tumor DNA and the normal DNA are calculated and compared for each of the microsatellite markers using OncoMate® MSI Dx Interpretive Software. If the length of two or more of the five mononucleotide-repeat marker alleles is changed by ≥2.75 base pairs (bp), the tumor is classified as MSI-H; if the allele length is changed for only one marker, or if the difference in allele lengths at the five markers is <2.75bp, the tumor is classified as microsatellite stable (MSS). The sizes of the Penta C and Penta D pentanucleotide-repeat marker alleles are compared as an identity check between the normal and tumor DNA samples.

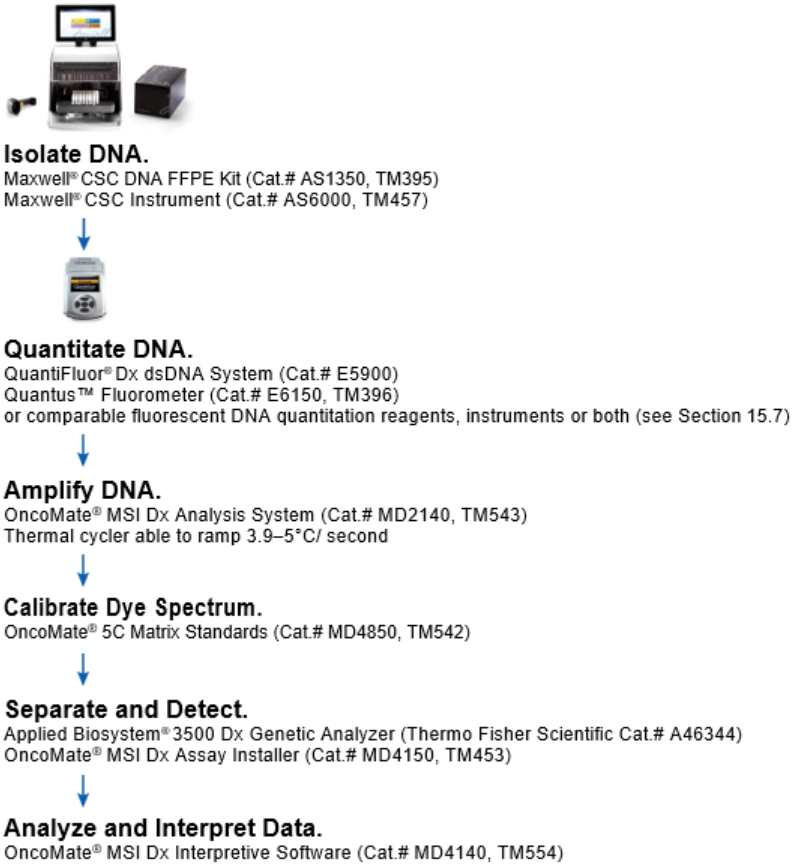


Figure 19. OncoMate® MSI Dx Analysis System assay workflow for identifying Lynch syndrome testing candidates.

15.7 Principles of the Procedure

The OncoMate® MSI Dx Analysis System workflow involves DNA extraction from FFPE tissue samples, quantification of double-stranded DNA (dsDNA) using a fluorescent dsDNA-binding dye, amplification of specific microsatellite markers via multiplex PCR, analysis of amplified DNA fragments by capillary electrophoresis and analysis of CE fragment data using an interpretive software. This section reviews the technical foundation for each of these steps in the assay workflow.

Prior to DNA extraction with the Maxwell® CSC Instrument, the FFPE tissue section is manually preprocessed with lysis buffer, proteinase K, mineral oil and heat. Initial incubation of the sample at 56°C with proteinase K deparaffinizes the sample, digests proteins and releases nucleic acids. A subsequent 80°C incubation acts to release nucleic acids crosslinked to each other and to proteinaceous components. A room-temperature RNase treatment of the lysate digests RNA from the sample. Samples are centrifuged to separate aqueous and mineral oil/paraffin phases, and the aqueous phase is transferred to the Maxwell® CSC Instrument for nucleic acid binding, washing and elution into Nuclease-Free Water. Extraction of dsDNA from FFPE samples is performed using the Maxwell® CSC DNA FFPE Kit. The Maxwell® CSC Instrument extracts nucleic acids using paramagnetic particles, which provide a mobile solid phase to capture, wash and elute dsDNA. This system efficiently binds dsDNA to paramagnetic particles in the first well of a cartridge prefilled with purification reagents. The bound nucleic acid is then moved through distinct wells of the cartridge, mixing during processing. Once eluted, the extracted dsDNA is ready for analysis.

DNA extracts are then quantified using fluorescent DNA-binding dyes. Fluorescent DNA-binding dyes enable the sensitive quantitation of small amounts of DNA in a purified sample. These dyes are selective for dsDNA, and the signal is linear over a wide range of DNA inputs. Prior to analysis, fluorescent dyes are diluted and mixed with DNA and control samples, and DNA binding occurs within minutes. A standard curve is prepared and analyzed in parallel with the dye-stained dsDNA, and fluorescence is measured using a compatible fluorometer. The results of this analysis determine DNA concentration and inform the sample volume requirement for subsequent PCR analysis.

PCR is an enzymatically driven and temperature-dependent in vitro method to amplify specific, targeted tracts of DNA from a broader DNA sample. During PCR, short DNA sequences (primers) bind to flanking regions of the targeted DNA sequence and initiate amplification. Tightly controlled temperature variations within the thermal cycler promote: 1) denaturation of double-stranded DNA, 2) primer annealing and 3) synthesis of the complementary DNA strand by a DNA polymerase enzyme. Temperature cycling is repeated many times, resulting in an exponential increase in the abundance of the targeted DNA sequence. During multiplex PCR, several distinct DNA targets are copied in parallel within the same reaction. When primers are conjugated with a fluorescent dye molecule, the PCR products generated are also dye-labeled. To analyze dye-labeled PCR products, the amplified double-stranded DNA is heat-denatured in formamide. The resulting single-stranded DNA is electrokinetically injected into a capillary electrophoresis instrument, where the DNA fragments are separated based on size and detected through the incorporated fluorescent label. A size standard is added to the formamide and amplified sample mixture prior to denaturation and CE analysis to permit accurate sizing of the amplified DNA fragments.

The OncoMate® MSI Dx Analysis System is a fluorescent, multiplex PCR-based test to detect DNA sequence length changes in microsatellite regions of colorectal tumor cell DNA relative to the same regions from the patient's normal cells. Microsatellites are short, DNA-repeat regions [e.g., $(A)_n$, $(CA)_n$, $(AAT)_n$, $(AGAT)_n$, $(AAAAG)_n$,] that are distributed throughout the human genome and are prone to insertion and deletion copying errors during DNA replication. Normally, copying errors are repaired by the cellular DNA MMR system. MSI is observed when MMR function is deficient and DNA replication errors are not repaired, resulting in different lengths of microsatellite alleles in MMR-deficient tissue and normal tissue samples. Among microsatellites, mononucleotide repeats are the most likely to show instability (9,10).

The OncoMate® MSI Dx Analysis System amplification kit includes fluorophore-labeled primers for co-amplification of seven microsatellite markers: five mononucleotide repeat markers (BAT-25, BAT-26, NR-21, NR-24 and MONO-27) and two pentanucleotide repeat markers (Penta C and Penta D, Table 39, Figure 20). The mononucleotide-repeat markers are analyzed to determine MSI status and were selected for high sensitivity and specificity to alterations in repeat lengths in samples containing mismatch repair defects. These markers are quasi-monomorphic; almost all individuals are homozygous for the same common alleles. The pentanucleotide-repeat markers were selected for their high level of polymorphism and low degree of MSI. These markers are included as an identity check between individual normal and tumor sample pairs to confirm that the sample pairs were derived from the same individual (9).

Table 39. Expected Amplified Size Ranges and Detection Channels for the Markers Included in the OncoMate® MSI Dx Analysis System.

Mononucleotide Markers	Repeat Structure	Detection Channel	Amplified Size Range
BAT-26	$A_{(26)}$	Blue	83 to 121bp
NR-21	$A_{(21)}$	Green	83 to 108bp
BAT-25	$A_{(25)}$	Green	110 to 132bp
MONO-27	$A_{(27)}$	Green	134 to 168bp
NR-24	$A_{(24)}$	Yellow	103 to 138bp

Pentanucleotide Markers	Repeat Structure	Detection Channel	Amplified Size Range
Penta D	$AAAGA_{(2–17)}$	Blue	123 to 253bp
Penta C	$AAAAC_{(4–17)}$	Yellow	140 to 228bp

During capillary electrophoresis (CE), OncoMate® MSI Dx Analysis System (Cat. #MD2140) amplification products are separated and analyzed alongside fluorescently labeled DNA fragments of known size, the Size Standard 500. Following CE, the resulting DNA fragment data (.fsa files) are simultaneously imported and analyzed by the OncoMate® MSI Dx Interpretive Software. During this process, data quality control (QC) checks are performed, and DNA fragments amplified from seven microsatellite regions are sized with reference to the size standard fragments using the Local Southern method (16). For each sample pair (normal and tumor) or positive control analyzed, the software employs an allele-calling routine to distinguish true microsatellite alleles from PCR "stutter" artifacts, which occur due to "slippage" of the DNA polymerase enzyme while copying repetitive DNA sequences (4). For each of the five mononucleotide-repeat markers, the smallest DNA fragment (in bp) with the greatest peak intensity (in relative fluorescence units, RFU) relative to neighboring stutter peaks is assigned as the allele of interest for subsequent comparisons.

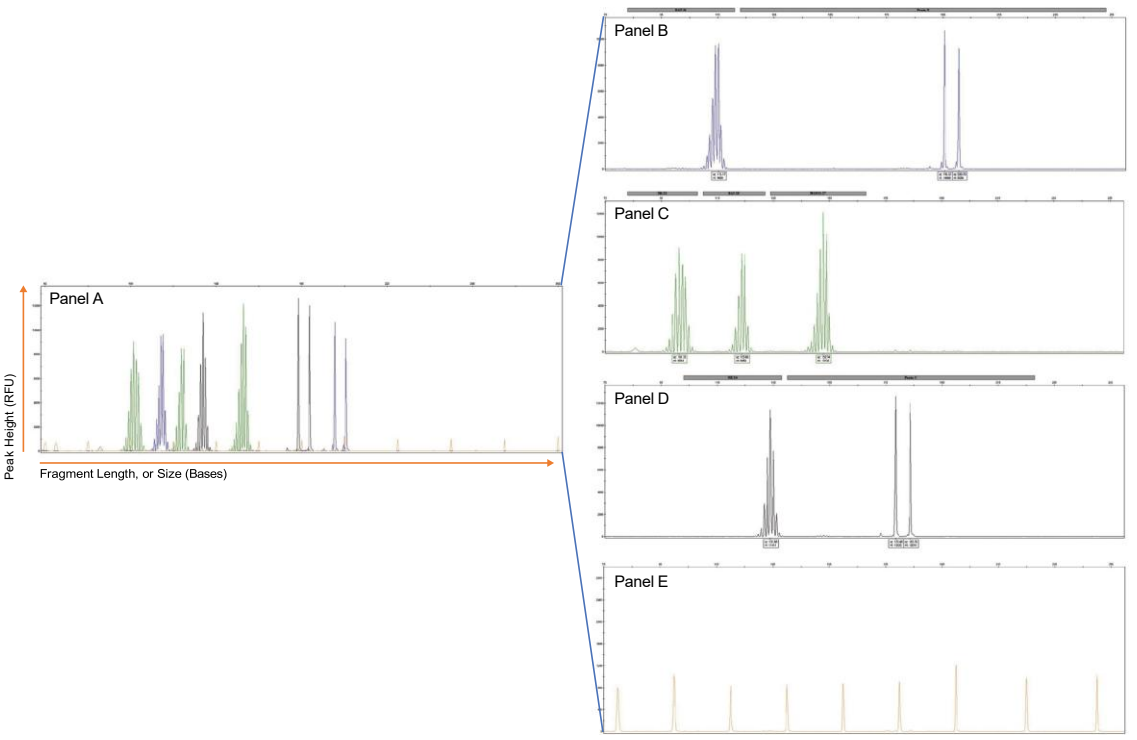


Figure 20. OncoMate® MSI Dx Analysis System data example. A single genomic DNA template (1ng) was amplified using the OncoMate® MSI Dx Analysis System, and the PCR products were analyzed using the Applied Biosystems® 3500 Dx Genetic Analyzer with POP-7® polymer and 50cm capillary array. **Panel A.** An electropherogram showing the simultaneous detection of all fluorescently labeled DNA fragments. **Panels B–E.** Microsatellite data displayed by detection channel, allowing easier interpretation. Panel E contains size standards.

The OncoMate® MSI Dx Interpretive Software requires data from paired normal and CRC tumor samples to determine tumor MSI status. The size difference (in bp) between the allele of interest in the normal and tumor samples is calculated to determine the stability of each of the five mononucleotide-repeat markers. A marker is interpreted as 'Unstable' when this size difference is at least 3bp (implemented in the software as ≥2.75bp to account for the sizing precision of capillary electrophoresis) (Figure 21). A tumor sample is interpreted as MSI-H when two or more markers are 'Unstable'. A tumor sample is interpreted as MSS when fewer than two markers are interpreted as 'Unstable' (4). A sample may be interpreted as 'No Call' or 'Invalid' in response to specific QC failures.

Two pentanucleotide-repeat markers are analyzed by the software as an identity check between the normal and tumor DNA samples. When all alleles detected in the normal sample are also present in the tumor sample, the sample identity check passes.

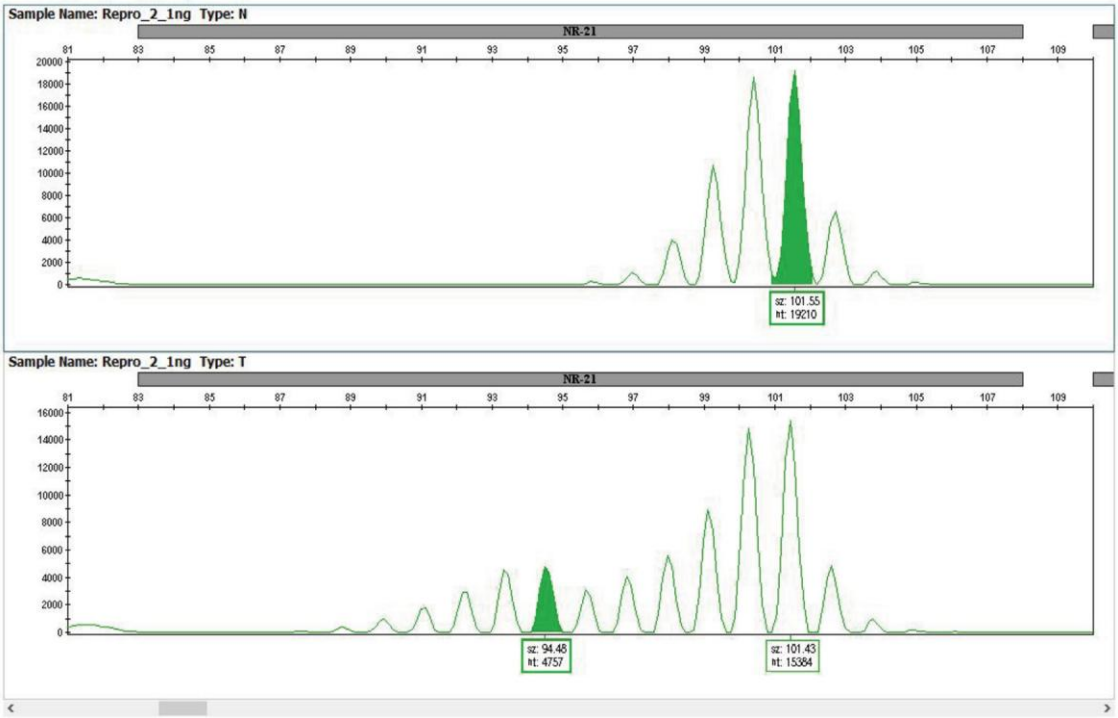


Figure 21. Stability assessment of mononucleotide markers. Stability assessment of mononucleotide-repeat markers. In the example above, the microsatellite alleles of interest are highlighted. For a mononucleotide marker, if the size difference between the new allele in the tumor sample and the reference allele in the normal sample is at least 2.75bp, the marker is interpreted as unstable. In this example, the reference allele in the normal sample (top electropherogram) is 101.55bp, while the new allele in the tumor sample (bottom electropherogram) is 94.48bp. The size difference between these two alleles is 7.07bp; this difference is ≥2.75bp, therefore the NR-21 marker is interpreted as 'Unstable'.

15.8 Assay Limitations

1. For in vitro diagnostic use only.
2. For professional use only.
3. The OncoMate® MSI Dx Analysis System is intended for use with DNA extracted from formalin-fixed, paraffin-embedded (FFPE) tissue samples from colorectal cancer patients. It is not intended for use with DNA from fresh cancer tissues or from other types of specimens or fixatives.
4. Normal and tumor tissue from the same patient must be tested at the same time, and data from both samples must be available to generate a result.
5. This assay is validated for use on the Applied Biosystems® 3500 Dx Genetic Analyzer.
6. This assay has been validated for use with the Maxwell® CSC DNA FFPE Kit.
7. This assay has not been validated for assessment of expansion of repeats in the target loci.
8. The assay has been validated for a DNA input of 1ng. Using less than this amount of DNA may lead to false negative results.
9. This assay has been validated for use with the OncoMate® 5C Matrix Standard.
10. For tumor samples, tumor content must be ≥30%, based on standard pathological characterization. Tumor samples that do not meet these criteria are not suitable for use with the OncoMate® MSI Dx Analysis System.
11. For tumor samples exhibiting instability at a single locus (1/5 alleles unstable), assess the tumor content and examine electropherograms, and consider a retest by enriching tumor content for the sample or orthogonal testing to rule out a false-negative test result.
12. Performance of the OncoMate® MSI Dx Analysis System was validated using the procedures described in this manual. Modifications to these procedures may alter the performance of the assay.
13. Test results obtained using the product must be interpreted by healthcare professionals in conjunction with other clinical findings, family history and other laboratory data.
14. The clinical performance of this device to guide treatment decision for MSI-high patients has not been established.

16. Product Components and Storage Conditions

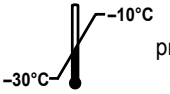
16.1 Materials Provided with the OncoMate® MSI Dx Analysis System (Cat.# MD2140)

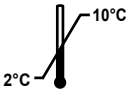
Σ 100 This product contains sufficient reagents to perform 100 reactions (50 paired reactions).

The following materials are included:

COMPONENT	SIZE	PART #
OncoMate® MSI 5X Primer Mix	**200µl**	**MD705A**

Includes: Fluorophore-labeled and unlabeled primers for BAT-26, Penta D, NR-21, BAT-25, MONO-27, NR-24 and Penta C in a buffered solution.

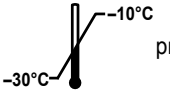
Storage Conditions: Pre-amplification area; 

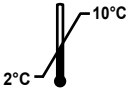
prior to use; 

following first use.

COMPONENT	SIZE	PART #
OncoMate® MSI 5X Master Mix	**200µl**	**MD280A**

Includes: GoTaq® MDx Hot Start DNA Polymerase, dNTPs, magnesium chloride and salts in a buffered solution with stabilizers.

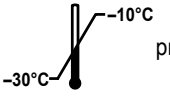
Storage Conditions: Pre-amplification area; 

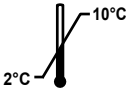
prior to use; 

following first use.

COMPONENT	SIZE	PART #
2800M Control DNA, 10ng/µl	**25µl**	**MD810A**

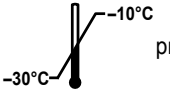
Includes: Cell-line derived male genomic DNA standard in a buffered solution.

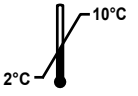
Storage Conditions: Pre-amplification area; 

prior to use; 

following first use.

COMPONENT	SIZE	PART #
Water, Amplification Grade	**1.25ml**	**MD193A**

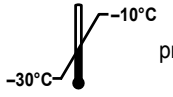
Storage Conditions: Pre-amplification area; 

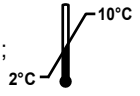
prior to use; 

following first use.

COMPONENT	SIZE	PART #
Size Standard 500	**100µl**	**MD500A**

Includes: Fluorophore-labeled DNA fragments in a buffered solution.

Storage Conditions: Post-amplification area; 

prior to use; 

following first use.

16.2 Storage and Handling of the OncoMate® MSI Dx Analysis System

Upon receipt, store all components at –30°C to –10°C in a nonfrost-free freezer. Before first use, store the 2800M Control DNA at 2°C to 10°C for at least 8 hours. After the first use, store the OncoMate® MSI Dx Analysis System at 2°C to 10°C for up to 3 months. Do not refreeze. Store the OncoMate® MSI 5X Primer Mix and Size Standard 500 protected from light. Store pre-amplification and post-amplification reagents in separate rooms and use with dedicated pipettes, tube racks, etc. Components stored under conditions other than those stated on the labels may not perform properly and may adversely affect results.

16.3 Materials Not Provided

Laboratory Reagents

- Maxwell® CSC DNA FFPE Kit (Cat.# AS1350)
- OncoMate® MSI Dx 5C Matrix Standard (Cat.# MD4850)
- Hi-Di™ Formamide 3500 Dx Series (Thermo Fisher Scientific Cat.# 4404307)
- Fluorescent-dye-based dsDNA quantification reagents
- Nuclease-Free Water (Cat.# MC1191)

  Note: It is critical to use high-quality Hi-Di™ Formamide. Freeze Hi-Di™ Formamide in aliquots at –30°C to –10°C. Multiple freeze-thaw cycles or long-term storage at 2–10°C may cause formamide breakdown. Poor-quality formamide may contain ions that compete with DNA during injection, resulting in lower peak heights and reduced sensitivity.

Laboratory Equipment

Note: The following laboratory equipment is required in two distinct areas of the laboratory: one for pre-amplification procedures and one for post-amplification procedures.

- Set of calibrated precision pipettes capable of delivering 1µl to 1,000µl
- Aerosol-resistant pipette tips (10µl to 1,000µl)
- 1.5ml microcentrifuge tubes
- MicroAmp® Optical 96-Well Reaction Plate with Barcode (Thermo Fisher Scientific Cat.# 4306737)
- MicroAmp® 8-Cap Strip, clear (Thermo Fisher Scientific Cat.# N8010535) (pre-amplification only)
- Personal microcentrifuge (“mini centrifuge”)
- Centrifuge compatible with 96-well plates (e.g., “mini plate spinner centrifuge”)
- Microcentrifuge tube racks
- Vortex mixer
- Nonfrost-free freezer at –30°C to –10°C
- Refrigerator at 2°C to 10°C
- Crushed ice (post-amplification only)

Instruments and Accessories

- Maxwell® CSC Instrument (Cat.# AS6000)
- Fluorometer compatible with fluorescent-dye-based dsDNA quantification reagents
- Applied Biosystems® 3500 Dx Genetic Analyzer (Thermo Fisher Scientific Cat.# A46344)
- Thermal cycler compatible with 96-well plates or reaction tubes
- 3500 Dx Capillary Array 50cm (Thermo Fisher Scientific Cat.# 4404684)
- 3500 Dx Series Septa 96-Well (Thermo Fisher Scientific Cat.# 4410700)
- POP-7® Performance Optimized Polymer 3500 Dx Series (Thermo Fisher Scientific Cat.# 4393709, 4393713)
- Anode Buffer Container 3500 Dx Series (Thermo Fisher Scientific Cat.# 4393925)
- Cathode Buffer Container 3500 Dx Series (Thermo Fisher Scientific Cat.# 4408258)
- 3500 Dx Series Septa Cathode Buffer Container (Thermo Fisher Scientific Cat.# 4410716)
- Conditioning Reagent 3500 Dx Series (Thermo Fisher Scientific Cat.# 4409543)

Software

- OncoMate® MSI Dx Assay Installer (Cat.# MD4150)
- OncoMate® MSI Dx Interpretive Software (Cat.# MD4140)

17. Before You Begin

17.1 Warnings and Precautions

Chemical Safety Warning: Some reagents used with fragment analysis are potentially hazardous. Handle and dispose of hazardous materials according to the guidelines established by your institution. Formamide is an irritant and a teratogen; avoid inhalation and contact with skin. Read the warning label and take appropriate precautions when handling this substance. Always wear gloves and safety glasses when working with formamide. Consult the safety data sheet for formamide, available from the Thermo Fisher Scientific Technical Services Department, prior to use.

