TECHNICAL MANUAL

OncoMate™ MSI Dx Analysis System

Instructions for Use of Product
MD2140
For Prescription Use Only.

Promega

Promega

INSTRUCTIONS FOR USE OF PRODUCT
MD2140

Rev0
TM543

PROMEGA
2800 Woods Hollow Rd.
Madison, WI USA

OncoMate™ MSI Dx Analysis System

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1. Introduction

1.1 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 4, 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 4 to analyze amplified products by capillary electrophoresis and in Sections 5 and 6 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

1.2 Product Name

OncoMate™ MSI Dx Analysis System
Cat.# MD2140, 100 reactions Σ 100

Common Name
Fluorescent, multiplex PCR reagents

1.3 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

1.4 Key to Symbols Used

Symbol	Explanation	Symbol	Explanation
IVD	In Vitro Diagnostic Medical Device		Protect from light
2°C 10°C	Store at 2°C to 10°C	PROMEGA 2800 Woods Hollow Rd. Madison, WI USA	Manufacturer
	Caution		Irritant
	Use by	Σ	Contains sufficient for <n> tests
2	Do not reuse		Warning. Biohazard
i	Consult instructions for use	REF	Catalog number
LOT	Lot number	SN	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) 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® 3500Dx 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.

1.6 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 1). 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.

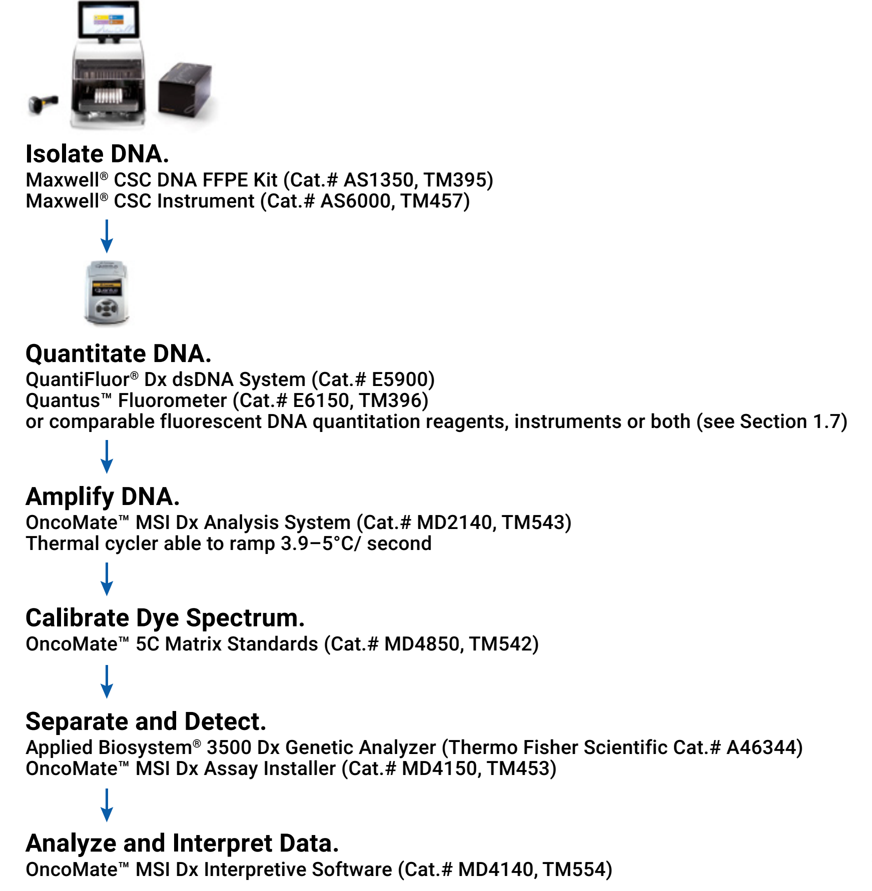


Figure 1. OncoMate™ MSI Dx Analysis System assay workflow.