Safety Data Sheet Statement: Important information regarding the safe handling, transport and disposal of this product is contained in the Safety Data Sheet (SDS). SDSs for all reagents provided in the kits are available online at: **www.promega.com/resources/msds/** or upon request from Promega Technical Services at: **genetic@promega.com**

Biosafety Precautions: Formalin-fixed paraffin-embedded tissues are acceptable for use in the OncoMate® MSI Dx Analysis System. Follow the guidelines established by your institution for the handling and disposal of these tissues.

PCR Precautions and Good Laboratory Practices: The quality of purified DNA, small changes in buffers, ionic strength, primer concentrations, reaction volume, choice of thermal cycler and thermal cycling conditions can affect PCR success. Therefore, the OncoMate® MSI Dx Analysis System requires strict adherence to the recommended procedures for amplification and fluorescence detection described in this manual.

PCR-based microsatellite analysis is subject to contamination by very small amounts of human DNA. Extreme care should be taken to avoid cross-contamination when preparing template DNA, handling kit components, assembling amplification reactions and analyzing amplification products. Store and use reagents and materials used prior to amplification

(OncoMate® MSI 5X Master Mix, OncoMate® MSI 5X Primer Mix, 2800M Control DNA and Water, Amplification Grade) in a separate room from those used following amplification (Size Standard 500). Prepare amplification reactions in a room dedicated for reaction setup. Use equipment and supplies dedicated for amplification setup. Always include a negative control reaction (i.e., no template) to detect reagent contamination and a positive control reaction to verify reagent performance. Wear gloves and use aerosol-resistant pipette tips to prevent cross-contamination.

17.2 Specimen Preparation and Review

The OncoMate® MSI Dx Analysis System is intended for use with FFPE colorectal tissue samples collected from colorectal cancer patients. Prepare colorectal FFPE tissue samples using 10% neutral-buffered formalin following standard pathology practices. Complete a pathology review on prepared tissue sections to confirm that the material is appropriate for downstream use. Tissue sections suitable for use in the assay contain sufficient nucleated cells and a tumor content ≥30% tumor cells and range in volume from 0.1mm^3 to 2.0mm^3. Example dimensions include a 5mm × 5mm piece of embedded tissue in a 10µm-thick section. Store FFPE blocks and slides at room temperature.

Notes:

1. Tumors are heterogeneous in terms of both genotype and phenotype. Mutation-positive tumors can contain wildtype DNA.
2. Obtaining sufficient high-quality DNA from FFPE tissues can be problematic. DNA may be degraded due to prolonged or unsuitable fixation of the tissue sample before embedding in paraffin. The performance of OncoMate® MSI Dx Analysis System amplification reactions may be affected by the use of insufficient or poor-quality DNA. Accordingly, adhere to best practices to fix and process FFPE tissues.

17.3 DNA Quantification System and Fluorometer Requirements

The OncoMate® MSI Dx Analysis System is intended for use with DNA isolated with the Maxwell® CSC DNA FFPE Kit (Cat.# AS1350) and quantified using dsDNA-binding dyes. Prior to first analysis, you must select a fluorescence-based DNA quantification system consisting of reagents and a fluorometer. The OncoMate® MSI Dx Analysis System was developed using quantification systems capable of accurately measuring dsDNA concentrations as low as 0.05ng/µl. Assay performance may be negatively affected by the use of less sensitive reagents and instrumentation. Suitable fluorescent dsDNA quantification kits are available from several manufacturers. When selecting a fluorometer, follow the recommendations of the fluorescent reagent manufacturer to ensure compatibility.

Note: UV-absorbance measurements are unreliable for determining dsDNA concentration in DNA extracts from FFPE colorectal tissue samples.

17.4 Thermal Cycler Requirements for OncoMate® MSI Dx Analysis System Amplification Reactions

The OncoMate® MSI Dx Analysis System was developed and tested using thermal cyclers that meet the following specifications:

Maximum Block Ramp Rate: 3.9°C/second to 5°C/second

Temperature Accuracy: ±0.25°C (at ≥90°C)

Temperature Uniformity: <0.5°C (at ≥90°C)

Heated lid capable of reaching 103°C to 105°C

The performance of this assay may be negatively affected by the use of thermal cyclers with specifications outside of the indicated ranges.

After confirming that the thermal cycler selected meets the required performance criteria, preprogram the instrument with the protocol provided in Section 18.6.

17.5 Capillary Electrophoresis Instrument Configuration and Requirements

17.5.1 Instrument Configuration

OncoMate® MSI Dx Analysis System amplification products are analyzed by capillary electrophoresis using the Applied Biosystems® 3500 Dx Genetic Analyzer in 'Diagnostic Mode' using POP-7® 3500 Dx Series Polymer and a 3500 Dx Series Capillary Array, 50cm. If necessary, use the "Change Polymer Type" wizard to install POP-7® 3500 Dx Series Polymer on the instrument. Conditioning Reagent 3500 Dx Series is required when changing polymer type.

17.5.2 Assay Installation

OncoMate® MSI Dx Analysis System amplification products are analyzed using the 'Promega_OncoMate_MSI_Dx_Assay'. Prior to first analysis, the 'Promega_OncoMate_MSI_Dx_Assay' must be installed on the Applied Biosystems® 3500 Dx Genetic Analyzer using the OncoMate® MSI Dx Assay Installer. OncoMate® File Name Convention and Results Group files also are installed for your convenience. However, customized File Name Convention and Results Group files may be created to meet a laboratory's specific needs. Refer to Section 27.1 of this manual for complete assay installation instructions.

17.5.3 Spectral Calibration

Prior to first use, spectral calibration of the Applied Biosystems® 3500Dx Genetic Analyzer using the OncoMate® 5C Matrix Standard (Cat.# MD4850) is required. Spectral calibration is performed using the 'OncoMate_MSI' dye set, which is installed on the instrument using the OncoMate® MSI Dx Assay Installer. Perform a new spectral calibration after any major maintenance on the system, such as changing the laser, calibrating or replacing the CCD camera or changing the polymer type or capillary array. In addition, perform a new spectral calibration after the instrument is moved to a new location or is serviced by the manufacturer. In some instances, a software upgrade may also necessitate generation of a new spectral calibration.

17.5.4 Use of Conditioning Reagent 3500 Dx Series

Conditioning Reagent 3500 Dx Series (Thermo Fisher Scientific Cat.# 4409543) is used during routine maintenance of the 3500 Dx Genetic Analyzer and when changing the polymer type on the instrument. During execution of the 3500 Dx Genetic Analyzer "Change Polymer Type" and "Wash Pump and Channels" wizards, complete the optional bubble purge steps ('bubbles are observed before' and 'bubbles are observed after') and the 'Fill Array' step when installing or reinstalling polymer. If the bubble purge and array-fill steps are not completed, a known amplification artifact in NR-21 (see Section 20.5) may not be filtered by the OncoMate® MSI Dx Interpretive Software and 'Invalid' results may be observed.

Refer to the *OncoMate® 5C Matrix Standard Technical Manual* #TM542 for detailed protocols and additional information on spectral calibration.

Refer to the *Applied Biosystems® 3500 Dx Genetic Analyzer and 3500xL Dx Genetic Analyzer IVD User Guide* for additional information on instrument preparation and maintenance.

17.6 OncoMate® MSI Dx Interpretive Software Requirements

OncoMate® MSI Dx Interpretive Software is required for data analysis. Refer to the *OncoMate® MSI Dx Interpretive Software Reference Manual* #TM554 for supporting information, including instructions for installing the software, navigating the various screens and performing administrative tasks. Use of the interpretive software (as described in Section 19) requires that the following actions, described in TM554, have been completed:

- Installation of the OncoMate® MSI Dx Interpretive Server
- Installation of the OncoMate® MSI Dx Interpretive Client
- Registration of the OncoMate® MSI Dx Interpretive Software
- Licensing of the OncoMate® MSI Dx Interpretive Software
- **Optional:** Configuration of the OncoMate® MSI Dx Interpretive Software
- **Optional:** Creation of user accounts and user roles

The OncoMate® MSI Dx Interpretive Software groups samples and controls into batches for analysis. A batch is defined by the individual CE plate from which the samples and controls were injected. To import and analyze data using the interpretive software, the following minimum requirements must be met:

- The batch must have both a positive control and a negative control, and these controls must be identified accordingly in the corresponding .fsa files (See Section 18.9). Any time a new sample batch is prepared, including for trouble-shooting QC issues, the matched normal or tumor sample(s) and the Positive and Negative Amplification Controls also must be analyzed. It is acceptable to have more than one of each control type; however, each control in a batch must conform to Quality Control requirements (see Section 20.4, Table 45).
- The samples in the batch must be identified in the .fsa file as Samples. Additionally, each sample must have the designation of N or T in user-defined field 1 (UDF1) to indicate whether the sample is a normal or a tumor sample (see Section 18.9).
- Samples must exist as matched pairs of normal and tumor samples with the same Sample ID (see Section 18.9).

Within the OncoMate® MSI Dx Interpretive Software Client, each user is assigned a role with specific permissions to perform workflow tasks. Permissions to review samples, perform final review of samples and approve samples are used to define the user's responsibilities within the review and approval workflow. For more information on creating users and setting roles and permissions within the software, refer to the *OncoMate® MSI Dx Interpretive Software Reference Manual* #TM554.

18. Assay Protocol

18.1 DNA Extraction from FFPE Tissue Sections

Once the tissue sample is confirmed to be appropriate for the OncoMate® MSI Dx Analysis System, extract DNA through a process of deparaffinization and cell lysis followed by automated purification using the Maxwell® CSC DNA FFPE Kit (Cat.# AS1350) and the Maxwell® CSC Instrument (Cat.# AS6000). Store Maxwell® CSC DNA FFPE Kit extracts at 2°C to 10°C for up to 24 hours; store extracts at –30°C to –10°C for up to 5 months.

Refer to the *Maxwell® CSC DNA FFPE Kit Technical Manual* #TM395 for details and comprehensive instructions on DNA extraction.

18.2 Quantification of Double-Stranded DNA in Extracts from FFPE Tissue Sections

Following DNA extraction, quantify dsDNA using a method based on fluorescent dsDNA-binding dyes.

Prior to quantification, vortex the DNA extract three times for 5 seconds each at maximum speed, and centrifuge briefly (1 to 2 seconds) in a mini centrifuge to collect the liquid at the bottom of the tube and to pellet any residual resin from DNA purification. When quantifying DNA, use ≥1µl sample to improve accuracy.

For specific instructions on DNA quantification, refer to the technical manuals for the fluorescent dsDNA quantification kit and instrument selected.

Note: It is common for residual resin from the Maxwell® CSC DNA FFPE Kit to carry over into the final DNA extract. The resin will not interfere with downstream analyses.

18.3 Dilution of FFPE Tissue DNA Extracts

The OncoMate® MSI Dx Analysis System was developed with a dsDNA input of 1.0ng, delivered in a volume of 1µl to 6µl. We recommend diluting DNA to a constant concentration across samples so that the 1ng DNA input is added to each reaction in a constant volume. During method validation of the OncoMate® MSI Dx Analysis System, all DNA templates were diluted to 0.5ng/µl and 2µl of diluted template DNA was added to each reaction. Complete the following steps for DNA samples that require dilution prior to amplification:

1. Vortex DNA sample three times for 5 seconds each at maximum speed, and centrifuge briefly (1 to 2 seconds), if necessary, in a mini centrifuge to collect the sample at the bottom of the tube.

   Note: Failure to adequately mix DNA samples and dilutions may result in quantification errors and OncoMate® MSI Dx Analysis System assay failure.

2. Dilute DNA sample in Nuclease-Free Water (Cat.# MC1191) so that 1.0ng of dsDNA is added to each amplification reaction in the desired template volume (see Section 18.5). To maximize accuracy, pipet volumes of ≥1µl when diluting sample DNA.

   Note: DNA extracts from the Maxwell® CSC DNA FFPE Kit are eluted in Nuclease-Free Water. Use Nuclease-Free Water if sample dilution is required. PCR amplification efficiency can be greatly altered by changes in pH (due to added Tris HCl) or available magnesium concentration (due to chelation by EDTA) in the amplification reaction when Tris or Tris-EDTA-based diluents are used. Prepare fresh sample DNA dilutions for each experiment. Storing diluted sample DNA for future use may result in poor amplification and assay failure.

18.4 2800M Control DNA Dilution

The OncoMate® MSI Dx Analysis System was developed with a 2800M Control DNA input of 1.0ng, delivered in a volume of 1.0µl to 6.0µl. Store the 2800M Control DNA, 10ng/µl, at 2°C to 10°C for a minimum of 8 hours before first use. We recommend diluting the control DNA to the same constant concentration as the test samples. During method validation of the OncoMate® MSI Dx Analysis System, 2800M Control DNA was diluted to 0.5ng/µl and 2µl of diluted control DNA was added to each reaction.

1. Vortex the 2800M Control DNA three times for 10 seconds each at maximum speed.
2. Dilute 2800M Control DNA in Nuclease-Free Water (Cat.# MC1191) or Water, Amplification Grade, so that 1.0ng is added to the positive control reaction in the desired volume (1–6µl). See Table 40 for example dilutions. To ensure accuracy, pipet volumes ≥1µl when preparing 2800M Control DNA dilutions.

Table 40. Diluting the 2800M Control DNA.

Volume of DNA Template Per Reaction	Volume of 2800M Control DNA (10ng/µl)	Volume of Water[1]
1.0µl	2.0µl	18µl
2.0µl	2.0µl	38µl
3.0µl	2.0µl	58µl
4.0µl	2.0µl	78µl
5.0µl	2.0µl	98µl
6.0µl	2.0µl	118µl

[1]Nuclease-Free Water or Water, Amplification Grade

Notes:

1. Prepare a fresh 2800M Control DNA dilution for each experiment. Storing diluted 2800M Control DNA for future use may result in poor amplification of the positive control and assay failure.
2. The OncoMate® MSI Dx Interpretive Software requires at least one 2800M Control DNA positive control reaction to be amplified and analyzed per plate ("Batch"). Failure to include a positive control reaction or use of a DNA other than 2800M Control DNA as the positive control will result in batch failure and invalid results for all samples in that batch.

18.5 Preparation of OncoMate® MSI Dx Analysis System Amplification Reactions

Keep all pre-amplification and post-amplification reagents in separate rooms. Prepare amplification reactions in a room dedicated for reaction setup. Use equipment and supplies dedicated for amplification setup. Wear gloves and use aerosol-resistant pipette tips to prevent DNA cross-contamination. Use a fresh pipette tip when adding each DNA sample, the 2800M Control DNA and Water, Amplification Grade (for negative controls), to amplification reactions.

1. If necessary, dilute the template DNA to the desired DNA concentration. See Section 18.3 for more information.
2. At the first use, thaw Water, Amplification Grade, OncoMate® MSI 5X Primer Mix and OncoMate® MSI 5X Master Mix completely. After the first use, store the reagents at 2°C to 10°C.

   Note: A precipitate may form in the OncoMate® MSI 5X Master Mix. Presence of the precipitate will not affect DNA amplification using the OncoMate® MSI Dx Analysis System.
3. Centrifuge tubes briefly (1 to 2 seconds) in a mini centrifuge to bring contents to the bottom, and vortex reagents three times for 3 seconds each at maximum speed. Do not centrifuge after vortexing, as this may cause the re-agents to form a concentration gradient in the tube.
4. Label a new MicroAmp® Optical 96-Well Reaction Plate with Barcode.
5. Determine the number of reactions to be assembled. This must include at least one positive amplification control and one negative amplification control reaction for each plate processed. Add additional reactions to the calculation to compensate for pipetting error. While this approach consumes a small amount of each reagent, it ensures that sufficient PCR amplification mix is available for all samples.
6. Assemble the PCR amplification mix as described in Table 41. Add the final volume of Water, Amplification Grade, OncoMate® 5X Master Mix and OncoMate® 5X Primer Mix to a clean, 1.5ml tube. The template DNA will be added to each reaction well individually at Step 8.

Table 41. Assembly of PCR Amplification Mix.

PCR Amplification Mix Component[1]	Volume Per Reaction	×	Number of Reactions	=	Final Volume
Water, Amplification Grade	to a final volume of 10µl	×		=	
OncoMate® MSI 5X Master Mix[2]	2µl	×		=	
OncoMate® MSI 5X Primer Mix[3]	2µl	×		=	
Template DNA (1.0ng)	up to 6µl				
Total Reaction Volume	10µl				

[1]Combine Water, Amplification Grade, OncoMate® 5X Master Mix and OncoMate® 5X Primer Mix in a new 1.5ml tube. The template DNA will be added to each reaction well individually at Step 8.

[2]A precipitate may form in OncoMate® MSI 5X Master Mix. Presence of the precipitate will not affect DNA amplification using the OncoMate® MSI Dx Analysis System.

[3]The OncoMate® MSI 5X Primer Mix is light sensitive and must be stored in the dark.

7. Vortex the PCR amplification mix three times for 3 seconds each at maximum speed, and then pipet the PCR amplification mix into each well of the reaction plate(s) used for samples and controls.

   Note: Failure to vortex the PCR amplification mix sufficiently can result in poor amplification or marker-to-marker imbalance. Add the PCR amplification mix to the wells of the reaction plate as soon as the mix is prepared. Proceed promptly with Steps 8 through 11, followed immediately by thermal cycling.

8. Vortex the diluted FFPE template DNA (prepared in Section 18.3) three times for 5 seconds each at maximum speed, and centrifuge briefly (1 to 2 seconds) in a mini centrifuge to collect the liquid at the bottom of the tube. Pipet 1.0ng of each sample into the designated well containing PCR amplification mix. Mix by pipetting several times.
9. Vortex the diluted 2800M Control DNA (prepared in Section 18.4) three times for 10 seconds each at maximum speed, and centrifuge briefly (1 to 2 seconds) in a mini centrifuge to collect the liquid at the bottom of the tube. Pipet 1.0ng of the 2800M Control DNA dilution into the well(s) reserved for the positive control reaction(s). Mix by pipetting several times.
10. For the negative amplification control, pipet Water, Amplification Grade, (instead of template DNA) into the well(s) reserved for the negative control reaction(s). Mix by pipetting several times.

    Note: Failure to amplify and analyze a negative control reaction will result in batch failure and 'Invalid' results for all patient samples during data analysis using the OncoMate® MSI Dx Interpretive Software.
11. Cap the wells with MicroAmp® 8-Cap Strips, and centrifuge briefly in a mini plate spinner centrifuge to bring contents to the bottom of the wells and to remove air bubbles.

18.6 Thermal Cycling

1. Ensure that the heated lid has reached the programmed temperature and place the reaction plate in a thermal cycler. Close the thermal cycler lid.
2. Select and run the specified protocol in Figure 22. Ensure that the reaction volume is set to 10µl. The total cycling time, including ramping, is approximately 1 hour and 15 minutes.

Thermal Cycling Protocol[1]
96°C for 1 minute, then:
96°C for 10 seconds
58°C for 1 minute
72°C for 30 seconds
for 29 cycles, then:
60°C for 10 minutes, then:
4°C hold

[1]Reaction volume: 10µl; Heated lid: 103° to 105°C

Figure 22. Thermal cycling protocol for the OncoMate® MSI Dx Analysis System.

3. After completion of the thermal cycling protocol, proceed with fragment analysis, or store amplification products protected from light overnight at 2°C to 10°C or long-term at –30°C to –10°C.

Note: Long-term storage of amplified samples at temperatures >–10°C may produce artifacts that interfere with data analysis.

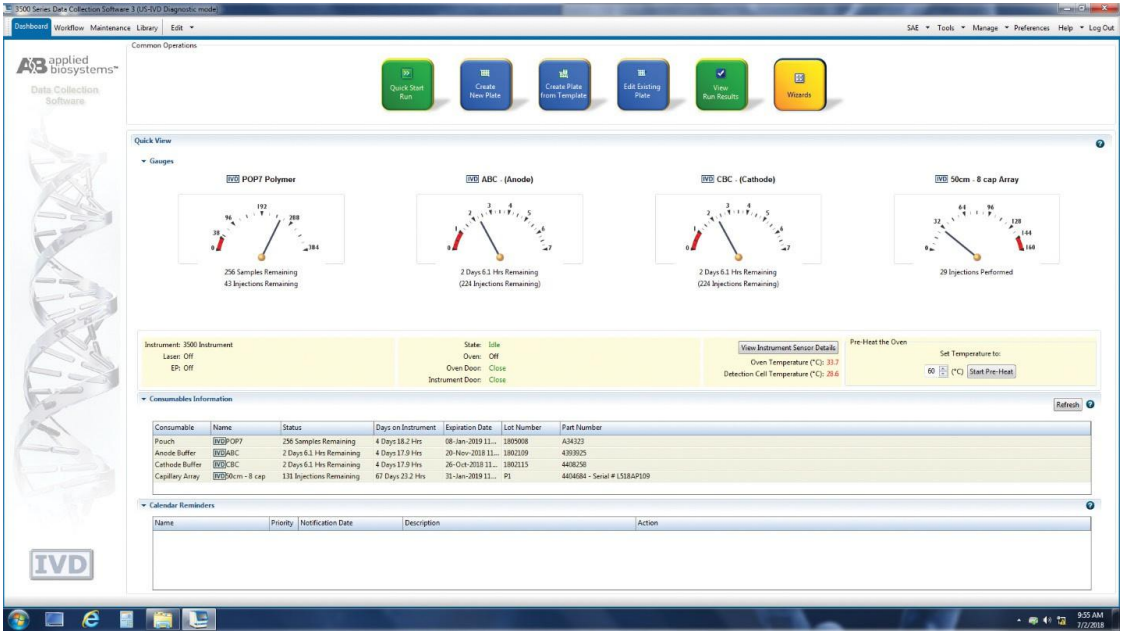
18.7 Preparation of Applied Biosystems® 3500 Dx Genetic Analyzer

1. Open the 3500 Series Data Collection Software, and select **Diagnostic Mode** upon login. Navigate to the 'Dashboard' screen (Figure 23).
2. Complete any instrument maintenance required under Calendar Reminders. Under Consumables Information, ensure that consumables are not expired and that a sufficient number of samples or injections are available to complete the planned analysis. Within the instrument, inspect the consumables to ensure that buffer levels are at their fill lines. Check the pump assembly for bubbles and run the Remove Bubble wizard if needed.

Refer to the *Applied Biosystems® 3500 Dx Genetic Analyzer and 3500xL Dx Genetic Analyzer IVD User Guide* for additional information on instrument preparation and maintenance.

3. Set the oven temperature to 60°C and then select **Start Pre-Heat**. Preheat the oven for at least 30 minutes before starting a run.

   Note: The oven will turn off after 2 hours of instrument inactivity.



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Figure 23. Dashboard on the Applied Biosystems® 3500 Dx Genetic Analyzer, installed with POP-7® polymer, a 50cm array and Data Collection Software Version 3.

18.8 Preparation of OncoMate® MSI Dx Analysis System Amplified Fragments for Capillary Electrophoresis

1. If amplified samples were stored at –30°C to –10°C, thaw them completely before proceeding. Vortex for 5 seconds and centrifuge the plate for 5 to 10 seconds in a mini plate spinner centrifuge to collect contents at the bottom of wells.
2. Determine the number of wells required to analyze all amplified samples, including the positive and negative control reactions. Add to this number any unused wells from which an injection will be initiated plus additional wells to compensate for pipetting error.
3. Vortex the Size Standard 500 three times for 3 seconds each at maximum speed, and prepare the capillary electro-phoresis loading cocktail as directed in Table 42.

Note: Formamide is an irritant and a teratogen; avoid inhalation and contact with skin. Read the warning label and take appropriate precautions when handling this substance. Always wear gloves and safety glasses when working with formamide.

Table 42. Capillary Electrophoresis Loading Cocktail Preparation.

Loading Cocktail	Volume Per Well	×	Number of Wells	=	Final Volume
Hi-Di™ 3500 Dx Series Formamide	9.5µl	×		=	
Size Standard 500	0.5µl	×		=	
Total Volume	10µl				

4. Vortex the loading cocktail three times for 3 seconds each at maximum speed, and centrifuge briefly (1 to 2 seconds) in a mini centrifuge to collect the reagents at the bottom of the tube.
5. Pipet 10µl of loading cocktail into each required well of a MicroAmp® Optical 96-Well Reaction Plate with Barcode.

   Note: Loading cocktail or Hi-Di™ formamide must be added to every well from which an injection is initiated, whether amplified products are also added to the well or not. Failure to add loading cocktail or Hi-Di™ formamide to a well that is injected may result in damage to the capillary array and run failure.
6. Add 1µl of amplified sample or control reaction to each designated well.
7. Cover wells with 3500 Dx Series Septa.
8. Centrifuge the plate for 5 to 10 seconds in a mini plate spinner centrifuge to bring the formamide-sample mixture to the bottom of each well and to remove air bubbles.
9. Denature samples at 95°C for 3 minutes in a thermal cycler, and then immediately chill the plate on crushed ice for at least 3 minutes. Denature samples just prior to loading the plate onto the Applied Biosystems® 3500 Dx Genetic Analyzer.

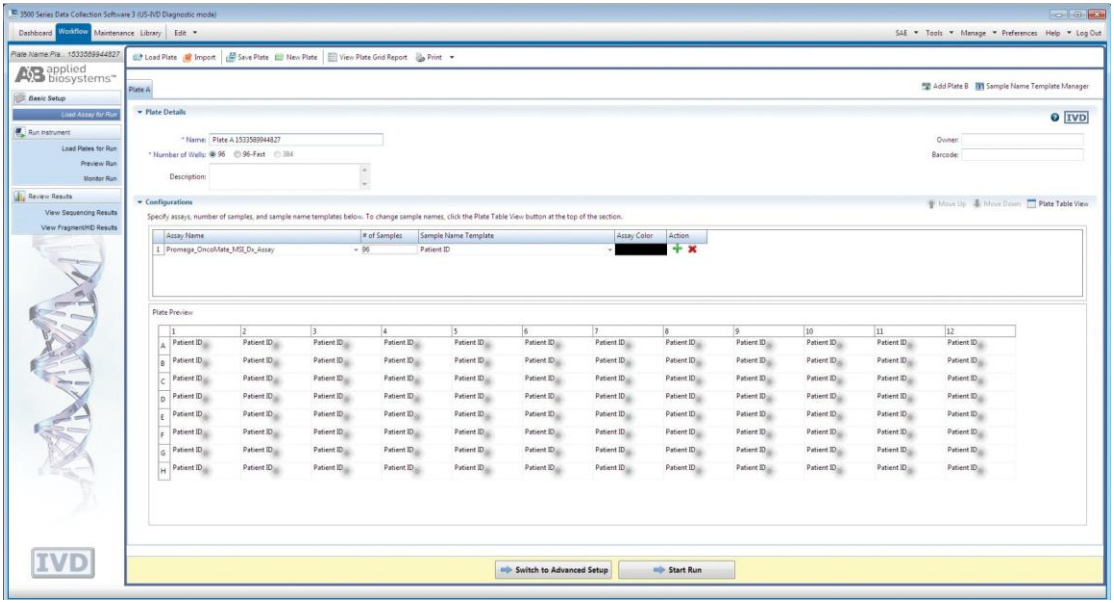
   Note: Do not close the heated lid of the thermal cycler, as this may melt the plate septa.
10. Place the plate in the 96-well plate base and cover with the plate retainer. Load the plate onto the Applied Biosystems® 3500 Dx Genetic Analyzer. Ensure that the oven is preheated to 60°C.

18.9 Detection of Amplified Fragments Using the Applied Biosystems® 3500 Dx Genetic Analyzer

The Applied Biosystems® 3500 Dx Genetic Analyzer Data Collection Software (DCS) employs an application-specific "Assay" that defines run parameters during sample analysis. Separation and fluorescence-based detection of PCR products generated using the OncoMate® MSI Dx Analysis System amplification kit is accomplished using the Promega_OncoMate_MSI_Dx_Assay. If this assay is not yet installed on the Applied Biosystems® 3500 Dx Genetic Analyzer, refer to Section 27.1 for installation instructions.

The Promega_OncoMate_MSI_Dx_Assay is preconfigured with all necessary parameters to separate and detect amplified fragments (e.g., dye set, injection time, injection voltage). These parameters cannot be changed.

1. In the Applied Biosystems® 3500 Dx Genetic Analyzer Data Collection Software, navigate to the 'Workflow' tab. Select **Switch to Advanced Setup** if Basic Setup is displayed under the Applied Biosystems logo in the navigation pane (Figure 24).



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Figure 24. 'Workflow' tab, Load Assay for Run screen.

2. Select **Define Plate Properties** in the navigation pane (Figure 25). Under Plate Details, assign the plate a unique Name, and set the Number of Wells to **96** and the Plate Type to **Fragment**. Verify that the Capillary Length and Polymer are set to **50cm** and **POP7**, respectively.

   Note: The plate Name assigned at this step is used by the OncoMate® MSI Dx Interpretive Software to name the sample batch.

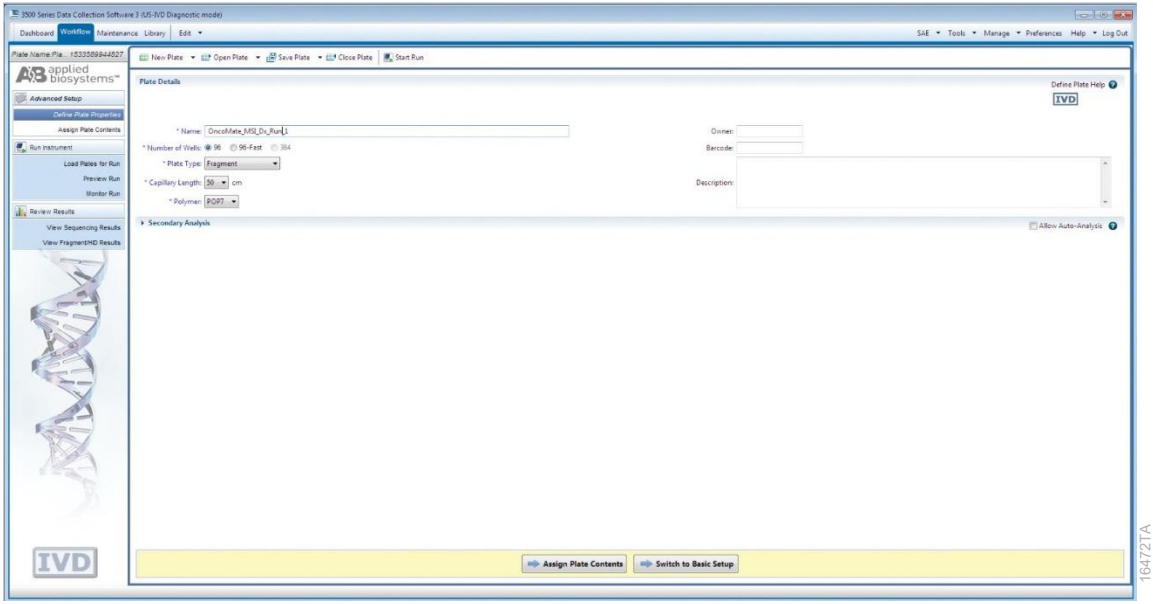
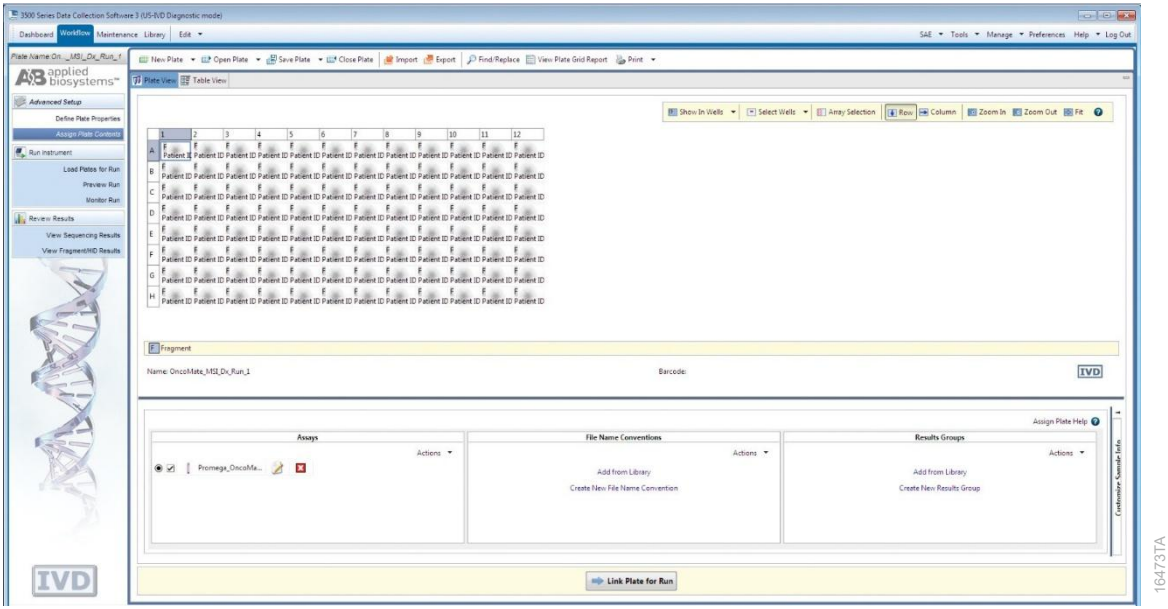


Figure 25. 'Define Plate Properties' screen.

3. Select **Assign Plate Contents** in the navigation pane or at the bottom of the screen (Figure 25).

4. With the 'Plate View' tab selected (Figure 26), use the **Add from Library** links under the Assays, File Name Conventions and Results Groups headers to add the corresponding OncoMate® MSI Dx files to the plate. The OncoMate_MSI_DX File Name Convention and Results Group files are provided for convenience; customized files can be created and used in their place to suit the unique needs of different laboratories.



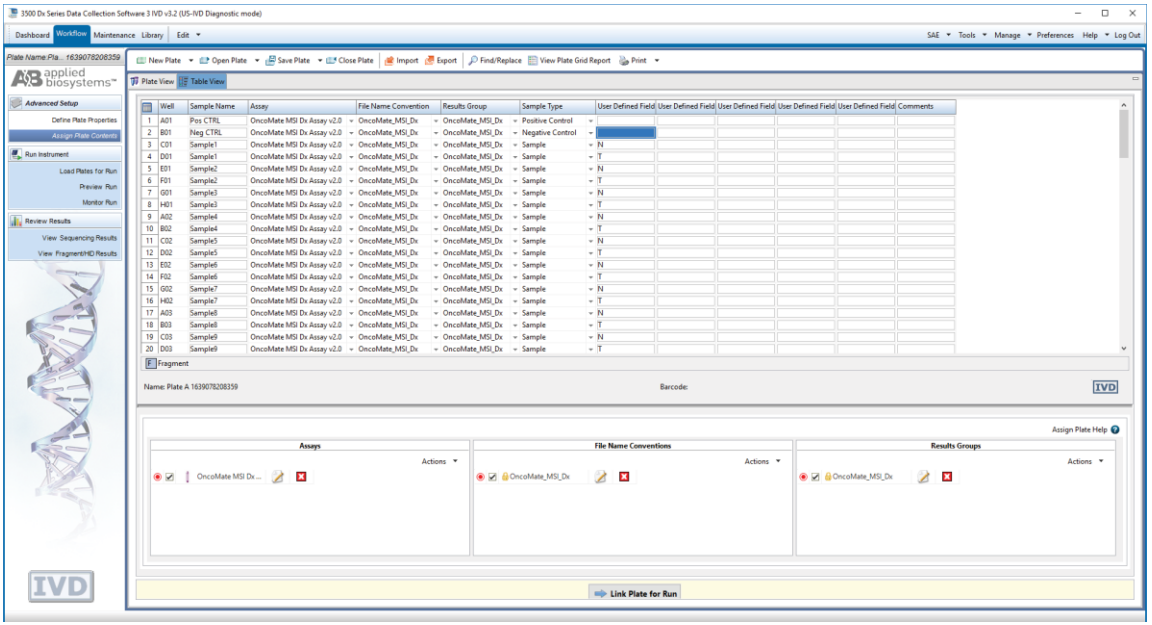
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Figure 26. 'Plate View' tab, Assign Plate Contents screen.

5. Switch to the 'Table View' tab, and enter information for the samples and controls to be analyzed (Figure 27). Source-matched normal and tumor patient samples are required to obtain a valid result during downstream data analysis using the OncoMate® MSI Dx Interpretive Software. The normal and tumor samples for a given patient must have an identical Sample Name, otherwise the resulting sample data files will not be imported into the software. In the field marked User Defined Field 1, enter an "N" for normal tissue samples or a "T" for tumor samples; these entries are required for patient samples. Leave this field empty for control samples. For all samples and controls:

   - Select **Promega_OncoMate_MSI_Dx_Assay** for the Assay.
   - Select **OncoMate_MSI_Dx** for both the File Name Convention and the Results Group, unless customized versions were created.
   - Assign the Sample Type as **Sample**, **Positive Control** or **Negative Control**.

Note: Sample and run information also can be entered using the **Import** function. Refer to the *Applied Biosystems® 3500 Dx Genetic Analyzer and 3500xL Dx Genetic Analyzer IVD User Guide* for information on creating and using plate templates to assign and import plate contents.



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Figure 27. 'Table View' tab, Assign Plate Contents screen.

6. Select **Link Plate for Run**.
7. The 'Load Plate' window will launch. Select **Yes** to acknowledge plate changes (if applicable), and **OK** to acknowledge the Plate loaded successfully message.
8. When the 'Run Information' screen launches (Figure 28), change the Run Name, if desired, and select **Start Run**.

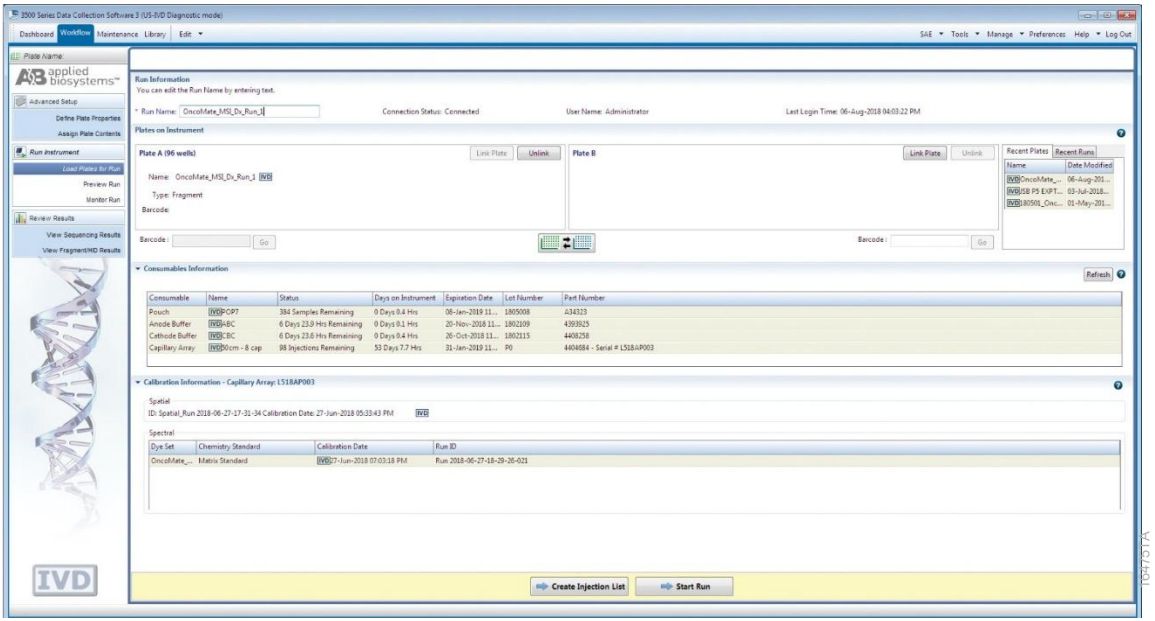


Figure 28. Run Information screen.

Note: A **Reinject** option is available through the Applied Biosystems® 3500 Dx Genetic Analyzer DCS while the analysis of a sample batch is in progress. If the **Reinject** option is used, the data generated are considered part of the same sample batch as the original injection.

Data (.fsa) files created using the **Reinject** option have a "_1" suffix applied to the file name, but the Sample Name within the .fsa files is identical to the original injection. The OncoMate® MSI Dx Interpretive Software uses the Sample Name to identify sample pairs, and sample names must be unique. Because data (.fsa) files created using the **Reinject** option have nonunique sample names, they are excluded during batch import by the interpretive software.

To analyze the original and re-injected data in the same batch, use the **Rename** option in the DCS to rename the samples. Samples can be renamed following completion of CE according to the instructions provided in the *Applied Biosystems® 3500 Dx Genetic Analyzer and 3500xL Dx Genetic Analyzer IVD User Guide*. The renamed samples must have unique matching Sample Names for the normal and tumor sample pair.

19. Data Analysis using the OncoMate® MSI Dx Interpretive Software

19.1 Introduction

Separation and detection of OncoMate® MSI Dx Analysis System amplification products by capillary electrophoresis result in data (.fsa files) that require downstream analysis. The instructions below describe the use of OncoMate® MSI Dx Interpretive Software to analyze .fsa files from matched normal and tumor sample pairs and controls. The interpretation of software results is discussed in Section 20.

Data analysis using the OncoMate® MSI Dx Interpretive Software is more sophisticated than the basic analysis performed within the Applied Biosystems® 3500 Dx Genetic Analyzer DCS. Quality warnings displayed in the DCS may be triggered by broad peaks, signal spikes, etc. that are filtered by the OncoMate® software. Therefore, all samples should be analyzed using the OncoMate® MSI Dx Interpretive Software as the final assessment of data quality.

The data analysis workflow in the OncoMate® MSI Dx Interpretive Software Client (Figure 29) entails:

- Starting the interpretive software server and client applications and logging into the client
- Importing controls and matched tumor and normal sample pairs to create a sample batch
- Automated fragment sizing, allele filtering and quality control verification for each sample pair and control in the batch (during import)
- Automated interpretation of MSI status for each sample pair (during import)
- Reviewing MSI results for each sample pair in the batch
- Approving MSI results for each sample pair in the batch
- Creating reports and export files for each batch

Within the OncoMate® MSI Dx Interpretive Software Client, each user is assigned a role with specific permissions to perform workflow tasks. Permissions to review samples, perform final review of samples and approve samples are used to define the user's responsibilities within the review and approval workflow.

Note: Refer to the *OncoMate® MSI Dx Interpretive Software Reference Manual* #TM554 for instructions describing the installation and configuration of the interpretive software, including steps for creating users and setting their roles and permissions.

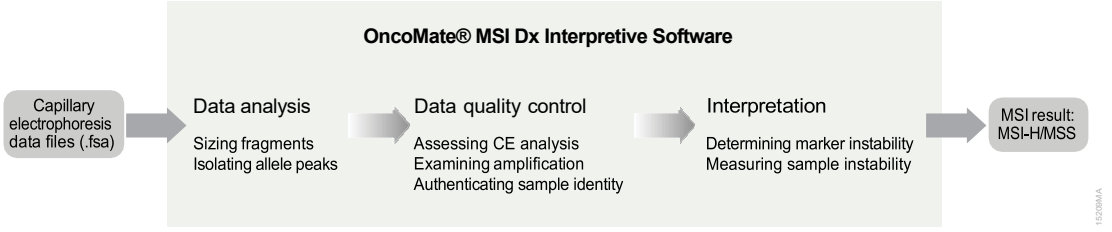


Figure 29. The OncoMate® MSI Dx Interpretive Software automated workflow.

19.2 Standard Client Login Procedure

1. Double-click the icon 

for the OncoMate® MSI Dx Interpretive Software Client on the computer desktop.
Note: If there is a problem connecting to the OncoMate® MSI Dx Interpretive Software Server, you will see a warning message (Figure 30). Refer to the *OncoMate® MSI Dx Interpretive Software Reference Manual* #TM554 for information on starting the server. If the server is running on a different computer than the client, check that the network connection is active and that the server is running.

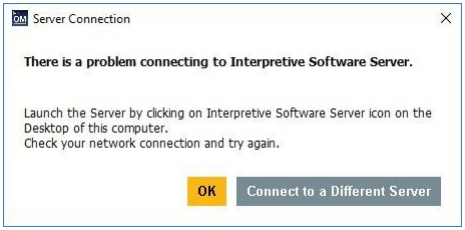


Figure 30. Server connection problem.

2. At the 'Login' screen, enter your User Name and Password (case-sensitive) to activate the **Log In** button. Select **Log In** to enter the 'Home' screen of the OncoMate® MSI Dx Interpretive Software Client. To exit the application without logging in, press **Exit Application**.

19.3 Importing Sample Data (.fsa files) for Automated Analysis

1. From the 'Home' screen of the OncoMate® MSI Dx Interpretive Software Client (Figure 31), click **Import Samples** to open the 'Import Samples' file browser.