1.7 Principles of the Procedure

The OncoMate™ MSI Dx Analysis System workflow involves DNA extraction from FFPE tissue samples, 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, Figure 1). 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 1. 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 (displayed black)	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 (displayed black)	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.

Panel B

Panel C

Panel A

Peak Height (RFU)

Fragment Length, or Size (Bases)

Panel D

Panel E

Figure 2. 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 3). 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 3. 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'.

1.8 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 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.

2. Product Components and Storage Conditions

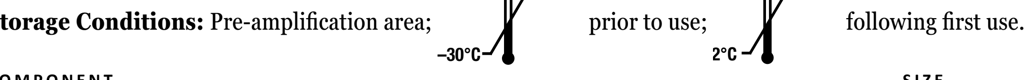
2.1 Materials Provided with the OncoMate™ MSI Dx Analysis System (Cat.# MD2140)

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; –30°C to –10°C prior to use; 2°C to 10°C following first use.

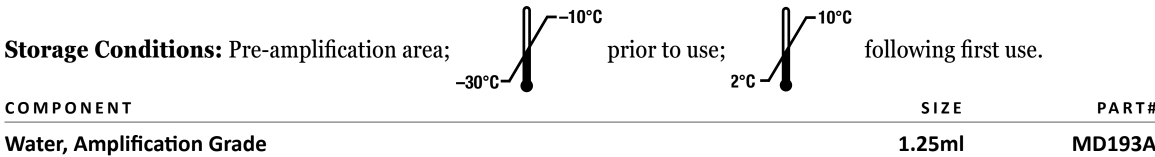
COMPONENT	SIZE	PART#
OncoMate™ MSI 5X Master Mix	**200µl**	**MD280A**

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

Storage Conditions: Pre-amplification area; –30°C to –10°C prior to use; 2°C to 10°C following first use.

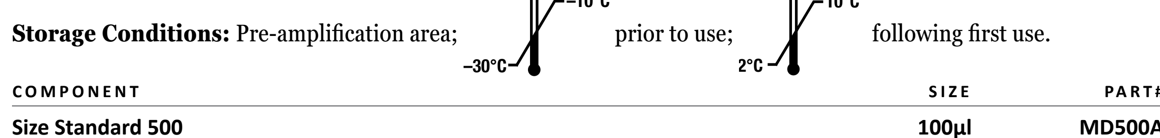
COMPONENT	SIZE	PART#
2800M Control DNA, 10ng/µl	**25µl**	**MD810A**

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



Storage Conditions: Pre-amplification area; –30°C to –10°C prior to use; 2°C to 10°C following first use.

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



Storage Conditions: Pre-amplification area; –30°C to –10°C prior to use; 2°C to 10°C 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; –30°C to –10°C prior to use; 2°C to 10°C following first use.

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 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.

2.3 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)

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 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.

3.2 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.

3.3 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.

3.4 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 4.6.

3.5 Capillary Electrophoresis Instrument Configuration and Requirements

3.5.1 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.

3.5.2 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 13.1 of this manual for complete assay installation instructions.

3.5.3 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.

3.5.4 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 6.5) 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.

3.6 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 5) 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 4.9). Any time a new sample batch is prepared, including for troubleshooting 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 6.4, Table 7).
- 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 4.9).
- Samples must exist as matched pairs of normal and tumor samples with the same Sample ID (see Section 4.9).

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.

4. Assay Protocol

4.1 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.

4.2 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.

4.3 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 4.5). 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.

4.4 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 2 for example dilutions. To ensure accuracy, pipet volumes ≥1µl when preparing 2800M Control DNA dilutions.

Table 2. 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.

4.5 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 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 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 reagents 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 3. 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 3. 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 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(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.

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 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.

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.