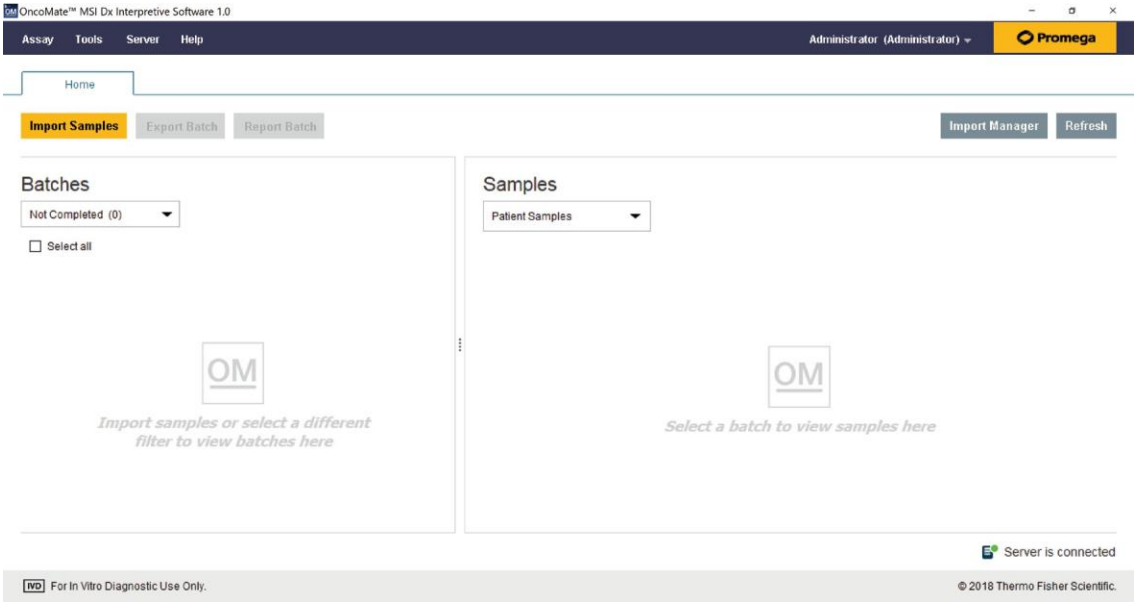


Figure 31. The OncoMate® MSI Dx Interpretive Software 'Home' screen.

2. Browse to the folder containing the sample files (or injection folders containing sample files) that you wish to import (Figure 32). All .fsa files in the selected folder, including those within subfolders of the selected folder, will be imported.

Notes:

1. Import selection is through a folder browser; individual .fsa files will not be displayed. It is not possible to select individual .fsa files for import.
2. Only a single folder at a time can be selected for import. Ensure that the folder selected contains all .fsa files and subfolders containing .fsa files that you wish to import.
3. The OncoMate® MSI Dx Interpretive Software Client will attempt to import the selected samples and assign them to a sample batch. Minimally, two controls, a positive and a negative, and one sample, comprising a matched pair of normal and tumor .fsa data files, are required for successful batch creation. The sample pair must share the same Sample ID, and UDF1 in the .fsa files must identify the samples as N for normal or T for tumor (See Section 18.9 for instructions on sample labeling using UDF1). Import will fail for sample files that do not conform to these requirements.

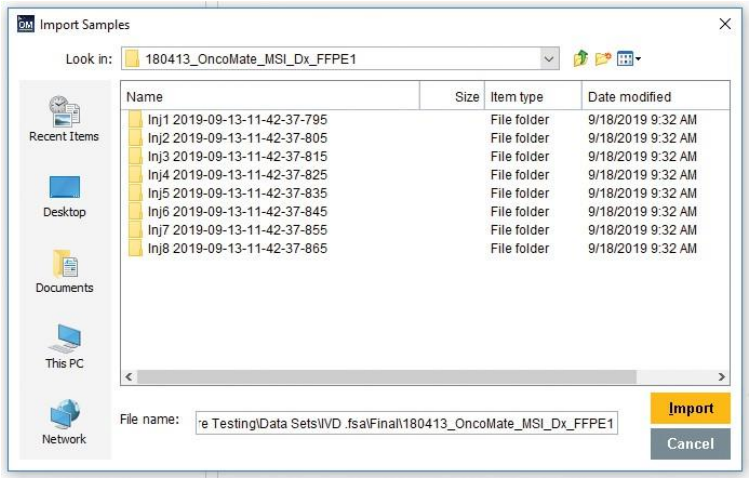


Figure 32. The OncoMate® MSI Dx Interpretive Software 'Import Samples' screen.

3. Press **Import** to initiate the import and analysis process, or press **Cancel** to cancel the import. If presented with the 'Confirmation' screen, press **Continue** to import files from the selected subfolders, or press **Cancel** to cancel import.
4. When the import process is finished, confirm that all samples intended for analysis were imported and are displayed in the Samples pane on the 'Home' screen.

Notes:

1. While samples are importing, an Active notification and rotating status icon are displayed in the notification bar at the bottom right of the screen.



2. As sample files are imported into the OncoMate® MSI Dx Interpretive Software Client, they are assigned to a sample batch based on the capillary electrophoresis plate name assigned by the user and stored in the .fsa file. After successful import of sample files, batches of samples are listed in the Batches pane of the 'Home' screen.
3. Details associated with sample or batch import failures can be found by clicking the **Import Manager** button at the top-right side of the 'Home' screen.
4. Once imported, each tumor and normal sample pair is tracked within the interpretive software as a single Sample.

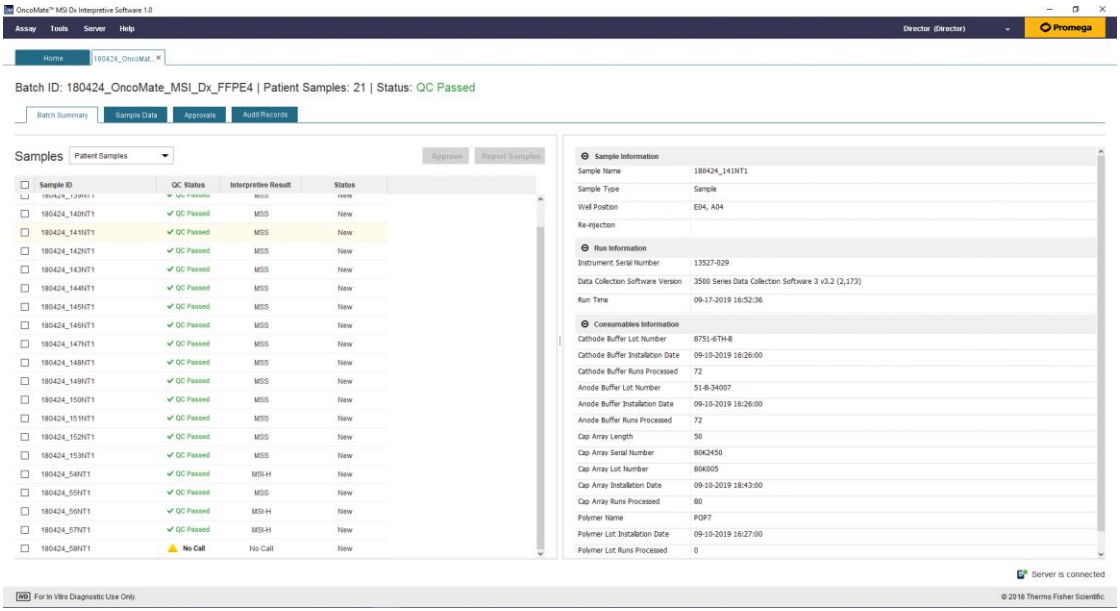
19.4 Reviewing Sample Results

After sample import, it is the responsibility of the initial reviewer to mark samples as Reviewed. A user that has a role with Review permission will complete the following steps:

1. Once a batch of samples is created in the OncoMate® MSI Dx Interpretive Software Client, double click the batch name in the Batches pane to open a batch screen (Figure 33) and view the associated data, including sample electropherograms, approvals and audit records.

   Notes:

   1. The title bar at the top of the batch screen displays the name of the batch, the number of samples in the batch and the QC status of the batch.
   2. Batches that have a status of QC Failed are considered invalid and are not subject to the standard review and approval process. For a QC Failed batch, it is only necessary to approve the batch, and no sample review is possible. Skip to Section 19.6 for instructions to approve a QC Failed batch.



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Figure 33. The batch screen opens to the 'Batch Summary' tab and presents information associated with the selected batch of samples, including electropherograms, approvals and audit records.

2. Select the 'Sample Data' tab (Figure 34) to display results for the matched normal and tumor sample pair selected in the Samples pane. The MSI interpretive result for the sample is displayed at the upper right of the 'Sample Data' tab.

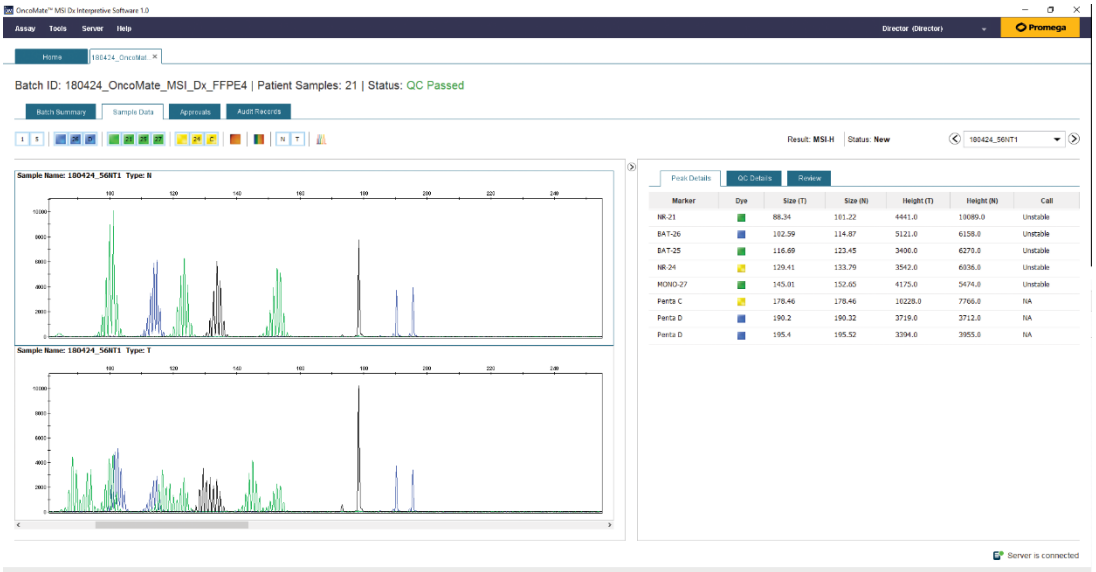


Figure 34. The 'Sample Data' tab displays sample electropherograms and provides Peak Details.

3. Review the sample data. If the MSI interpretive result is Invalid or No Call, select the 'QC Details' tab (Figure 35) to view any quality control flags that apply to the sample.

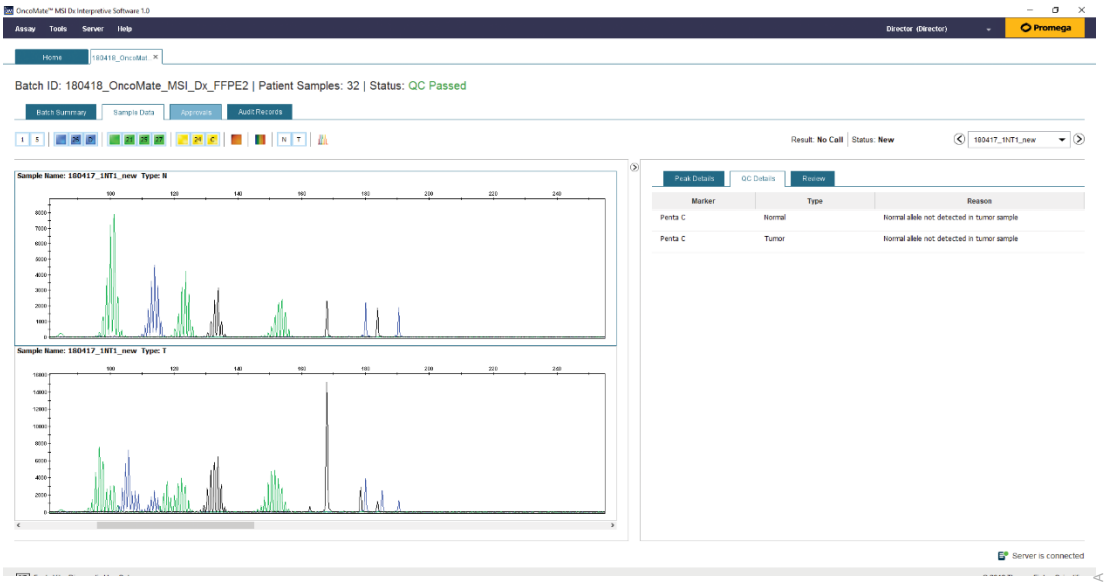


Figure 35. The 'QC Details' tab.

4. Select the 'Review' tab (Figure 36). To add comments to the review for the displayed sample, press **Add Comment**. Refer to Section 20 for information on notable MSI results that may warrant additional review (e.g., MSS with a single unstable locus).
5. Record the initial review of the sample by pressing **Accept**. Once a sample is accepted, the sample Status is updated to Under Review on the 'Batch Summary' tab.
6. Use the drop-down menu, **Left Arrow** or **Right Arrow** buttons at the upper right of the 'Sample Data' tab to access the remaining samples in the batch. For each sample, repeat Section 19.4, Steps 3–6, to continue the sample review process.

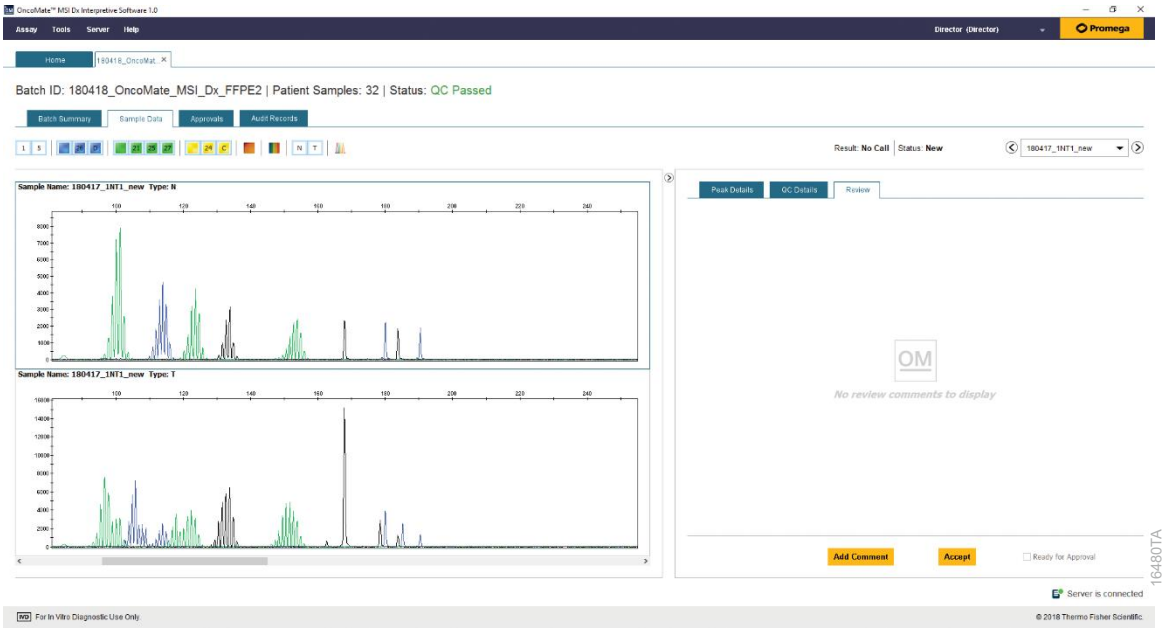


Figure 36. The 'Review' tab in the Details pane.

19.5 Marking Results as Ready for Approval

After initial review of one or more samples is completed, it is the responsibility of the final reviewer to mark samples as Ready for Approval. A user that has permission as a Final Reviewer will complete the following steps:

1. Starting from the 'Home' screen of the software, double-click the desired sample batch to review. This will open a batch screen in the software (Figure 33).
2. Select the 'Sample Data' tab (Figure 34) to display results for the sample highlighted in the Samples pane.

3. Review the sample data. If the MSI interpretive result is Invalid or No Call, select the 'QC Details' tab (Figure 35) to view any quality control flags that apply to the sample.

   Note: If the batch status is QC Failed, all samples in the batch will have an MSI interpretive result of Invalid. In this case, the 'QC Details' tab will not display any quality control flags for the individual samples. Quality control flags for controls are accessed by returning to the 'Batch Summary' tab (Figure 33), selecting **Control Samples** from the Samples drop-down menu and then returning to the 'Sample Data' tab to view the control sample electropherograms and 'QC Details' tab.

4. Select the 'Review' tab (Figure 36).

5. Complete this step if different users perform the reviews described in Sections 19.4 and 19.5: Press **Accept** to record the final review of the displayed matched sample pair, and press **Add Comment** to record any notes related to the sample review.

6. Check the **Ready for Approval** checkbox to complete the review process for the matched sample pair. The sample Status is updated to Ready for Approval on the 'Batch Summary' tab.

7. Use the drop-down menu, **Left Arrow** or **Right Arrow** buttons at the upper right of the 'Sample Data' tab to access the remaining samples in the batch. For each sample, repeat Section 19.5, Steps 3 through 7, to complete the sample review process.

19.6 Approving Results

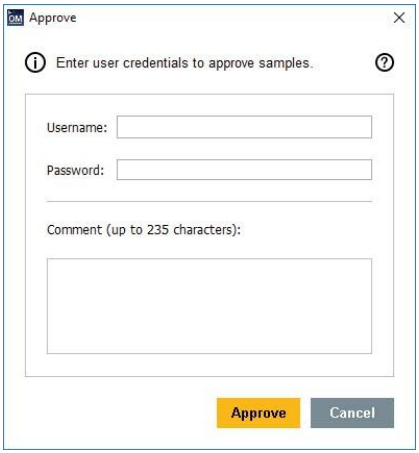
After sample review is completed, it is the responsibility of a user with permission to approve samples to approve the MSI interpretive result for each sample. This user will perform the following steps:

1. If starting from the 'Home' screen of the software, double-click the name of sample batch requiring approval. This will open a batch screen in the software (Figure 33). Within the batch screen, the 'Batch Summary' tab indicates which samples have completed the review process and have a status of Ready for Approval.

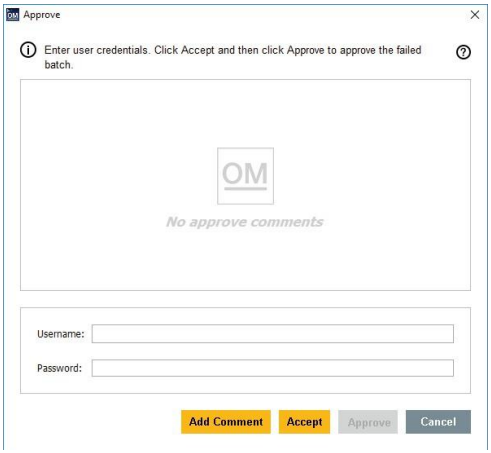
2. If the batch has a status of QC Passed, press **Approve** at the top right of the Samples pane to open the 'Approve' screen (Figure 37, Panel A).

3. On the 'Approve' screen enter the user name and password and any notes for this approval, then press **OK**. All samples marked as Ready for Approval will be approved.

A.



B.



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Figure 37. Panel A. 'Approve' screen shown for samples marked as Ready for Approval. **Panel B.** 'Approve' screen shown for batches marked as QC Failed.

4. If the batch has a status of QC Failed, all samples in the batch will have an MSI interpretive result of Invalid, and an individual sample review is not required before approval. After opening the batch, press **Approve** to open the 'Approve' screen for failed batches (Figure 37, Panel B). Press **Add Comment** to add any comments to the batch approval. Press **Accept** to mark all samples in the batch with an action of Accepted. Next enter a user name and password, and then press **Approve** to approve the entire batch of samples.

   Note: For QC Failed batches, the 'QC Details' tab will not display any quality control flags for the individual samples. Quality control flags for controls are accessed by returning to the 'Batch Summary' tab (Figure 33), selecting **Control Samples** from the Samples drop-down menu and then returning to the 'Sample Data' tab to view the Control Sample data and QC details.

19.7 Creating Reports and Export Results

Once all samples in the batch are reviewed and approved, results may be exported.

1. Create a Batch Export file. From the 'Home' screen, check the box to the left of batch for which an export file will be generated and press **Export Batch**. After a batch export file is generated, the sample batch will have a status of Completed in the OncoMate® MSI Dx Interpretive Software.

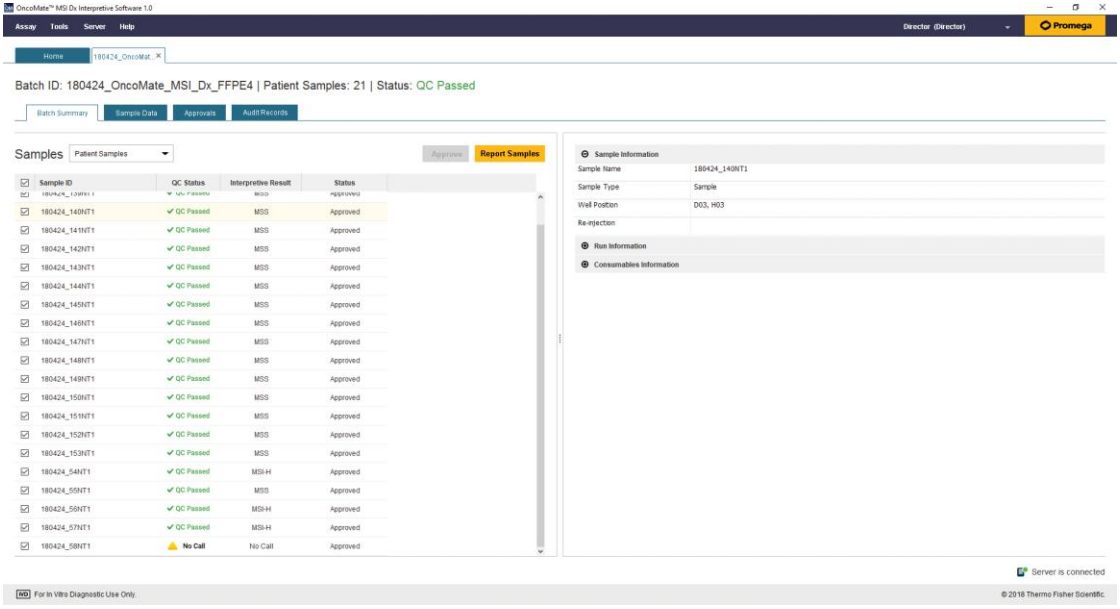
   Note: The batch export file is a comma-delimited text file (.csv) that includes the following information:

   - Date and time of export file creation
   - Batch ID
   - Sample ID(s)
   - Well(s)
   - QC status
   - Sample review status
   - MSI interpretive result for each sample
   - Mononucleotide-repeat marker calls for each sample

2. Create Sample Reports. From the 'Home' screen, double click a batch to open a batch screen. On the 'Batch Summary' tab (Figure 38), select one or more samples for which a report will be generated by checking the boxes to the left of the matched sample pair list. Press **Report Samples** to create a sample report (.pdf file) for each selected matched sample pair.

   Note: Sample reports contain a summary of the information and electropherograms for an individual sample. Contained within the sample report are sections that describe:

   - Result Information: Summary of the results for the sample.
   - Marker Stability Information: Individual marker calls for each mononucleotide marker and a pass/fail indication for pentanucleotide sample identity.
   - Run and Analysis Information: Summary of run and approval information.
   - Data Summary: Electropherogram plots for each marker.
   - Peak Details: Allele sizes and marker calls for each marker.
   - Sample QC Details: A list of any quality flags that were observed for each marker.
   - Review Comments: Comments added to the sample during the review and approval workflow.

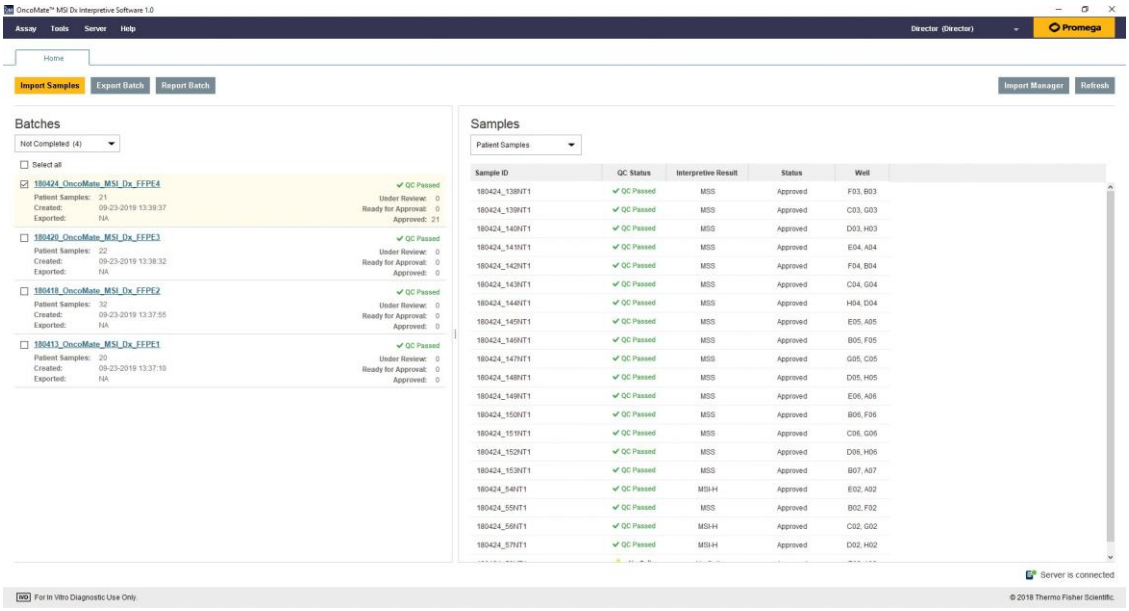


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Figure 38. Sample reports are created by clicking Report Samples from the 'Batch Summary' tab.

3. Create Batch Reports. From the 'Home' screen, select one or more batches for which a report will be generated by checking the boxes to the left of the batch list. Press **Report Batch** to create a batch summary report (.pdf file) for each selected batch (Figure 39).

   Note: Batch reports can be generated only when all samples within a batch are approved. This report provides a high-level summary of controls and samples within a batch. QC Status and Well are reported for both controls and samples. Interpretive Result, Action, Status and Approval Date and Time are reported for all samples.



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Figure 39. From the 'Home' screen, select one or more batches and press Report Batch to create batch summary reports.

20. Interpretation of Results

20.1 Introduction

This section reviews the logic employed by the OncoMate® MSI Dx Interpretive Software to analyze .fsa files from matched normal and tumor sample pairs and controls. In addition, information on specific QC Flags is summarized, and guidance on user interpretation of No Call samples is provided.

For instructions describing the installation, configuration and navigation of the OncoMate® MSI Dx Interpretive Software, consult the *OncoMate® MSI Dx Interpretive Software Reference Manual* #TM554.

20.2 Understanding OncoMate® MSI Dx Analysis System Data

The OncoMate® MSI Dx Analysis System generates size (i.e., DNA fragment length) data for microsatellite regions amplified from matched normal and CRC tumor sample pairs. These data are analyzed using the OncoMate® MSI Dx Interpretive Software to determine tumor sample MSI status. Five mononucleotide-repeat markers (BAT-25, BAT-26, NR-21, NR-24 and MONO-27) and two pentanucleotide-repeat markers (Penta C and Penta D) are evaluated. Mononucleotide-repeat markers produce a distribution of "stutter" peaks during PCR amplification (9). Within any individual stutter peak distribution, the peak with the highest fluorescence value is considered an allele. More than one stutter peak distribution and allele may be present per marker for both normal (when heterozygous) and tumor (when heterozygous or unstable) tissue samples. The OncoMate® MSI Dx Interpretive software uses a peak-filtering algorithm to isolate the alleles of interest (i.e., those useful for making an MSI determination). When a marker in a tumor sample is unstable, one or more novel alleles that are distinct from the normal allele(s) will be detected.

Pentanucleotide-repeat markers produce one or more distinct peaks during PCR amplification that are separated by ≥5bp in approximately 5bp intervals. In typical normal and tumor tissue samples, one or two pentanucleotide alleles will be identified by the software for homozygous or heterozygous individuals, respectively. In some tumor samples, fewer or additional alleles may be identified relative to the normal sample. The presence of low-intensity n–5bp, n–1bp, n+1bp and n+5bp stutter peaks flanking the main allele(s) is common, although these peaks will typically be ignored (i.e., not called as alleles) by the OncoMate® MSI Dx Interpretive Software during data analysis.

20.3 Determination of Sample MSI Status

The analysis of mononucleotide-repeat markers in paired normal and CRC tumor samples determines tumor MSI status. For each of the five mononucleotide-repeat markers, the smallest allele (in bp) identified is considered the allele of interest for subsequent comparisons. The size difference (bp) between the allele of interest in the normal and tumor samples is calculated to determine marker stability. A tumor sample is interpreted as MSI-H when two or more markers are 'Unstable'. A tumor sample is interpreted as MSS when fewer than two markers are interpreted as 'Unstable' (4).

Pentanucleotide-repeat markers are analyzed by the software as an identity check between the normal and tumor DNA samples. The identity check will pass if all alleles identified in the normal sample are also identified in the tumor sample.

A sample may be interpreted as 'No Call' or 'Invalid' in response to specific data quality issues. For example, within tumor samples the pentanucleotide markers may lose or display additional alleles that complicate interpretation of identity with normal samples (see Section 20.6).

Table 43 summarize the marker stability calls and sample interpretive results, respectively, returned by the interpretive software.

Table 43. Marker-Level Stability Calls Provided by the OncoMate® MSI Dx Interpretive Software.

Value	Description
Stable	Microsatellite instability was not detected for the marker. The difference in allele size of the mononucleotide-repeat region analyzed was less than 2.75bp for the normal and tumor sample pair.
Unstable	Microsatellite instability was detected for the marker. The difference in allele size of the mononucleotide-repeat region analyzed differed by at least 2.75bp for the normal and tumor sample pair.
No Call	No Call indicates that the marker stability could not be determined due to a data quality issue (see Section 20.4). View the 'QC Details' tab for information about failed quality attributes for the sample. See Section 20.6 for guidance on interpreting No Call results. See the troubleshooting section of this manual for guidance on resolving QC failures that lead to No Call results.
Invalid	Invalid indicates that the quality of the sample data is unacceptable due to a critical QC failure (see Section 20.4) and cannot be used to determine an MSI interpretive result. View the 'QC Details' tab for the sample or the controls for information about failed quality attributes. See the troubleshooting section of this manual for guidance on resolving QC failures that lead to invalid results.
NA	For pentanucleotide markers, stability is not assessed, and not applicable (NA) is reported.

Table 44. Sample-Level Interpretive Results Provided by the OncoMate® MSI Dx Interpretive Software.

Value	Description
MSI-H	MSI-H (MSI high) indicates that two or more mononucleotide-repeat markers were identified as unstable.
MSS	MSS (MSI stable) indicates that fewer than two mononucleotide-repeat markers were identified as unstable.
No Call	No Call indicates that an automated interpretive result could not be assigned to the sample due to a data quality issue (see Section 20.4). View the 'QC Details' tab for information about failed quality attributes for the sample. See Section 20.6 for guidance on interpreting No Call results. See the troubleshooting section of this manual for guidance on resolving QC failures that lead to No Call results.
Invalid	Invalid indicates that the quality of the sample data is unacceptable due to a critical QC failure (see Section 20.4). No MSI interpretation can be made from these data. When a batch is marked QC Failed due to an issue with a Control sample, all samples within that batch are marked as Invalid. View the 'QC Details' tab for the sample or for the controls for information about failed quality attributes. See the troubleshooting section of this manual for guidance on resolving QC failures that lead to invalid results.

20.4 Summary of Batch- and Sample-Level Data Quality Attributes

This section provides an overview of QC flags that may be observed following data analysis using the OncoMate® MSI Dx Interpretive Software.

See the *OncoMate® MSI Dx Interpretive Software Reference Manual* #TM554 for a comprehensive discussion of batch- and sample-level quality control measures.

The OncoMate® MSI Dx Interpretive Software evaluates several quality attributes associated with control and patient samples to determine whether the batch and sample pairs are of sufficient quality for MSI interpretation. These quality attributes are described in Table 45.

Table 45. QC Details Messages Displayed by the OncoMate® MSI Dx Interpretive Software in Response to Quality Issues Affecting Control or Patient Samples. When a quality issue affects the positive or negative control, a batch status of QC Failed is displayed and Invalid results are returned for all patient samples in the batch. When no quality issues are observed for control samples, Invalid or No Call MSI results are returned for patient samples exhibiting a quality issue.

			MSI Result When a Sample Fails the Corresponding QC Test:	
QC Details Message	**Description of QC Test**	**Samples Evaluated[1]**	**Control Sample Fails QC[2]**	**Patient Sample Fails QC[3]**
Poor sizing quality	The observed pattern of Size Standard 500 peaks must match the expected pattern.	+, –, N, T	All samples Invalid	Invalid
Spectral issues detected	Peaks that are aligned by length in separate dye channels are evaluated for spectral pull-up (i.e., signal bleedthrough between dye channels). When a peak in one dye channel has a signal intensity greater than 10% of an aligned peak in a separate dye channel, the lower-intensity peak is flagged as spectral pull-up.	+, N, T	All samples Invalid	Invalid
Marker peak height too high to evaluate	The intensity (RFU) of peaks in a given sample must not exceed the maximum detectable range of the Applied Biosystems® 3500 Dx Genetic Analyzer.	+, N, T	All samples Invalid	Invalid
Broad peak shape detected	The width of peaks must not exceed the value assigned for MSI analysis.	+, N, T	All samples Invalid	Invalid
No allele detected	At least one allele above 175RFU must be present within each marker.	+, N, T	All samples Invalid	No Call
Unexpected allele count detected	For each pentanucleotide marker, there can be no more than two alleles present in the normal sample.	+, N	All samples Invalid	No Call
Unexpected peaks detected	For the positive amplification control, the alleles present in the pentanucleotide markers must match the expected alleles for the 2800M control DNA within 1.5 base pairs. For negative amplification controls, there must be no peaks detected above the calling threshold (175RFU).	+, –	All samples Invalid	No Call

QC Details Message	Description of QC Test	Samples Evaluated[1]	MSI Result When a Sample Fails the Corresponding QC Test: Control Sample Fails QC[2]	MSI Result When a Sample Fails the Corresponding QC Test: Patient Sample Fails QC[3]
Normal allele not detected in tumor sample	For each pentanucleotide marker, the alleles identified in the normal sample must be present in the tumor sample (within 1.5 base pair).	T	Controls not evaluated	No Call
Low allele peak height detected	For mononucleotide markers that have been interpreted as Stable, allele peak height(s) in the tumor sample must be greater than 700RFU to ensure assay sensitivity.	T	Controls not evaluated	No Call

[1]N, normal sample; T, tumor sample; +, positive control; –, negative control

[2]The MSI interpretive result is Invalid for all patient samples in the batch when a control sample exhibits a QC issue and the batch status is QC Failed.

[3]The MSI interpretive results are Invalid or No Call for an individual patient sample exhibiting a QC issue when the batch status is QC Passed.

20.5 Summary of Known Amplification Artifacts and Capillary Electrophoresis Anomalies

A known amplification artifact is observed within the NR-21 marker as a single broad peak in the size range of 83 to 87.7 base pairs. The OncoMate® MSI Dx Interpretive Software will not call this peak as an allele or consider this peak when determining the stability of the NR-21 marker. Although the peak may appear on the electropherogram, it will not affect automated MSI interpretation.

Note: During execution of the Applied Biosystems® 3500 Dx Genetic Analyzer "Change Polymer Type" and "Wash Pump and Channels" wizards, complete the optional bubble purge steps (bubbles are observed before and bubbles are observed after) and the 'Fill Array' step when installing or re-installing polymer. When the bubble purges and array-fill steps are not completed, the amplification artifact in NR-21 (see Section 20.5) may no longer be filtered by the OncoMate® MSI Dx Interpretive Software.

Other amplification artifacts may occur when too much DNA is used as input to the OncoMate® MSI Dx Analysis System. The baseline signal in the BAT-26 marker (blue channel) may become elevated and jagged. Except for extreme inputs (e.g., ≥4ng 2800M Control DNA), the OncoMate® MSI Dx Interpretive Software will filter these artifacts when determining the stability of the BAT-26 marker.

The OncoMate® MSI Dx Interpretive Software minimizes the impact of known, but random, anomalies that may be observed during capillary electrophoresis. Three such rare anomalies predominate: failed injections, broad peaks and signal spikes.

1. When an injection fails, little or no sample DNA is injected into capillary array. In these cases, a Sizing Quality QC failure will be observed due to the lack (or poor quality) of Size Standard 500 peaks, and the sample interpretive result will be Invalid.
2. A peak may be detected during capillary electrophoresis that exhibits a broad (i.e., not sharp) morphology. A broad peak may originate from polymer crystals or other aberrant material migrating through the capillary array. When the interpretive software detected a Broad Peak, the sample interpretive result will be Invalid.
3. A signal "spike" may be observed during capillary electrophoresis in the form of a near-zero width peak that spans all color channels. Such spikes are detected and ignored by the interpretive software and will not interfere with automated data analysis.

20.6 Data Review of Software Interpretative Result

Commonly observed genomic events in tumor tissues have been considered in the development of the OncoMate® MSI Dx Interpretive Software. Cancerous tissues can display general genomic instability independent of microsatellite instability, such as loss of heterozygosity (LOH). Additionally, microsatellite instability may manifest in a spectrum of profiles, from subtle alterations from the normal profile to distinct, novel alleles several bases removed from the normal allele. Due to the individual nature of cancer development and progression, rare tumor profiles may challenge the algorithms used in the interpretive software. We recommend a data review of interpretive results to identify samples with data profiles your laboratory may want to evaluate further based on current professional standards.

MSI-H and MSS:

The OncoMate® MSI Dx Interpretive Software provides an automated interpretive result, either MSI-H or MSS, when no data QC issues are observed for a sample. A tumor sample is interpreted as MSI-H when two or more markers are 'Unstable'. A tumor sample is interpreted as MSS when one or zero markers are interpreted as 'Unstable' (4).

Note: The interpretive software defines marker instability as a 3bp change (implemented as ≥2.75bp to account for the sizing precision of CE). The interpretive software will score as stable novel alleles that are shifted less than 3bp from the normal reference allele as well as fragment profiles without a distinct novel allele (i.e., those with a "shoulder" or "trailing" stutter peak profile). We recommend a data review of MSS samples for subtle allele shifts that your laboratory may want to evaluate further based on current professional standards. For tumor samples exhibiting instability at a single locus (1/5 alleles unstable), assess tumor content and examine electropherograms. If tumor content is low (i.e., around the recommended 30%), consider a retest by enriching tumor content for the sample or orthogonal testing to rule out a false-negative test result. In addition, the samples with a single unstable marker should be interpreted by healthcare professionals in conjunction with other clinical findings, family history and other laboratory data according to your laboratory's procedures and current professional standards.

Invalid:

When an Invalid sample result is returned, the data are deemed unacceptable and no sample interpretation is possible. Refer to Section 24, Troubleshooting, for information on resolving Invalid results.

No Call:

A No Call Interpretive result is returned with certain data QC messages (see Section 19.4). The following subsections describe scenarios for which a manual MSI determination may be possible for No Call samples. See Section 24, Troubleshooting, for guidance on resolving No Call sample results that cannot be manually interpreted.

No Call resulting from low peak intensity:

DNA recovered from FFPE samples is often highly fragmented, and overestimation of template DNA concentration can lead to low allele signal. Low signal may be compounded for longer markers because longer DNA fragments are typically in lower abundance than shorter fragments in FFPE DNA samples. The peak intensities for the longer markers may not be adequate for data evaluation. Such samples may exhibit "Low allele peak height detected" or "No allele detected" data QC flags in the OncoMate® MSI Dx Interpretive Software 'QC Details' tab.

A manual MSI status may be determined if the following conditions are true for No Call samples resulting from low peak intensity:

1. Low marker peak heights could not be rectified by a) increasing the DNA input to the OncoMate® MSI Dx Analysis System amplification reaction or b) re-analyzing a different FFPE tissue section.
2. No QC Details are observed for either pentanucleotide-repeat marker.
3. The only QC Details reported for the No Call mononucleotide-repeat markers are "Low allele peak height detected" or "No allele detected" (i.e., those associated with poor PCR performance).
4. Two or more mononucleotide-repeat markers were interpreted by the software. An interpreted marker is one for which a stable or unstable call was provided by the software.

If these conditions are met, the sample may be analyzed with the remaining, valid data using the following guidelines (17):

1. If two or more mononucleotide-repeat markers are unstable, interpret the tumor sample as MSI-H.
2. If four or more mononucleotide-repeat markers are stable, interpret the tumor sample as MSS.
3. Otherwise, the interpretive result will remain No Call.

No Call due to sample authentication:

Common genetic events observed with tumor tissue include LOH and generalized genomic instability, which may interfere with sample authentication (i.e., interpretation of identity between matched normal and tumor samples). Pentanucleotide-repeat markers may display a different number of alleles in a tumor sample relative to the matched normal sample. The presence of additional alleles in a tumor sample will not prompt a QC flag.

On the other hand, cases of pronounced allelic imbalance (e.g., LOH) may result in a pentanucleotide allele that is present in normal sample but is not called an allele in a tumor sample. Severe allelic imbalance is observed when one of the normal pentanucleotide alleles is lost (i.e., is greatly diminished in peak height and not identified as an allele) in the tumor. This state can cause a “Normal allele not detected in tumor” (sample authentication) QC flag for the sample, resulting in a No Call MSI Status. To confirm if a “lost” normal allele is present above the calling threshold, open the sample and hover the cursor over the "lost" peak in the electropherogram for the tumor sample. If this peak is present in the correct dye channel and above the software calling threshold, a vertical black line will appear on the electropherogram, indicating that the peak was detected but filtered by the interpretive software allele-calling algorithm (Figure 40).

A manual MSI status may be determined under the following conditions for No Call samples exhibiting the “Normal allele not detected in tumor” QC message:

1. The QC Details message “Normal allele not detected in tumor” was observed for a single pentanucleotide-repeat marker (i.e., for Penta C or Penta D, not both).
2. The “lost” normal allele is present in the Tumor sample above the 175RFU calling threshold but was filtered as stutter by the software’s allele-calling algorithm, due to the much greater height of another allele in the same panel.
3. No QC Details messages are observed for the mononucleotide-repeat markers.

If these conditions are met, data can be interpreted using the following thresholds:

1. If two or more mononucleotide-repeat markers are unstable, interpret the tumor sample as MSI-H.
2. If four or more mononucleotide-repeat markers are stable, interpret the tumor sample as MSS.

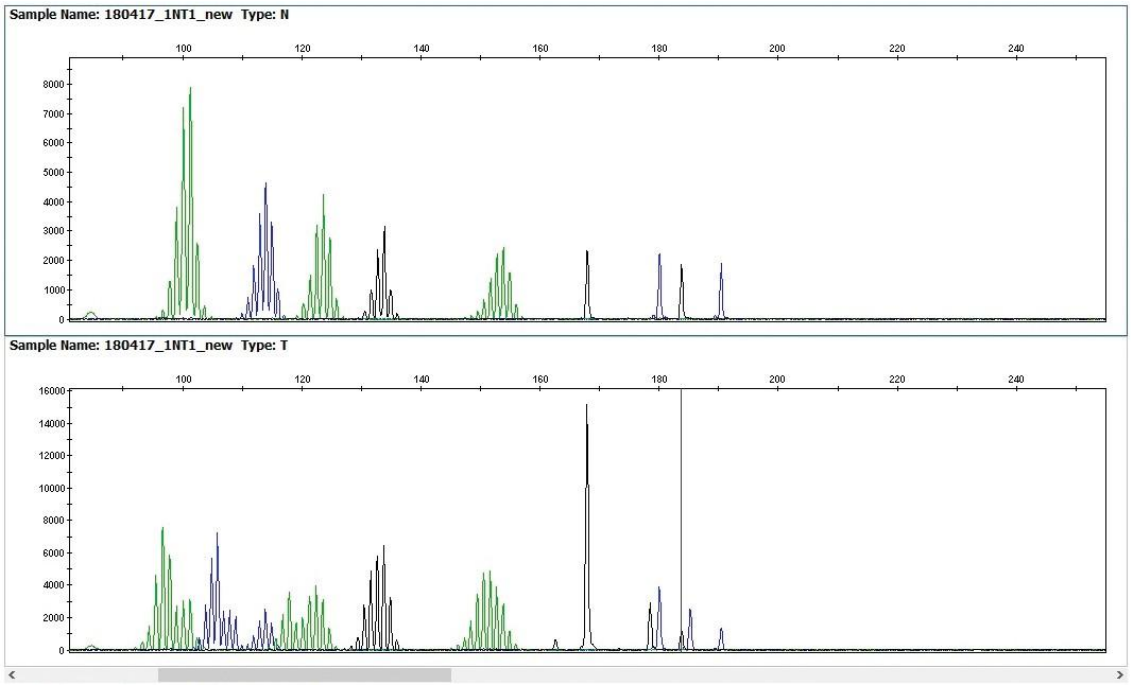


Figure 40. A peak that was detected but filtered by the interpretive software allele-calling algorithm. In cases of pronounced allelic imbalance in pentanucleotide-repeat loci, one of the normal alleles in the tumor sample may be filtered by the software as stutter. If this "lost" normal peak is present above the software calling threshold, a vertical black line is displayed when the cursor is hovered over it in the tumor sample electropherogram. In the tumor sample shown, the normal allele peak at 183bp was filtered as stutter due to the much greater height of the 168bp allele in the same panel.

21. Assay Quality Controls

21.1 Spectral Calibration

During capillary electrophoresis, dye-labeled OncoMate® MSI Dx Analysis System amplification products are separated and detected using the Applied Biosystems® 3500 Dx Genetic Analyzer. Prior to analysis, the Applied Biosystems® 3500 Dx Genetic Analyzer is calibrated with matrix standards so that the fluorescent signals resulting from the set of specific dyes used in the assay can be distinguished. The OncoMate® 5C Matrix Standard consists of DNA fragments labeled with five different fluorescent dyes (fluorescein, JOE, TMR-ET, CXR-ET and WEN) in one tube. The calibration is performed using the 'OncoMate_MSI' dye set, which is installed on the Applied Biosystems® 3500 Dx Genetic Analyzer using the OncoMate® MSI Dx Assay Installer. Once generated, the spectral calibration file is applied automatically during sample detection to account for the spectral overlap among the dyes and to separate the raw fluorescent signals into individual dye signals.