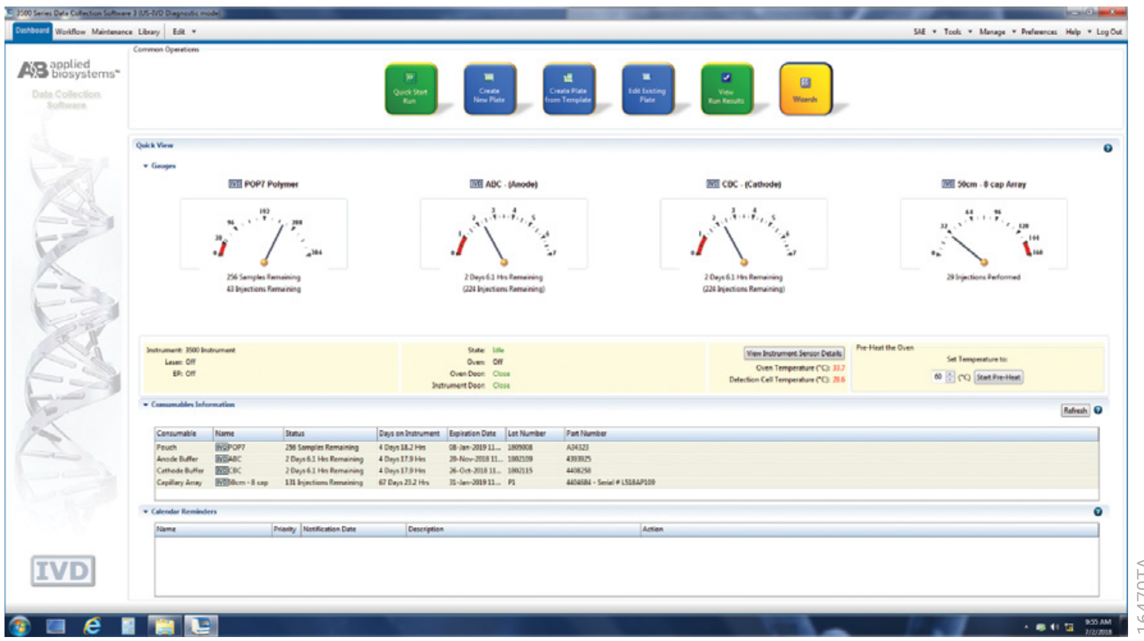


Figure 5. Dashboard on the Applied Biosystems® 3500 Dx Genetic Analyzer, installed with POP-7® polymer, a 50cm array and Data Collection Software Version 3.

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 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 electrophoresis loading cocktail as directed in Table 4.

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 4. 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.

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 Promega_OncoMate_MSI_Dx_Assay. If this assay is not yet installed on the Applied Biosystems® 3500 Dx Genetic Analyzer, refer to Section 13.1 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 6).