21.2 Matched Normal Tissue Sample

A matched normal tissue sample must be processed in parallel with every tumor sample. Mononucleotide-repeat markers can show heterozygosity or variation in normal-tissue allele length between individuals. To account for such variations in normal alleles, the interpretive software uses normal tissue as an allelic reference for novel tumor alleles. For this reason, the OncoMate® MSI Dx Interpretive Software requires a source-matched tumor and normal sample pair for analysis. Unmatched samples will be excluded from analysis. If the analysis of a tumor or normal sample must be repeated for any reason, the paired tumor or normal sample must be rerun as well.

21.3 Positive and Negative Controls

Positive and no-template ("negative") control amplification reactions using 2800M Control DNA and Water, Amplification Grade, respectively, must be analyzed concurrently with patient samples to verify assay performance. At least one 2800M Control DNA amplification reaction and one negative control amplification reaction must be completed for each plate (i.e., batch) of patient samples analyzed using the OncoMate® MSI Dx Interpretive Software. The negative control reaction is analyzed to ensure that no unexpected amplification occurred in no-template reactions, which would indicate the presence of DNA contamination and lead to an Invalid assay result. The positive control reaction is analyzed to demonstrate that the amplification chemistry performed as expected. See Table 46 for expected 2800M Control DNA results. No-template controls should not have amplified peaks above the 175RFU calling threshold.

Table 46. Expected Amplification and Analysis Results Using 1ng of 2800M Control DNA.

Marker Name	2800M Alleles (bp)[1,2]
NR-21	101
BAT-26	115
BAT-25	124.5
NR-24	134
MONO-27	152.5
Penta C	178.5, 184
Penta D	195.5, 200.5

[1]Allele sizes were determined using the Applied Biosystems® 3500 Dx Genetic Analyzer with POP-7® polymer and a 50cm capillary.

[2]Instrument-to-instrument and day-to-day variability in the performance of capillary electrophoresis instruments may result in a ±bp difference in the allele sizes for 2800M Control DNA. We observed ±1.5bp difference in the analytical studies when using 1ng of Control 2800M DNA. Ninety-seven percent of alleles were within 1bp.

21.4 Capillary Electrophoresis Standards

All analyzed samples and controls must contain Size Standard 500 (added prior to CE). Size Standard 500 contains a series of 21 DNA fragments of known lengths (60, 65, 80, 100, 120, 140, 160, 180, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500bp), also referred to as a DNA ladder. Each fragment is labeled with WEN dye and is detected separately (as a fourth color, orange) in the presence of OncoMate® MSI Dx Analysis System-amplified products using the Applied Biosystems® 3500 Dx Genetic Analyzer. For each sample or control, amplified DNA fragments are sized with reference to the size standard fragments using the Local Southern method (16). The size standard controls for capillary-to-capillary variations in sizing precision during CE and allows direct comparison of samples across the CE run. Only the 60-base to 300-base fragments are analyzed for fragment sizing in the OncoMate® MSI Dx Interpretive Software.

21.5 Quality Control Requirements for Data Interpretation

The OncoMate® MSI Dx Interpretive Software evaluates the quality of capillary electrophoresis data to ensure that a valid MSI determination can be made (see Section 20.4, Table 45, for a summary of QC metrics evaluated). Patient samples must be identified in the Applied Biosystems® 3500 Dx Genetic Analyzer data collection software as 'Samples' and imported into the software as matched normal and tumor pairs with the same sample ID. Additionally, UDF1 in the DCS must be populated with 'N' or 'T' to identify patient samples as normal or tumor, respectively. Positive and negative amplification controls must be analyzed in the same plate as corresponding patient samples during the CE separation run, and these controls must be identified as 'Positive Control' and 'Negative Control' in the Applied Biosystems® 3500 Dx Genetic Analyzer DCS. If amplification controls are not processed with patient samples, the batch status will be QC Failed and all samples in the batch will display an interpretive result of Invalid.

22. Expected Values

The OncoMate® MSI Dx Analysis System determines microsatellite instability status based on results generated for five mononucleotide repeat markers (BAT-25, BAT-26, NR-21, NR-24 and MONO-27). A tumor sample is interpreted as MSI-H when two or more markers are 'Unstable'. A tumor sample is interpreted as MSS when fewer than two markers are interpreted as 'Unstable'. A sample may be interpreted as 'No Call' or 'Invalid' in response to specific QC failures.

During the Method Comparison study, the most common observation for MSI-H and MSS samples was for all or none of the markers to be unstable (147/154 cases, 95%), which is consistent with published literature. Most MSI-H samples (46/47 cases, 98%) exhibited three or more unstable markers, while only 2% (2 of 107) of MSS cases had a single unstable marker. Samples with a single unstable marker should be interpreted by healthcare professionals in conjunction with other clinical findings, family history and other laboratory data according to your laboratory's procedures and current professional standards.

23. Performance Characteristics

All analytical studies followed the procedure outlined in the OncoMate® MSI Dx Analysis System instructions, unless noted otherwise in the study results section.

23.1 Extraction

Suitability of the Maxwell® CSC DNA FFPE Kit using the Maxwell® CSC Instrument for DNA extraction was demonstrated by performing DNA extractions from FFPE curls (0.1–2.0mm^3 tissue) obtained from four MSI-H, three MSS tumor samples and matched normal samples. The MSI-H samples were at 20–30% tumor content, and the MSS samples were at 20–60% tumor content. DNA extraction was performed by each of two operators using three lots of the Maxwell® CSC DNA FFPE Kit. The DNA FFPE kit lot was alternated between operators (e.g., Operator 1 used Lot 1 on Day 1 and Operator 2 used Lot 2 on Day 1). Once isolated, the extracted DNA was quantified using the QuantiFluor® dsDNA System and amplified using the OncoMate® MSI Dx Analysis System amplification kit. The amplified DNA was subjected to capillary electrophoresis using an Applied Biosystems® 3500 Dx Genetic Analyzer and analyzed with the OncoMate® MSI Dx Interpretive Software.

A total of five (5) samples initially yielded Invalid results. After reinjection of all five samples, one sample was resolved. The remaining four (4) samples were resolved by reamplification.

In the study, 96.4% (81/84) of the individual FFPE curls extracted produced results that were concordant with the predetermined MSI status. The 95% confidence intervals (CI) for percent correct and percent incorrect results were 89.9–99.3% and 0.7–10.1%, respectively.

The study demonstrated that the Maxwell® CSC Instrument using the Maxwell® CSC DNA FFPE Kit for DNA extraction met extraction capabilities for use with the OncoMate® MSI Dx Analysis System.

23.2 Normal Range and Cutoff

The OncoMate® MSI Dx Analysis System is intended to measure changes in amplified fragment length. The Normal Range and Cutoff study was conducted to verify the system's capability to resolve amplicons that differ by ≥3 base pairs. Two sets of seven synthetic DNA fragments ("resolution markers") were analyzed during this study. These fragments consist of dye-labeled amplicons of known size that are separated by 1bp within each set, with the two sets designed to bracket the upper (Large) and lower (Small) ends of the amplicon size range of the MSI markers (83–168bp).

The resolution markers were subjected to capillary electrophoresis using an Applied Biosystems® 3500 Dx Genetic Analyzer and analyzed with OncoMate® MSI Dx Interpretive Software. Resolution markers were analyzed either mixed only with the Size Standard 500 or mixed separately with two MSS tumor samples and the Size Standard 500. While the OncoMate® MSI Dx Interpretive Software was not designed to identify the resolution markers, it was critical to demonstrate the System software's ability to determine fragment size and precision. The sizing precision of individual resolution fragments was characterized (Table 47), and size differences between all fragments separated by 3bp were calculated and averaged. Observed mean differences were compared with predicted values.

There were no invalid results or samples requiring re-injection or re-amplification testing for this study.

Resolution fragments were sized precisely, with standard deviations ranging from 0.07–0.13bp. Mean absolute differences calculated for fragments separated by 3bp were also precise, ranging from 3.06–3.40bp and 3.05–3.35bp for observed and predicted values, respectively. Accordingly, the study met the objective of ≥3 base pair resolution and, in fact, demonstrated measurement precision of individual resolution markers (standard deviations ≤0.13 base pairs) that was sufficient to detect single-base-pair differences in size.

Table 47. Descriptive Statistics for Resolution Marker Base Pair Size.

Resolution Marker	N	Minimum	Maximum	Median	Mean	Standard Deviation	95% CI	
							Lower Limit	Upper Limit
Large	20	180.60	180.95	180.77	180.75	0.10	180.71	180.80
Large_2	20	181.67	182.02	181.89	181.83	0.10	181.78	181.88
Large_3	20	182.75	182.97	182.85	182.86	0.08	182.83	182.90
Large_4	20	183.70	184.04	183.88	183.85	0.09	183.80	183.89
Large_5	20	184.66	184.99	184.83	184.80	0.09	184.76	184.85
Large_6	20	185.73	186.06	185.90	185.88	0.10	185.83	185.93
Large_7	20	186.80	187.01	186.91	186.91	0.07	186.88	186.94
Small	20	84.29	84.71	84.62	84.57	0.13	84.51	84.63
Small_2	20	85.52	85.95	85.83	85.79	0.13	85.72	85.85
Small_3	20	86.66	87.14	86.95	86.92	0.13	86.86	86.98
Small_4	20	87.71	88.15	87.99	87.97	0.13	87.91	88.03
Small_5	20	88.73	89.18	89.04	89.02	0.12	88.96	89.07
Small_6	20	89.96	90.40	90.25	90.21	0.13	90.15	90.27
Small_7	20	91.06	91.55	91.39	91.34	0.13	91.28	91.41

23.3 Limit of Blank

A Limit of Blank study was conducted to confirm a blank (an MSS sample in this study) did not produce positive MSI-H results. The study tested four known MSS samples across three amplification kit lots, two operators and 60 replicates of each MSS sample—a total of 1,440 test results.

There were two samples for which the initial test result was invalid. These samples were reinjected per protocol, and after reinjection were resolved. No reamplification testing was required for this study.

All tests (1,440/1,440; 100%) resulted in MSS final interpretive results. The 1,440 test results represent 7,200 mononucleotide locus allele calls. For the mononucleotide loci, 99.99% (7,199/7,200) of the marker stability calls were "Stable". There was a single instance of one locus, NR-21, being called unstable. A single unstable locus results in an MSS final interpretive result, and the one unstable locus did not affect the final test result. In conclusion, the OncoMate™ MSI Dx Analysis System provides MSS results that are highly reproducible and were not affected by lot or operator.

23.4 Limit of Detection

The analytical sensitivity of the OncoMate® MSI Dx Analysis System was determined using extracted DNA isolated from six MSI-H tumor and matched normal samples, as well as a titration series of the 2800M Control DNA. The 2800M Control DNA samples were treated as MSS samples for analysis using the OncoMate® MSI Dx Interpretive Software. To create 20% tumor content (the minimum tumor content required), the tumor samples were mixed with the matched normal samples as needed. A subsequent study investigated higher tumor content. The MSI-H samples and the MSS samples were tested at 0.2, 0.5, 1.0, 2.0 and 2.5ng DNA per amplification reaction with 20 replicates for each sample and DNA amount (five MSI-H cases × 20 replicates). To evaluate the influence of tumor content on limit of detection (LOD), one tumor sample was combined with the matched normal sample to simulate a tumor content of 5%, 10%, 15% and 20%.

Each sample in the study was tested on at least one instrument with two OncoMate® MSI Dx Analysis System lots at six different concentrations by two operators (two replicates/operator/sample) and over 5 days for a total of 20 replicates per sample to identify LOD. Because there were no failures based upon the amount of DNA tested, additional testing was necessary to identify the LOD. New sample dilutions were prepared to test a 0.1ng per reaction DNA input using samples with a 20% tumor content, as well as a 1ng per reaction DNA input using a sample containing 2.5% tumor.

A summary of the interpretive results for the MSI-H and MSS cases are summarized in Table 48 for the DNA input study, and for MSI-H cases in the tumor content study in Table 49. Table 50 displays a summary of reference result vs. interpretive result and locus status by sample ID. The results for all samples tested at 1ng DNA input and 20% tumor content are shown. Table 51 displays a summary of reference result vs interpretive result and locus status by sample ID. The results for all samples tested at 0.5, 1.0 and 2.0ng DNA input and 20% and 30% tumor content are shown in Table 51.

The LOD for the OncoMate® MSI Dx Analysis System was determined across two dimensions: 1) the total amount of input DNA used for the assay and 2) the fraction of tumor DNA present in the sample. The LOD for the OncoMate® MSI Dx Analysis System was established at 30% tumor content based on concordance across all loci when using the recommended 1ng DNA input and samples where tumor content was adjusted by blending with DNA extracted from matched normal tissue.

Table 48. Interpretive Result Frequency for the DNA Input Study by Reagent Lot.

Lot by Final DNA Input (ng)		OncoMate® Interpretive Result (MSI-H Diluted Sample Set)			OncoMate® Interpretive Result (MSS Diluted Sample Set)		
		MSI-H	No Call	Total	MSS	No Call	Total
Lot 1	0.1	20	80	100	0	20	20
	0.2	99	1[1]	100	20	0	20
	0.5	100	0	100	20	0	20
	1.0	100	0	100	20	0	20
	2.0	100	0	100	20	0	20
	2.5	100	0	100	20	0	20
Lot 2	0.1	24	76	100	0	20	20
	0.2	100	0	100	20	0	20
	0.5	100	0	100	20	0	20
	1.0	100	0	100	20	0	20
	2.0	100	0	100	20	0	20
	2.5	100	0	100	20	0	20
All	0.1	44	156	200	0	40	40
	0.2	199	1	200	40	0	40
	0.5	200	0	200	40	0	40
	1.0	200	0	200	40	0	40
	2.0	200	0	200	40	0	40
	2.5	200	0	200	40	0	40

[1]One No Call due to low allele peak height detected.

Table 49. Interpretive Result Frequency for the Tumor Concentration Study, by Reagent Lot.

Lot by Percent Tumor Content		OncoMate® Interpretive Result		Total
		MSI-H	MSS	
Lot 1	2.5	0	20	20
	5	20	0	20
	10	20	0	20
	15	20	0	20
	20	20	0	20
Lot 2	2.5	0	20	20
	5	20	0	20
	10	20	0	20
	15	20	0	20
	20	20	0	20
All	2.5	0	40	40
	5	40	0	40
	10	40	0	40
	15	40	0	40
	20	40	0	40

Table 50. Summary of Reference Result vs. Interpretive Result and Locus Status with 95% Wilson-Score CI by Sample (1ng DNA Input and 20% Tumor Content).

	20% Tumor Content					
Sample (MSI Status)	DNA Input (ng)	NR-21 (n/N); Percent Concordant to Reference Result (95% CI)	BAT-26 (n/N); Percent Concordant to Reference Result (95% CI)	BAT-25 (n/N); Percent Concordant to Reference Result (95% CI)	NR-24 (n/N); Percent Concordant to Reference Result (95% CI)	MONO-27 (n/N); Percent Concordant to Reference Result (95% CI)
2800M (MSS)	1.0	40/40; 100% (91.2–100)	40/40; 100% (91.2–100)	40/40; 100% (91.2–100)	40/40; 100% (91.2–100)	40/40; 100% (91.2–100)
CRC-066 (MSI-H)	1.0	40/40; 100% (91.2–100)	40/40; 100% (91.2–100)	40/40; 100% (91.2–100)	40/40; 100% (91.2–100)	40/40; 100% (91.2–100)
CRC-076 (MSI-H)	1.0	40/40; 100% (91.2–100)	40/40; 100% (91.2–100)	40/40; 100% (91.2–100)	17/40; 42.5% (28.5–57.8)	40/40; 100% (91.2–100)
CRC-079 (MSI-H)	1.0	40/40; 100% (91.2–100)	40/40; 100% (91.2–100)	40/40; 100% (91.2–100)	40/40; 100% (91.2–100)	40/40; 100% (91.2–100)
CRC-081 (MSI-H)	1.0	36/40; 90% (77.0–96.0)	40/40; 100% (91.2–100)	40/40; 100% (91.2–100)	40/40; 100% (91.2–100)	39/40; 97.5 (87.1–99.6)
CRC-084 (MSI-H)	1.0	40/40; 100% (91.2–100)	40/40; 100% (91.2–100)	6/40; 15% (7.1–29.1)	2/40; 5% (1.4–16.5)	40/40; 100% (91.2–100)
CRC-213 (MSI-H)	1.0	40/40; 100% (91.2–100)	40/40; 100% (91.2–100)	40/40; 100% (91.2–100)	40/40; 100% (91.2–100)	40/40; 100% (91.2–100)

Table 51. Summary of Reference Result vs. Interpretive Result and Locus Status with 95% Wilson-Score CI by Sample (Supplemental Study, All DNA Inputs, 20% and 30% Tumor Content).

Sample (All MSI-H)	Percent Tumor Content	DNA Input (ng)	NR-21 (n/N); Percent Concordant to Reference Result (95% CI)	BAT-26 (n/N); Percent Concordant to Reference Result (95% CI)	BAT-25 (n/N); Percent Concordant to Reference Result (95% CI)	NR-24 (n/N); Percent Concordant to Reference Result (95% CI)	MONO-27 (n/N); Percent Concordant to Reference Result (95% CI)
CRC-066	20	0.5	20/20; 100% (83.9–100)	20/20; 100% (83.9–100)	19/20; 95% (76.4–99.1)	18/20; 90% (69.9–99.2)	17/20; 85% (64.0–94.8)
		1.0	20/20; 100% (83.9–100)	20/20; 100% (83.9–100)	20/20; 100% (83.9–100)	20/20; 100% (83.9–100)	20/20; 100% (83.9–100)
		2.0	20/20; 100% (83.9–100)	20/20; 100% (83.9–100)	20/20; 100% (83.9–100)	20/20; 100% (83.9–100)	20/20; 100% (83.9–100)
CRC-066	30	0.5	20/20; 100% (83.9–100)	20/20; 100% (83.9–100)	20/20; 100% (83.9–100)	20/20; 100% (83.9–100)	20/20; 100% (83.9–100)
		1.0	20/20; 100% (83.9–100)	20/20; 100% (83.9–100)	20/20; 100% (83.9–100)	20/20; 100% (83.9–100)	20/20; 100% (83.9–100)
		2.0	20/20; 100% (83.9–100)	20/20; 100% (83.9–100)	20/20; 100% (83.9–100)	20/20; 100% (83.9–100)	20/20; 100% (83.9–100)
CRC-079	20	0.5	20/20; 100% (83.9–100)	20/20; 100% (83.9–100)	20/20; 100% (83.9–100)	20/20; 100% (83.9–100)	17/20; 85% (64.0–94.8)
		1.0	20/20; 100% (83.9–100)	20/20; 100% (83.9–100)	20/20; 100% (83.9–100)	20/20; 100% (83.9–100)	20/20; 100% (83.9–100)
		2.0	20/20; 100% (83.9–100)	20/20; 100% (83.9–100)	20/20; 100% (83.9–100)	20/20; 100% (83.9–100)	20/20; 100% (83.9–100)
CRC-079	30	0.5	20/20; 100% (83.9–100)	20/20; 100% (83.9–100)	20/20; 100% (83.9–100)	20/20; 100% (83.9–100)	20/20; 100% (83.9–100)
		1.0	20/20; 100% (83.9–100)	20/20; 100% (83.9–100)	20/20; 100% (83.9–100)	20/20; 100% (83.9–100)	20/20; 100% (83.9–100)
		2.0	20/20; 100% (83.9–100)	20/20; 100% (83.9–100)	20/20; 100% (83.9–100)	20/20; 100% (83.9–100)	20/20; 100% (83.9–100)
CRC-084	20	0.5	20/20; 100% (83.9–100)	20/20; 100% (83.9–100)	2/20; 10% (2.8–30.1)	0/20; 0% (0–16.1)	20/20; 100% (83.9–100)
		1.0	20/20; 100% (83.9–100)	20/20; 100% (83.9–100)	1/20; 5% (0.9–23.6)	0/20; 0% (0–16.1)	20/20; 100% (83.9–100)
		2.0	20/20; 100% (83.9–100)	20/20; 100% (83.9–100)	0/20; 0% (0–16.1)	0/20; 0% (0–16.1)	20/20; 100% (83.9–100)
CRC-084	30	0.5	20/20; 100% (83.9–100)	20/20; 100% (83.9–100)	20/20; 100% (83.9–100)	8/20; 40% (21.9–61.3)	20/20; 100% (83.9–100)
		1.0	20/20; 100% (83.9–100)	20/20; 100% (83.9–100)	19/20; 95% (76.4–99.1)	0/20; 0% (0–16.1)	20/20; 100% (83.9–100)
		2.0	20/20; 100% (83.9–100)	20/20; 100% (83.9–100)	20/20; 100% (83.9–100)	0/20; 0% (0–16.1)	20/20; 100% (83.9–100)

23.5 Analytical Specificity

Primer pairs for the seven OncoMate® MSI Dx Analysis System markers were checked for target specificity using the publicly available Primer BLAST search tool on the US National Center for Biotechnology Information web site (**https://ncbi.nlm.nih.gov/tools/primer-blast/**, accessed 01/14/2020; 27). The primers share 100% identity with their intended targets, and Primer BLAST results predicted specific target amplification.

An analytical specificity study evaluated primer specificity of the OncoMate® MSI Dx Analysis System and demonstrated compatibility of the system with different thermal cycler models.

The 2800M Control DNA was used, and replicates were treated as MSS samples for analysis using the OncoMate® MSI Dx Interpretive Software. Samples were amplified in duplicate with the OncoMate® MSI Dx Analysis System amplification kit using 1ng, 2ng or 4ng of DNA on each of three different thermal cycler models. The thermal cyclers (Applied Biosystems Veriti® 96-Well Thermal Cycler, Eppendorf MasterCycler® Nexus SX1 Thermal Cycler and BioRad C1000 Touch™ Thermal Cycler) all fall within the following required performance specifications:

Maximum Block Ramp Rate: 3.9°C/second to 5°C/second

Temperature Accuracy: ±0.25°C (at ≥90°C)

Temperature Uniformity: <0.5°C (at ≥90°C)

Heated lid capable of reaching 103–105°C

There were no invalid results or samples requiring reinjection or reamplification testing for this study.

There was 100% agreement (36/36) between the expected and observed MSS call for all samples. There were no artifacts observed that interfered with the system's ability to provide the expected interpretive result when using different thermal cyclers.

23.6 Interfering Substances

A study was performed to establish the potential influence of interfering substances on the performance of the OncoMate® MSI Dx Analysis System, specifically chaotropic salts, alcohol, proteinase K treatment time, necrotic tissue, hemoglobin, triglycerides and mucin. DNA was extracted from sample curls (0.1–2mm^3 tissue). DNA extraction was performed for each sample at each condition tested using the Maxwell® CSC Instrument and Maxwell® CSC DNA FFPE Kit.

In the first series of experiments, lysates from four tumor and matched normal samples were spiked with hemoglobin (2mg/ml final concentration), triglycerides (37mM final concentration) or mucin (1mg/ml final concentration) prior to DNA extraction. Following analysis with the OncoMate® MSI Dx Analysis System, all samples yielded the expected result.

Four tumor and matched normal samples (two replicates per sample for a total of 16 extractions) were incubated at 56°C in the presence of proteinase K for 20 minutes, 30 minutes (recommended condition) or 40 minutes prior to purification. All samples yielded the expected result.

Twelve tumor samples with necrotic tissue ranging from 0–75% and matched normal samples were tested. All samples yielded the expected result.

To evaluate the impact of potential carry-over alcohol or guanidine salts from the DNA purification process, aliquots of the extracted DNA from tumor and matched normal samples with varying amounts of tissue necrosis (0–75%) were spiked with ethanol (5% final concentration), guanidine hydrochloride (50µM final concentration) or water prior to amplification and analysis. All samples yielded the expected result.