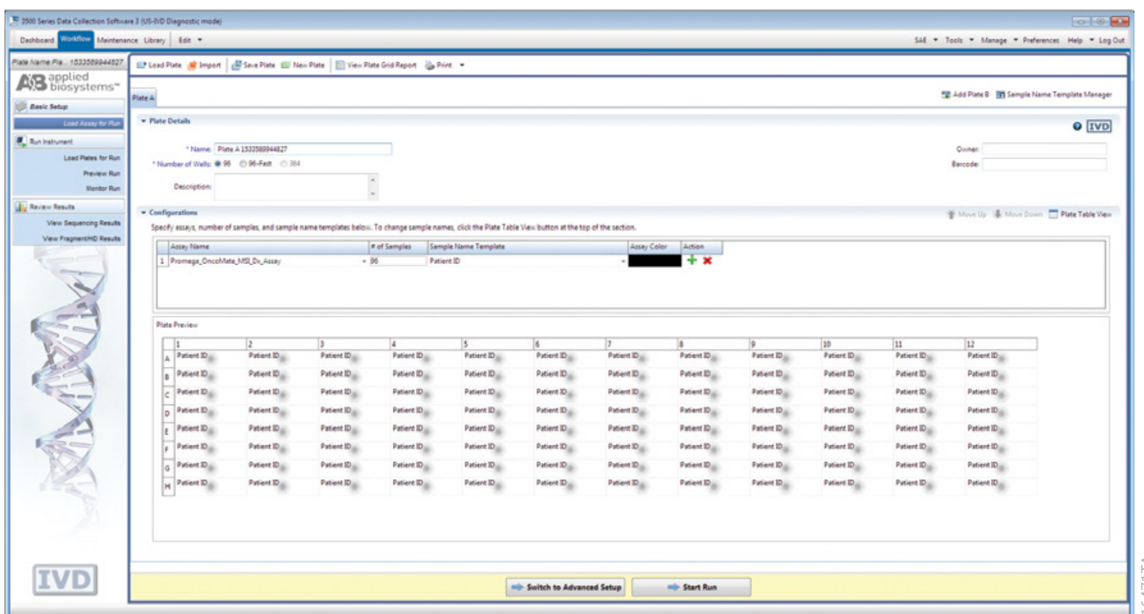


Figure 6. 'Workflow' tab, Load Assay for Run screen.

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 Interpretive Software to name the sample batch.

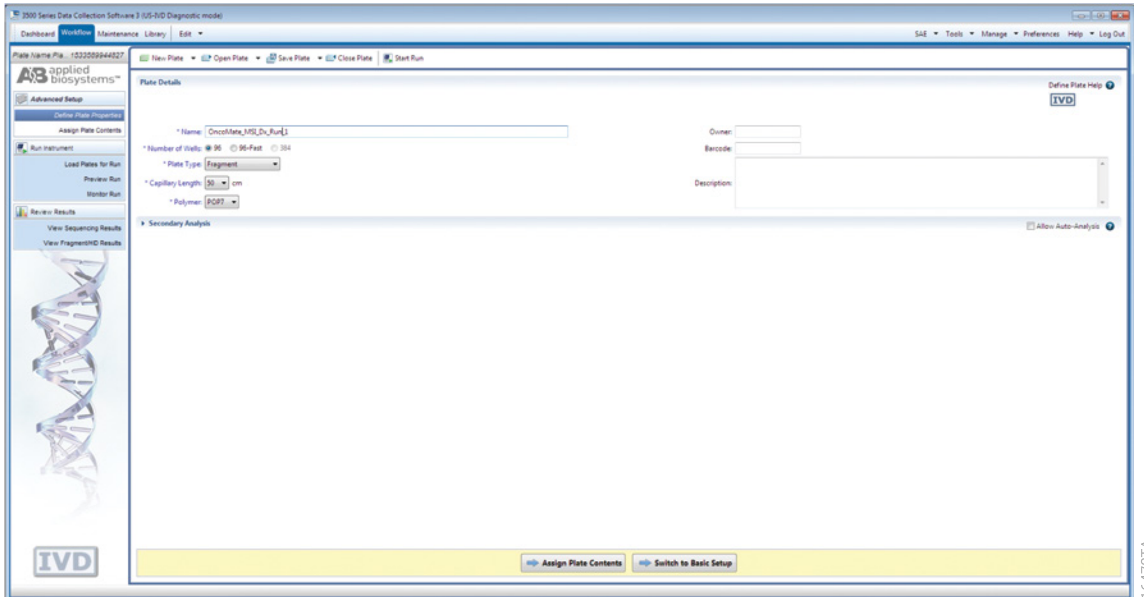


Figure 7. 'Define Plate Properties' screen.

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 **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.