There were 13 Invalid results initially obtained across the Interfering Substances testing. All 13 samples were reinjected, resulting in five samples being resolved. The remaining eight samples were resolved after reamplification.

In conclusion, the OncoMate® MSI Dx Analysis System showed no statistically significant impact on assay performance by the interfering substances, specifically chaotropic salts (50µM guanidine hydrochloride), ethanol (5%), necrotic tissue (0–75%), hemoglobin (2mg/ml), triglycerides (37mM), and mucin (1mg/ml) or proteinase K digestion time [20, 30 (standard) or 40 minutes] tested in this study (Table 52).

Table 52. OncoMate® MSI Dx Analysis System Sample Treatment by Interpretative Result.

	Interpretive Result[1]				Total	
	MSI-H		MSS			
Sample Treatment	**N**	**%**	**N**	**%**	**N**	**%**
20 minutes at 56°C	4	50.0	4	50.0	8	100
30 minutes at 56°C	4	50.0	4	50.0	8	100
40 minutes at 56°C	4	50.0	4	50.0	8	100
Ethanol spike	8	50.0	8	50.0	16	100
Guanidine spike	8	50.0	8	50.0	16	100
Hemoglobin	4	50.0	4	50.0	8	100
Mucin	4	50.0	4	50.0	8	100
Necrosis	14	58.3	10	41.7	24	100
Triglycerides	4	50.0	4	50.0	8	100
Water spike	8	50.0	8	50.0	16	100
Total	62	51.7	58	48.3	120	100

[1]All samples returned the expected result (MSS or MSI-H) in interfering substances experiments.

23.7 Cross-Contamination

The sample-to-sample cross-contamination in the OncoMate® MSI Dx Analysis System was evaluated using extracted DNA from an MSI-H sample and an MSS sample, including both the tumor and its matched normal sample. The samples and reagent blanks were interspersed in a grid design across 96-well plates. In this plate layout, amplification, capillary electrophoresis and analysis were performed each day for a total of 10 days. Expected results included MSI-H, MSS and No Call test results. The No Call test result was expected for the reagent blanks. The results were not averaged, and any observed carryover was reported.

There were a total of three Invalid results initially obtained in the study. After reinjection, all three sample results were resolved. No reamplification was performed for this study.

There was 100% concordance between the interpretative result and the expected results for the 470 samples analyzed, and no interference with data interpretation was observed. The study concluded the OncoMate® MSI Dx Analysis System was not susceptible to sample-to-sample cross-contamination.

23.8 Reproducibility

Precision and reproducibility for the OncoMate® MSI Dx Analysis System was evaluated across multiple sites, operators, runs, days, replicates and assay kit lots. Data were assessed for between-site, between-operator, between-run, between-day, within-run and between-lot repeatability and precision.

The test panel consisted of seven CRC samples (four MSI-H and three MSS cases), a negative amplification control (Water, Amplification Grade) and a positive amplification control (2800M Control DNA). Two of the MSI-H samples were adjusted to a 20% tumor burden by mixing DNA extracted from the tumor sample with matched normal DNA. The samples were blinded and distributed to the operators at each of the test sites. The panel of samples was tested by two operators located at each of three sites on three instruments (one at each site). Two external sites and one internal site served as the three test sites. Three reagent lots were used for two runs per day, on three nonconsecutive days.

The Positive Percent Agreements (PPAs) for MSI-H and Negative Percent Agreements (NPAs) for MSS interpretative results versus expected results (Table 53) were reproducible for site, operator, day, lot and run. The PPAs for site, operator, day, lot and run ranged from 89.6–97.9% and the NPAs ranged from 97.2–100%, demonstrating reproducibility for each factor tested. The overall reproducibility PPA (95% CI) and NPA (95% CI) were 95.5% (92.4–97.6%) and 99% (97.4–100%), respectively.

Table 54 provides the absolute and relative frequency of interpretive results by sample ID.

A summary of PPA with a 95% Wilson-Score CI for interpretive results and locus status vs reference result is shown in Table 55.

A total of 13 results were initially Invalid during testing. All 13 samples were re-injected, resulting in eight samples that resolved and five samples that were not resolved. Upon re-amplification, three of the five unresolved samples were resolved. A total of two samples remained Invalid after re-amplification and are included in the agreement analysis below.

In conclusion, this study demonstrated that the OncoMate® MSI Dx Analysis System and reagents are reproducible between and across sites, operators, days, lots and runs.

Table 53. Summary of PPA and NPA for Interpretative Result vs. Reference Result.

Factor	Item	PPA		NPA	
		Percent (#/n)	95% CI	Percent (#/n)	95% CI
Site	1	96.9 (93/96)	91.1–99.4	98.6 (71/72)	92.5–100
	2	92.7 (89/96)	85.6–97.0	100 (72/72)	95–100
	3	96.9 (93/96)	91.1–99.4	100 (72/72)	95–100
Operator	1	97.9 (47/48)	88.9–100	100 (36/36)	90.3–100
	2	95.8 (46/48)	85.8–99.5	97.2 (35/36)	85.5–99.9
	3	89.6 (43/48)	77.3–96.5	100 (36/36)	90.3–100
	4	95.8 (46/48)	85.8–99.5	100 (36/36)	90.3–100
	5	97.9 (47/48)	88.9–100	100 (36/36)	90.3–100
	6	95.8 (46/48)	85.8–99.5	100 (36/36)	90.3–100
Day	1	93.8 (90/96)	86.9–97.7	98.6 (71/72)	92.5–100
	2	95.8 (92/96)	89.7–98.8	100 (72/72)	95–100
	3	96.9 (93/96)	91.1–99.4	100 (72/72)	95–100
Lot*	1	95.8 (92/96)	89.7–98.8	100 (72/72)	95–100
	2	94.8 (91/96)	88.3–98.3	98.6 (71/72)	92.5–100
	3	95.8 (92/96)	89.7–98.8	100 (72/72)	95–100
Run*	A	94.4 (136/144)	89.4–97.6	100 (108/108)	96.6–100
	B	96.5 (139/144)	92.1–98.9	99.1 (107/108)	95.0–100
Total		95.5 (275/288)	92.4–97.6	99.5 (215/216)	97.4–100

*Not all lots tested in a run.

Table 54. Absolute and Relative Frequencies for Interpretative Results by Sample ID.

	Reference Result												
	MSI-H						MSS						
Interpretative Result	MSI-H		MSS		Invalid		MSI-H		MSS		Invalid		Total
Sample ID	N	%	N	%	N	%	N	%	N	%	N	%	N
AS_REP_01	59	81.9	12	16.7	1	1.4	0	0	0	0	0	0	72
AS_REP_02	72	100	0	0	0	0	0	0	0	0	0	0	72
AS_REP_03	72	100	0	0	0	0	0	0	0	0	0	0	72
AS_REP_04	72	100	0	0	0	0	0	0	0	0	0	0	72
AS_REP_05	0	0	0	0	0	0	0	0	71	98.6	1	1.4	72
AS_REP_06	0	0	0	0	0	0	0	0	72	100	0	0	72
AS_REP_07	0	0	0	0	0	0	0	0	72	100	0	0	72
Total	275	95.5	12	4.2	1	0.3	0	0	215	99.5	1	0.5	504

Table 55. Summary of PPA and 95% Wilson-Score CI for Interpretative Result and Locus Status.

Sample	Reference MSI Status	Agreement to Reference Status (n/N); Percent PPA (95% CI)	NR-21 (n/N); Percent Agreement (95% CI)	BAT-26 (n/N); Percent Agreement (95% CI)	BAT-25 (n/N); Percent Agreement (95% CI)	NR-24 (n/N); Percent Agreement (95% CI)	MONO-27 (n/N); Percent Agreement (95% CI)
AS_REP_01	MSI-H	59/71; 83.1% (72.7–90.1)	71/71; 100% (94.9–100)	71/71; 100% (94.9–100)	27/71; 38.0% (27.6–49.7)	71/71; 100% (94.9–100)	53/71; 74.6% (63.4–83.3)
AS_REP_02	MSI-H	72/72; 100% (94.9–100)	72/72; 100% (94.9–100)	72/72; 100% (94.9–100)	72/72; 100% (94.9–100)	72/72; 100% (94.9–100)	72/72; 100% (94.9–100)
AS_REP_03	MSI-H	72/72; 100% (94.9–100)	72/72; 100% (94.9–100)	72/72; 100% (94.9–100)	72/72; 100% (94.9–100)	72/72; 100% (94.9–100)	72/72; 100% (94.9–100)
AS_REP_04	MSI-H	72/72; 100% (94.9–100)	72/72; 100% (94.9–100)	72/72; 100% (94.9–100)	72/72; 100% (94.9–100)	72/72; 100% (94.9–100)	72/72; 100% (94.9–100)
AS_REP_05	MSS	71/71; 100% (94.9–100)	71/71; 100% (94.9–100)	71/71; 100% (94.9–100)	71/71; 100% (94.9–100)	71/71; 100% (94.9–100)	71/71; 100% (94.9–100)
AS_REP_06	MSS	72/72; 100% (94.9–100)	72/72; 100% (94.9–100)	72/72; 100% (94.9–100)	72/72; 100% (94.9–100)	71/71; 100% (94.9–100)	72/72; 100% (94.9–100)
AS_REP_07	MSS	72/72; 100% (94.9–100)	72/72; 100% (94.9–100)	72/72; 100% (94.9–100)	72/72; 100% (94.9–100)	72/72; 100% (94.9–100)	72/72; 100% (94.9–100)

23.9 Method Comparison Studies

The primary objective of the method comparison study was to evaluate the accuracy and usability of the OncoMate® MSI Dx Analysis System in identifying microsatellite instability in the clinical setting. Tumors from Lynch syndrome patients have a high likelihood of having an MSI-H status (2, 4, 17), and therefore MSI-H status can identify patients where further genetic testing for Lynch syndrome is recommended.

A method comparison was performed between the OncoMate® MSI Dx Analysis System and the VENTANA MMR IHC Panel, the predicate device to identify Lynch syndrome candidates, which stains for the presence or absence of DNA mismatch repair proteins. A comparison of the OncoMate® MSI Dx Analysis System result to germline Next Generation Sequencing for DNA mismatch repair genes (NGS MMR genes) was performed to confirm identification of Lynch syndrome patients.

The study was performed by testing a sequential series of 130 colorectal cancer patient samples that were enriched with a second set of 24 suspected Lynch syndrome samples, for a total of 154 cases. Sample curls, generated from FFPE tissue blocks, were provided to an external laboratory to perform immunohistochemistry. Extracted DNA aliquots of 154 matched CRC DNA samples of unknown MSI status were randomized then analyzed by the OncoMate® MSI Dx Analysis System and NGS.

Immunohistochemistry was performed on all 154 tumor samples to determine protein expression of the MHL1, MSH2, MSH6 and PMS2 genes using the VENTANA MMR IHC Panel on the provided sample curls, per the VENTANA instructions for use and the laboratory's Standard Operating Procedures. BRAF testing was only performed on cases with loss of staining for MLH1 protein.

DNA from the 154 samples were provided to a reference laboratory for Next Generation Sequencing. These samples underwent DNA sequencing to determine the presence or absence of pathogenic mutations of the mismatch repair genes (MLH1, MSH2, MSH6 and PMS2) and BRAF exon 15. Upon germline testing, 18 Lynch syndrome cases were confirmed. Only pathogenic or likely pathogenic mutations listed in the ClinVar database (22) were accepted as confirmed Lynch syndrome cases.

A total of two samples yielded Invalid results during initial testing. Both samples were resolved upon re-injection.

23.10 Method Comparison: OncoMate® MSI Dx Analysis System vs. IHC Results

The comparison results between the OncoMate® MSI Dx Analysis System and the VENTANA IHC MMR Panel for the 154 samples are listed in Table 56. A total of 106 samples were scored as MSS using the OncoMate® MSI Dx Analysis System and MMR Intact for all four MMR proteins using the VENTANA IHC MMR Panel. Forty-five samples exhibited a loss of IHC staining for at least one of the four MMR proteins (dMMR). Of these 45 samples, 44 samples were scored as MSI-H by the OncoMate® MSI Dx Analysis System.

Three samples were scored as MSI-H by the OncoMate™ MSI Dx Analysis System but were scored as MMR Intact by IHC staining for all four MMR proteins. The data are summarized in Table 56 and Table 57.

The Positive Percent Agreement (PPA) was 97.8% and the Negative Percent Agreement (NPA) was 97.2%, with an Overall Percent Agreement (OPA) of 97.4% between the two methods. Additional comparison and agreement analysis data stratified by sequential and enrichment cohort can be found in Table 57 and Table 58. The OncoMate® MSI Dx Analysis System effectively identified tumors with MMR deficiency and shows strong agreement with the VENTANA MMR IHC panel.

Table 56. Comparison and Agreement Analysis of the OncoMate® MSI Dx Analysis System Interpretative Results vs. Ventana MMR IHC (All Samples).

OncoMate® MSI Dx Analysis System	Ventana MMR IHC Results			
	MMR Loss	MMR Intact	Invalid	Total
MSI-H	44	3	0	47
MSS	1	106	0	107
Invalid	0	0	0	0
Total	45	109	0	154

	Agreement		
Type	n/N	Percent	95% CI
PPA	44/45	97.8	88.4–99.6
NPA	106/109	97.2	92.2–99.1
OPA	150/154	97.4	93.5–99.0

Table 57. Comparison and Agreement of the OncoMate® MSI Dx Analysis System Interpretative Results vs. Ventana MMR IHC (Sequential Cohort).

OncoMate® MSI Dx Analysis System	Ventana MMR IHC Results			
	MMR Loss	MMR Intact	Invalid	Total
MSI-H	23	0	0	23
MSS	1	106	0	107
Invalid	0	0	0	0
Total	24	106	0	130

	Agreement		
Type	n/N	Percent	95% CI
PPA	23/24	95.8	79.8–99.3
NPA	106/106	100.0	96.5–100.0
OPA	129/130	99.2	95.8–99.9

Table 58. Comparison and Agreement Analysis of the OncoMate® MSI Dx Analysis System Interpretative Results vs. Ventana MMR IHC (Enrichment Cohort).

OncoMate® MSI Dx Analysis System	Ventana MMR IHC Results			
	MMR Loss	MMR Intact	Invalid	Total
MSI-H	21	3	0	24
MSS	0	0	0	0
Invalid	0	0	0	0
Total	21	3	0	24

	Agreement		
Type	n/N	Percent	95% CI
PPA	21/21	100.0	84.5–100.0
NPA	0/3	0.0	0.0–56.1
OPA	21/24	87.5	69.0–95.7

23.11 Method Comparison to NGS Mismatch Repair Gene Mutations Results

A total of 18 samples tested positive for Lynch syndrome, based on detection of a pathogenic or likely pathogenic mutation in one of the mismatch repair genes (MLH1, MSH2, MSH6 or PMS2) and no mutations in BRAF exon 15, two of these 18 cases were from the sequential cohort. The data for all samples are summarized in Table 59. Table 60 and Table 61 summarize the data for the sequential cohort and enrichment cohort, respectively. Seventeen of the samples (17/18) tested MSI-H with the OncoMate® MSI Dx Analysis System. One of the samples (1/18) tested MSS with the OncoMate® MSI Dx Analysis System and exhibited no loss of MMR protein by IHC. This sample is referenced as having a likely pathogenic mutation in the PMS2 gene on the ClinVar database (22). This single nucleotide polymorphism (rs267608153) results in a c.903G>T variant that likely results in a splicing defect (25). All 16 of the enrichment cohort samples test MSI-H with the OncoMate® MSI Dx Analysis System.

The Positive Percent Agreement (PPA) was 94.4% and the Negative Percent Agreement (NPA) was 77.9% between the two methods for all samples. The PPA was 100% between the two methods for the enrichment cohort. The NPA is less informative than the PPA in a comparison of somatic microsatellite instability to germline mutations in MMR genes, because cases negative for germline, pathogenic Lynch syndrome mutations include MSI-H cases with sporatic, somatic causes for dMMR as well as MSS cases. Somatic mechanisms such as epigenetic silencing and biallelic somatic mutation can lead to dMMR and an MSI-H phenotype (26) without a germline MMR gene mutation. In conclusion, the OncoMate® MSI Dx Analysis System effectively identified tumors with confirmed germline MMR mutations indicative of Lynch syndrome.

Table 59. Comparison and Agreement of the OncoMate® MSI Dx Analysis System Interpretative Results vs. NGS MMR (All Samples).

	DNA Sequencing Results			
OncoMate® MSI Dx Analysis System	**Pathogenic Mutation**	**No Pathogenic Mutation**	**Invalid**	**Total**
MSI-H	17	30	0	47
MSS	1	106	0	107
Invalid	0	0	0	0
Total	18	136	0	154

	Agreement		
Type	**n/N**	**Percent**	**95% CI**
PPA	17/18	94.4	74.2–99.0
NPA	106/136	77.9	70.3–84.1
OPA	123/154	79.9	72.8–85.4

Table 60. Comparison and Agreement of the OncoMate® MSI Dx Analysis System Interpretative Results vs. NGS MMR (Sequential Cohort)

OncoMate® MSI Dx Analysis System	DNA Sequencing Results			
	Pathogenic Mutation	No Pathogenic Mutation	Invalid	Total
MSI-H	1	12	0	13
MSS	1	106	0	107
Invalid	0	0	0	0
Total	2	128	0	130

	Agreement		
Type	n/N	Percent	95% CI
PPA	1/2	50.0	9.5–90.5
NPA	106/128	82.8	75.3–88.4
OPA	107/130	82.3	74.8–87.9

Table 61. Comparison and Agreement of the OncoMate® MSI Dx Analysis System Interpretative Results vs. NGS MMR (Enrichment Cohort)

OncoMate® MSI Dx Analysis System	DNA Sequencing Results			
	Pathogenic Mutation	No Pathogenic Mutation	Invalid	Total
MSI-H	16	8	0	24
MSS	0	0	0	0
Invalid	0	0	0	0
Total	16	8	0	24

	Agreement		
Type	n/N	Percent	95% CI
PPA	16/16	100.0	80.6–100
NPA	0/8	0.0	0.0–32.4
OPA	16/24	66.7	46.7–82.0

24. Troubleshooting

For questions not addressed here, consult the other technical manuals associated with the OncoMate® MSI Dx Analysis System (listed in Section 15.1). Any time a new sample batch is prepared, including for troubleshooting QC Failed or No Call results, the matched normal or tumor sample(s) and the Positive and Negative Amplification Controls must also be analyzed (see Section 21, Assay Quality Controls). See Table 45 for information about QC Details messages displayed by the OncoMate® MSI Dx Interpretive Software. Where applicable, a summary of recommended actions is provided, describing the typical troubleshooting steps required to address QC failures reported by the interpretive software. Additional investigation into root causes may be necessary, following the information provided in the Causes and Comments column.

Contact information available at: **www.promega.com**; e-mail: **genetic@promega.com**.

Symptoms	Causes and Comments
Capillary electrophoresis (CE) run failed to start after selecting **Start Run** in the Applied instrument Biosystems® 3500 Dx Genetic Analyzer data collection software.	Bubbles were present in the instrument fluidics. Run the Remove Bubble wizard to clear the bubbles in the fluidics, and then restart the CE run.
	An error occurred on the system computer. Reboot the CE instrument and the instrument's computer following the manufacturer's instructions, and then restart the CE run.
	One or more of the instrument consumables was expired or has reached the sample limit. Replace the expired or exhausted instrument consumable(s), and then restart the CE run.
Invalid sample result obtained. Size Standard 500 fragments showed low resolution, fewer fragments than expected, unexpected peaks or if low peak intensities (amplicons also may be affected) OncoMate® MSI Dx Interpretive Software QC details: “Poor sizing quality”	**Recommended Actions:** 1. Repeat capillary electrophoresis. 2. See Causes and Comments below for additional guidance the error persists. Ensure that 11µl of loading cocktail containing Size Standard 500 is used during capillary electrophoresis.
	Bubbles were present in the instrument fluidics. Run the Remove Bubble wizard to clear the bubbles in the instrument fluidics, and then repeat CE analysis for the affected samples, including positive and negative controls. Review the electropherogram in the Applied Biosystems® 3500 Dx Genetic Analyzer DCS to troubleshoot the root cause of the sizing quality flag.

Symptoms	Causes and Comments
Invalid sample result obtained. Size Standard 500 fragments showed low resolution, fewer fragments than expected, unexpected peaks or low peak intensities (amplicons also may be affected) (continued) OncoMate® MSI Dx Interpretive Software	Contaminants or crystal deposits were present in the polymer. Repeat CE analysis for the affected samples, including positive and negative controls. Review the electropherogram available through the Applied Biosystems® 3500 Dx Genetic Analyzer DCS to troubleshoot the root cause of the sizing quality flag. When replenishing the polymer, ensure it is brought to room temperature as directed by the manufacturer.
QC details: "Poor sizing quality"	One or more capillaries were blocked. Refill the capillary array, and repeat CE analysis for the affected samples, including positive and negative controls. Installation of a new capillary array may be necessary. Review the electropherogram available through the Applied Biosystems® 3500 Dx Genetic Analyzer DCS to troubleshoot the root cause of the sizing quality flag.
	CE-related artifacts. Aberrant peaks may be observed during CE. When these affect the orange dye channel, the Size Standard 500 peaks may be obscured or mis-assigned by the software resulting in a "Poor Sizing Quality" flag. Repeat CE analysis for the affected samples, including positive and negative controls. Change instrument reagents if problem persists. Review the electropherogram available through the Applied Biosystems® 3500 Dx Genetic Analyzer DCS to troubleshoot the root cause of the sizing quality flag.
A quality warning was displayed in the Software Applied Biosystems® 3500 Dx Genetic Analyzer Data Collection Software, but sample or control data were analyzed successfully using the OncoMate® MSI Dx Interpretive Software.	Data analysis using the OncoMate® MSI Dx Interpretive is application-specific. Quality warnings displayed in the data collection software may be triggered by broad peaks, signal spikes, etc. that are ignored by the interpretive software or occur outside of the analysis range relevant for MSI determination. The OncoMate® MSI Dx Interpretive Software provides the final assessment of data quality.
Samples that were analyzed during the 3500 CE run were not imported into the OncoMate® MSI Dx Interpretive Software mismatched OncoMate® OncoMate® MSI Dx Interpretive Software observation: Notification displayed in the import manager	The Sample Name entered into the Applied Biosystems® Dx Genetic Analyzer DCS was not identical for the matched tumor and normal sample pair. Sample files with sample names will not be identified as a pair by the MSI Dx Interpretive Software and will not be imported. Open the .fsa files using the Applied Biosystems® 3500 Dx Genetic Analyzer DCS, edit the 'Sample Name' field and save the updated files. See the *Applied Biosystems® 3500 Dx Genetic Analyzer and 3500xL Dx Genetic Analyzer IVD User Guide* for information about using the Rename option.

Symptoms	Causes and Comments
Samples that were analyzed during the were not imported into the OncoMate® MSI Dx Interpretive Software (continued) MSI Dx Interpretive Software observation: Notification displayed in the import manager	The required 'N' or 'T' designations were not entered (or CE run entered incorrectly) into the Applied Biosystems® 3500 Dx Genetic Analyzer DCS for the matched tumor and normal sample pair. Matched samples that are not properly designated as Normal (N) or Tumor (T) in UDF1 of the Applied OncoMate® Biosystems® 3500 Dx Genetic Analyzer DCS will not be imported by the OncoMate® MSI Dx Interpretive software. Open the .fsa files using the Applied Biosystems® 3500 Dx Genetic Analyzer DCS, edit UDF1 to display the correct designation and save the updated files. See the *Applied Biosystems® 3500 Dx Genetic Analyzer and 3500xL Dx Genetic Analyzer IVD User Guide* for information about opening and updating fields in .fsa files.
	An injection was repeated during the CE run using the **Reinject** option in the Applied Biosystems® 3500 Dx Genetic Analyzer DCS. When selecting the **Reinject** option in the DCS, the name(s) of the resulting .fsa file(s) will have a "_1" suffix, but the Sample Name coded within the .fsa file(s) will be the same as the original injection. An import error will be displayed in the OncoMate® MSI Dx Interpretive Software Import Manager if re-injected samples are analyzed alongside samples from the original injection because of the presence of duplicate sample pairs with the same Sample Name. Open the sample file(s) and rename the sample(s) within the DCS. Repeat batch import into the OncoMate® MSI Dx Interpretive Software. If batch import is unsuccessful, archive and purge the batch from the interpretive software and repeat batch import.
Invalid result obtained for all samples. Poor amplification or marker-to-marker imbalance in allele intensities observed for the 2800M Control are DNA amplification reaction (samples also may be affected)	**Recommended Actions:** 1. Repeat amplification for all samples. Ensure that control DNA dilutions, reagents and mixtures vortexed according to the instructions for use.
OncoMate® MSI Dx Interpretive DNA Software QC details: "No allele detected" "Marker peak height too high to evaluate"	The 2800M Control DNA stock solution or 2800M Control dilution was not vortexed sufficiently. Low and/or excessive peak intensities may be observed, including excessive amplification of Penta D alleles. Repeat amplification reactions and CE analysis, including positive and negative controls.
	The amplification mixture prepared in Section 18.5 was not vortexed sufficiently. Repeat amplification reactions and CE analysis, including positive and negative controls.

Symptoms	Causes and Comments
No Call sample result obtained. Weak or inadequate fluorescent signal for allele peaks; tumor allele intensity less than 700RFU for a mononucleotide-marker interpreted as stable OncoMate® MSI Dx Interpretive Software QC details: "No allele detected" "Low allele peak height detected"	**Recommended Actions:** 1. Repeat DNA quantitation. If there is a quantitation error, repeat amplification using 1.0ng of template DNA. repeat 2. If there is no error in quantitation or dilution, then repeat the amplification reaction with more DNA. 3. If the failure is not resolved in the previous step, then repeat the assay with DNA prepared from a different tissue section. 4. See the Causes and Comments below for additional guidance if error persists. QC Failures may result from issues with equipment and reagents.
	Poor-quality or degraded DNA. Improper or prolonged fixation of paraffin-embedded samples can result in low DNA yields, poor-quality, cross-linked or degraded DNA, and poor amplification of longer DNA fragments. Improper storage of the DNA following successful DNA extraction also may result in DNA degradation. Repeat the amplification reaction with more DNA, or repeat DNA preparation using a different tissue section.
	Insufficient template DNA. Mononucleotide markers interpreted as stable must have an allele intensity ≥700RFU for the tumor sample to ensure assay sensitivity. Allele intensities for the normal sample must be ≥175RFU, the assay calling threshold. Make sure DNA was accurately quantitated and diluted, and repeat amplification reaction using 1.0ng of template DNA if a quantitation error is discovered. Otherwise, repeat the amplification reaction with more DNA. If the problem persists, repeat the assay with DNA prepared from a different tissue section.
	Impure DNA template. Impurities in DNA preparation may inhibit PCR. See the *Maxwell® CSC DNA FFPE Kit Technical Manual* #TM395 troubleshooting section for guidance.
	High salt concentration or altered pH. Do not dilute the FFPE DNA template in TE buffers. Repeat the assay with DNA prepared from a different tissue section and use only Nuclease-Free Water to dilute template DNA.

Symptoms	Causes and Comments
No Call sample result obtained. Weak or inadequate fluorescent signal for allele peaks; tumor allele is intensity less than 700RFU for a mononucleotide-repeat marker interpreted as stable (continued) OncoMate® MSI Dx Interpretive Software QC details: “No allele detected” peak height detected”	Thermal cycler or reaction plate problems (positive control reaction also was affected). Confirm that the PCR program correct and that the selected thermal cycler meets the requirements for the OncoMate® MSI Dx Analysis System (see Section 17.4). Calibration of heat block may be required. The assay was developed using the materials listed in Section 16.3 ; the use of other materials is not supported. Repeat “Low allele amplification reactions using supported instruments and materials.
	Poor-quality formamide was used. Use only Hi-Di™ 3500 Dx Series formamide.
	Poor capillary electrophoresis injection (Size Standard 500 peaks also are affected). Repeat capillary electrophoresis analysis for affected samples and positive and negative controls.
	Samples were not properly denatured before capillary electrophoresis. Heat-denature samples for 3 minutes and cool on crushed ice or in an ice-water bath for at least 3 minutes immediately prior to capillary electrophoresis.
	Amplification reaction components were not added to the bottom of the well during PCR setup. Prior to thermal cycling, centrifuge the plate briefly in a mini plate spinner centrifuge to bring contents to the bottom of the wells and remove air bubbles.

Symptoms	Causes and Comments
Invalid sample results obtained. Fluorescent signal for allele peaks exceeds the dynamic range of the Applied Biosystems® 3500 Dx Genetic Analyzer OncoMate® MSI Dx Interpretive Software CE QC details: “Poor Sizing Quality” “Marker peak height too high to evaluate”	**Recommended Actions:** 1. Repeat DNA quantitation. If there is a quantitation error, repeat amplification using 1.0ng of template DNA. 2. If no quantitation error is discovered, then dilute amplification product 1:8 in loading cocktail and repeat analysis using 1µl of the diluted amplification product. 3. If the issue was not resolved in Step 2 , then repeat amplification of saturated sample with less DNA. 4. If the failure was not resolved in Step 3, then then repeat the assay with DNA prepared from a different tissue section.
	Too much template DNA. For severely off-scale samples, a “Poor Sizing Quality” message may be the only QC detail displayed by the OncoMate® MSI Dx Interpretive Software. Interfering bleedthrough peaks in the orange dye channel can trigger the “Poor sizing quality” flag, which suspends additional quality assessment of affected samples. Make sure DNA is accurately quantitated and diluted. Repeat amplification reaction using 1.0ng of template DNA if a quantitation error is discovered. Otherwise, dilute amplification product 1:8 in loading cocktail (e.g., 1µl of amplification product plus 7µl of loading cocktail; see Table 42 for loading cocktail composition), and repeat CE analysis using 1µl of the diluted amplification product. If the issue was not resolved, then repeat the amplification reaction with less DNA. If the problem persists, repeat DNA preparation and analysis using a different tissue section.
	FFPE sample DNA was degraded or cross-linked. Degraded or cross-linked FFPE DNA templates may exhibit excessive amplification of smaller markers, particularly NR-21. Verify correct preparation conditions for FFPE samples and storage conditions for FFPE DNA extracts. Dilute amplification product 1:8 in loading cocktail (e.g., 1µl of amplification product plus 7µl of loading cocktail; see Table 42 for loading cocktail composition), and repeat CE analysis using 1µl of the diluted amplification product. If the issue was not resolved, then repeat the amplification reaction with less DNA. If problem persists, repeating DNA preparation and analysis using a different tissue section may be necessary.

Symptoms	Causes and Comments
Invalid sample results obtained. Fluorescent signal for allele peaks exceeds the dynamic range of the Applied Biosystems® 3500 Dx Genetic Analyzer (continued)	Samples were not properly denatured prior to loading. Heat-denature samples for 3 minutes, and cool on crushed ice or in an ice-water bath for at least 3 minutes immediately prior to capillary electrophoresis.
OncoMate® MSI Dx Interpretive Software during QC details: "Poor Sizing Quality" "Marker peak height too high to evaluate"	CE-related artifacts. Aberrant peaks may be observed CE. Ensure that POP-7® polymer is warmed to room temperature before installation and use. Repeat capillary electrophoresis. If the failure is not resolved, then repeat amplification.
	Contamination of control or patient sample with another template DNA or amplified product. Cross-contamination can be a problem. Use aerosol-resistant pipette tips and change gloves regularly. DNA contamination will manifest as a batch failure in the OncoMate® MSI Dx Interpretive Software if positive or negative amplification controls are affected.
	An unstable tumor tissue sample was mistakenly analyzed as a normal tissue reference sample. Open the .fsa files using the Applied Biosystems® 3500 Dx Genetic Analyzer DCS and verify that the 'N' and 'T' designations in the .fsa files were assigned correctly relative to your PCR plate layout. Edit UDF1 to display the correct designation if the normal and tumor samples were mis-assigned in the DCS. Save the updated files. See the *Applied Biosystems® 3500 Dx Genetic Analyzer and 3500xL Dx Genetic Analyzer IVD User Guide* for information about opening and updating fields in .fsa files. Repeat amplification and analysis of affected samples if issue cannot be resolved by correcting the 'N' and 'T' designations in the .fsa files for the matched sample pair. If the issue is not resolved with reamplification then repeat the assay with DNA prepared from a different tissue section.

Symptoms	Causes and Comments
Invalid or No Call sample result observed. Unexpected peaks visible in one or more dye colors OncoMate® MSI Dx Interpretive Software details: "Unexpected allele count detected" "Unexpected peaks detected" "Broad peak shape detected"	**Recommended Actions:** 1. Repeat capillary electrophoresis. 2. If the failure was not resolved in Step 1, then repeat amplification. 3. If the failure was not resolved in Step 2, then repeat the QC assay with DNA prepared from a different tissue section.
	Excess amount of DNA. Repeat capillary electrophoresis. If the failure was not resolved, then repeat amplification following the instructions for use. If the problem persists, then then repeat the assay with DNA prepared from a different tissue section.
Invalid sample results obtained. Unexpected peaks visible in one or more dye colors OncoMate® MSI Dx Interpretive Software QC details: "Spectral issues detected"	Pull-up or bleedthrough. Pull-up, also known as bleedthrough, can occur when peak heights are excessive or if the quality of the spectral calibration was poor. Repeat spectral calibration of Applied Biosystems® 3500 Dx Genetic Analyzer and then repeat spectral calibration. If the failures was not resolved, repeat DNA quantitation. If there is a quantitation error, repeat amplification reaction using 1.0ng of template DNA.
Invalid sample result obtained. Known amplification artifact in NR-21 is detected above 87.7bp and flagged (i.e., not filtered) in the OncoMate® software MSI Dx Interpretive Software QC details: "Broad peak shape Batch status of "QC Failed" if positive amplification control is affected	Residual Conditioning Reagent is present in the pump block. Perform a bubble purge with array fill. Repeat CE analysis for the affected samples, including positive and negative controls. If the problem persists, repeat the PCR. In general, OncoMate® following the completion of the "Change Polymer Type" or "Wash Pump and Channels" wizards, complete the optional bubble purge (bubbles are observed before and after) and fill-array step when installing or reinstalling the polymer.
No Call sample result obtained. One or more were pentanucleotide alleles present in the normal sample are absent from the tumor sample OncoMate® MSI Dx Interpretive Software sample QC details: "Normal sample not detected in tumor sample"	Normal and tumor DNA samples from different individuals analyzed as a sample pair. Verify that the PCR plate layout correctly corresponds with the sample information entered into the Applied Biosystems® 3500 Dx Genetic Analyzer DCS. Repeat CE analysis of affected samples with proper pairing, including positive and negative control reactions. If problem persists, repeat amplification reactions, ensuring proper sample pairing.
	Tumor sample exhibits loss of heterozygosity. See Section 20.6 ("No Call due to sample authentication") and Figure 40 for more information about resolving No Call sample results related to tumor sample loss of heterozygosity.

Symptoms	Causes and Comments
Invalid result obtained for all samples. Batch Status is QC Failed in the OncoMate® MSI Dx Interpretive Software	**Recommended Actions:** 1. Verify that a positive and a negative control sample was amplified with the batch and imported into the software. 2. If a control sample failed QC, see the Causes and Comments below to determine whether repeating capillary electrophoresis analysis or amplification reaction is required to address the QC failure.
	Positive or Negative Control data were not imported by the OncoMate® MSI Dx Interpretive Software. When the batch is opened, the positive or negative control reaction is not listed in the Samples pane of the 'Batch Summary' tab after selecting **Control Samples** from the drop-down menu. Verify that all .fsa files (including those in subfolders) from the CE run were imported. If .fsa files for controls samples are present but were not imported, open these files in the Applied Biosystems® 3500 Series Genetic Analyzer Data Collection Software and verify that the 'Sample Type' field was assigned correctly as Positive Control or Negative Control. Update and save the .fsa files as required. If a Positive or a Negative Control was not amplified with patient samples, repeat PCR for all samples, including both positive and negative controls.
	The QC Status of the Positive or Negative Control is QC Failed. To view the control samples, open the batch and select **Control Samples** from the drop-down menu in the Samples pane of the 'Batch Summary' tab. Select the control with a status of QC Failed, switch to the 'Sample Data' tab and review the electropherograms and the QC Details information. If the electropherogram is not displayed in the interpretive software, review the electropherogram available in the Applied Biosystems® 3500 Dx Genetic Analyzer DCS to troubleshoot the root cause of the failure. If the QC failure was due to a CE anomaly such as an aberrant peak or a failed injection (see Section 20.5), repeat CE run. You may need to load fresh reagents onto the Applied Biosystems® 3500 Dx Genetic Analyzer. If the QC failure was due to an amplification-related QC issue (see Table 45), repeat PCR and CE analysis for all samples and positive and negative controls, ensuring that good laboratory practices are followed to avoid sample contamination and that the 2800M Control DNA is properly prepared (see Section 18.4).

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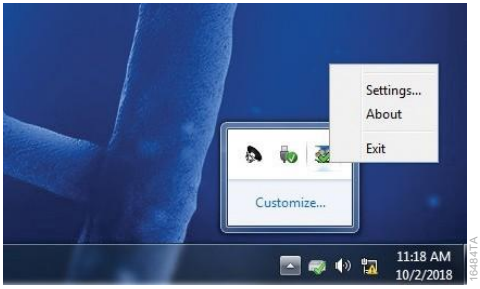
26. Additional Information

For technical assistance, call Promega Technical Services at: 1-800-356-9526 (toll-free) or 608-274-4330 or e-mail: **genetic@promega.com**

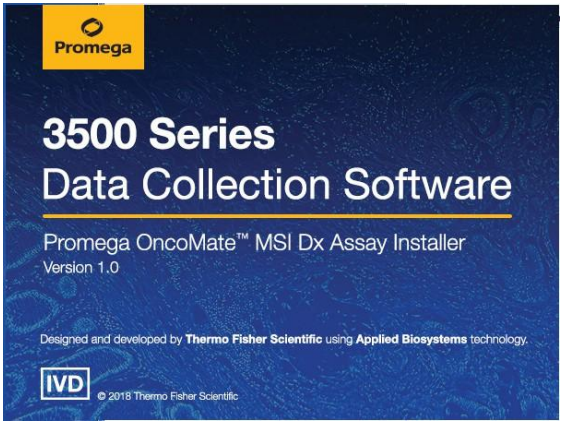
27. Appendix

27.1 OncoMate® MSI Dx Assay Installation

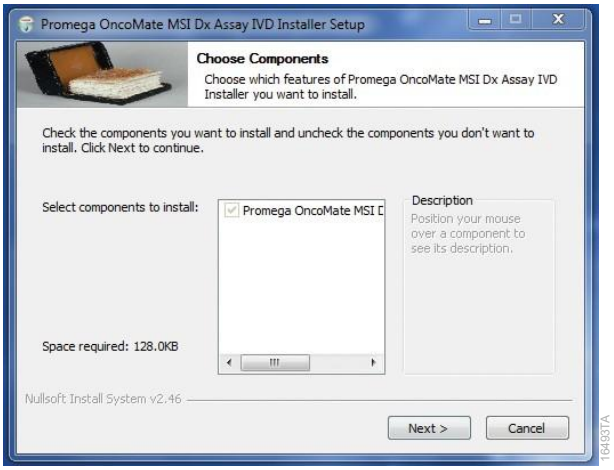
1. Copy the OncoMate_MSI_Dx_Assay_IVD_Installer.exe file to a folder on the target computer system or a memory stick.
2. Exit the Applied Biosystems® 3500 Series Data Collection Software.
3. Exit the services started by the data collection software by opening the system tray, right clicking the '3500 Server Monitor' (the icon with a bright-green check mark) and selecting **Exit** from the pop-up menu. This process will take approximately 30 seconds to complete.



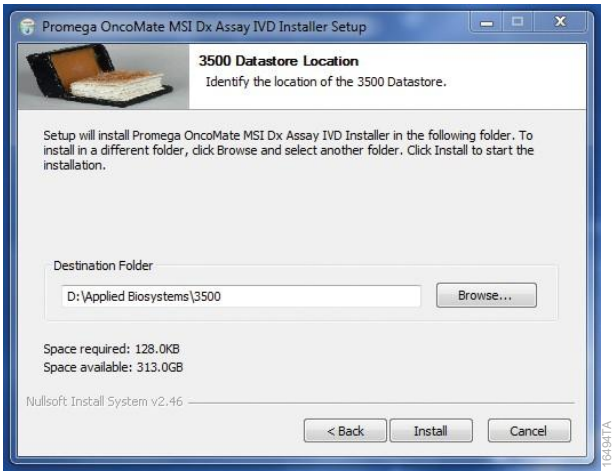
4. Double click the OncoMate_MSI_Dx_Assay_IVD_Installer.exe file to begin assay installation.
5. You will see the following splash screen:



6. When the following window appears, click **Next >**:



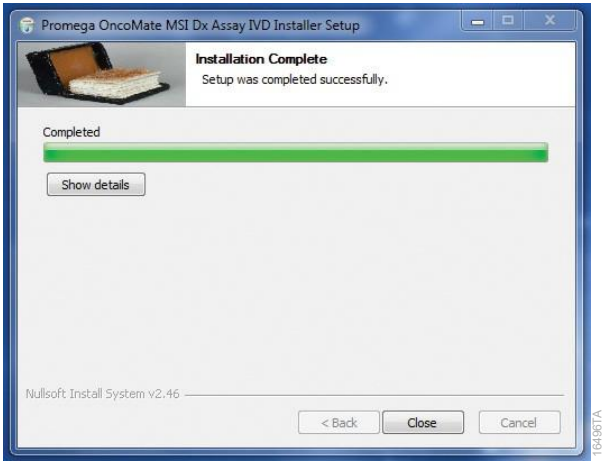
7. The destination folder for the Assay will be displayed. Click **Install**:



Note: If the Assay was previously installed, the following window will be displayed. Click **Yes** to re-install the assay:

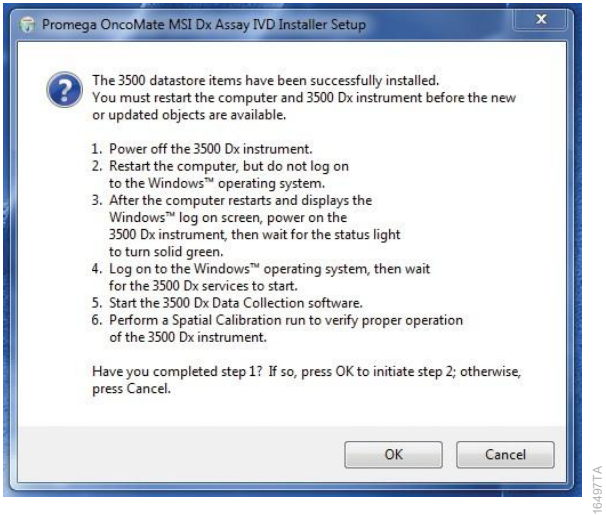


8. When the installation is completed, the following window will be displayed. Click **Close**:



9. A full system reboot is now required, and a window describing the reboot process will appear. Click **OK** and follow the system reboot instructions, or click **Cancel** and you will be reminded to restart the system later.

   Note: After **OK** is clicked, the system computer will automatically restart.



The Assay installation is logged in the file:

D:\Applied Biosystems\3500\Promega_OncoMate_MSI_Dx_Assay_Installer.log

The contents of this file appear as follows:

==========

Promega OncoMate MSI Dx Assay IVD Installation - 2020/01/06 16:39:53

==========

Install Path: D:\Applied Biosystems\3500

Output folder: D:\Applied Biosystems\3500

Rename: D:\Applied Biosystems\3500\configuration\display.properties->D:\Applied Biosystems\3500\configuration\display.properties.orig.06_01_2020_16_43_38

Copy to D:\Applied Biosystems\3500\configuration\display.properties

Delete file: D:\Users\ADMINI~1\AppData\Local\Temp\nso7D48.tmp

Extract: Promega_OncoMate_MSI_Dx_Assay_Indicator.txt

Output folder: D:\Applied Biosystems\3500

Output folder: D:\Applied Biosystems\3500\datastore\Assay

Extract: Promega_OncoMate_MSI_Dx_Assay.xml... 100%

Output folder: D:\Applied Biosystems\3500\datastore\Assay

Output folder: D:\Applied Biosystems\3500\datastore\CrAlgorithmParameter

Extract: 3500_MtxStd_50cm_OncoMate_MSI_POP7.xml... 100%

Output folder: D:\Applied Biosystems\3500\datastore\CrAlgorithmParameter

Output folder: D:\Applied Biosystems\3500\datastore\DyeSet

Extract: OncoMate_MSI.xml... 100%

Output folder: D:\Applied Biosystems\3500\datastore\DyeSet

Output folder: D:\Applied Biosystems\3500\datastore\FileNamingConventions

Extract: OncoMate_MSI_Dx.xml... 100%

Output folder: D:\Applied Biosystems\3500\datastore\FileNamingConventions

Output folder: D:\Applied Biosystems\3500\datastore\ResultsGroup

Extract: OncoMate_MSI_Dx.xml... 100%

Output folder: D:\Applied Biosystems\3500\datastore\ResultsGroup

Output folder: D:\Applied Biosystems\3500\datastore\SpectralCalibrations

Extract: 8Cap50cmOncoMate_MSIPOP7SpecCalibration.xml... 100%

Output folder: D:\Applied Biosystems\3500\datastore\SpectralCalibrations

Completed

End of Log - 2020/01/06 16:44:18

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Promega

28. Summary of Changes for Parts 1 and 2

Updates under PMA P250046 (approved TBD2026)

1. Updated Intended Use text in Section 1.5.
2. Edited Sections 1.6, 1.8, 3.2 and 12
3. Added new Section 10
4. Made minor text edits, renumbering figures and tables

Updates under PMA P240026 (approved 05NOV2025)

1. Specified Part 1 (Sections 1–14) as a companion diagnostic test protocol.
2. Replaced Intended Use text in Section 1.5.
3. Edited Sections 1.6, 1.7, 3.1, 3.5, 3.6.1, 3.6.2, 3.7.1, 4.2, 4.5, 4.9, 5.4 and 11.
4. Added Note to Section 4.
5. Updated Figure 1.
6. Added new Section 9.
7. Added the Lynch syndrome candidate protocol as Part 2 (Sections 15–27), renumbering figures and tables.
8. Made minor text edits.

[a] U.S. Pat. No. 9,139,868, European Pat. No. 2972229 and other patents pending.

[b] TMR-ET, CXR-ET and WEN dyes are proprietary.

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