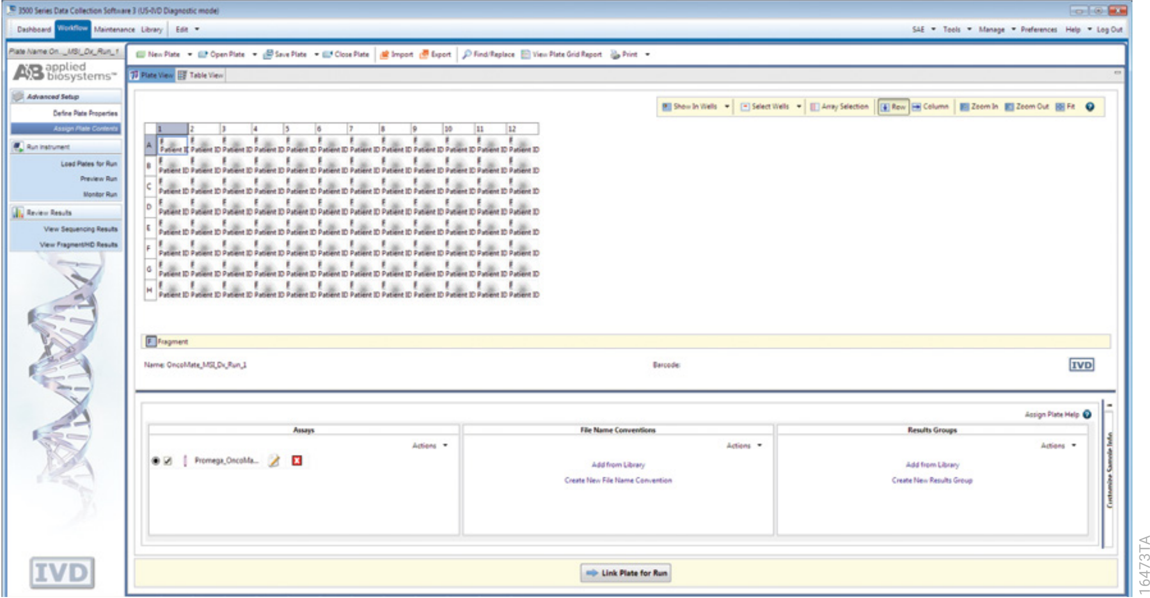


Figure 8. 'Plate View' tab, Assign Plate Contents screen.

5. Switch to the 'Table View' tab and enter information for the samples and controls to be analyzed (Figure 9). 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.

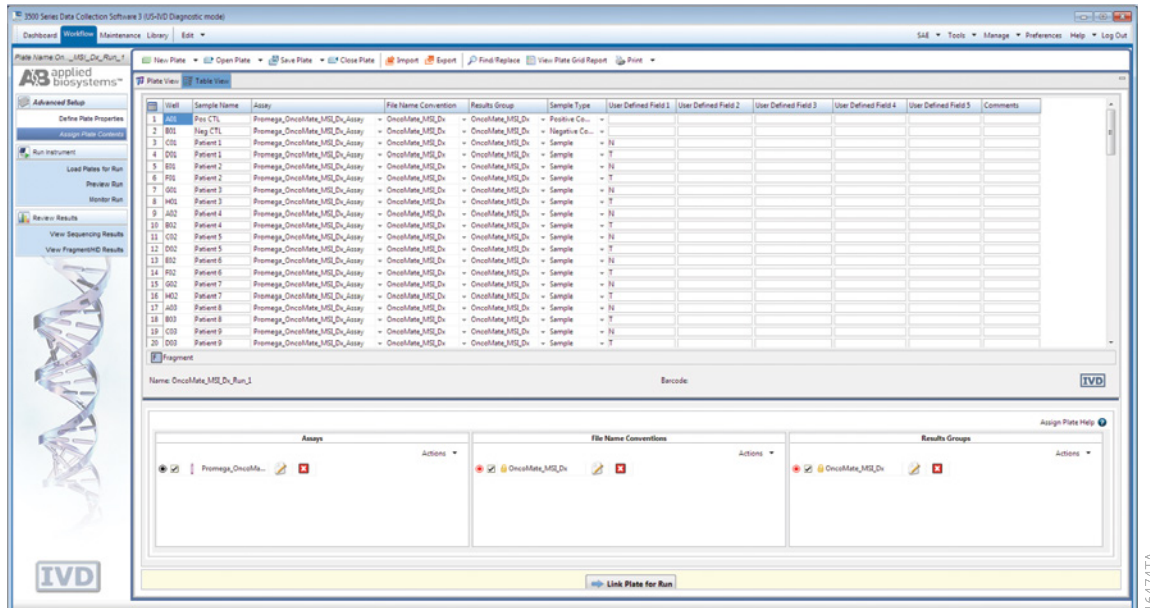


Figure 9. 'Table View' tab, Assign Plate Contents screen.

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 10), change the Run Name, if desired, and select **Start Run**.

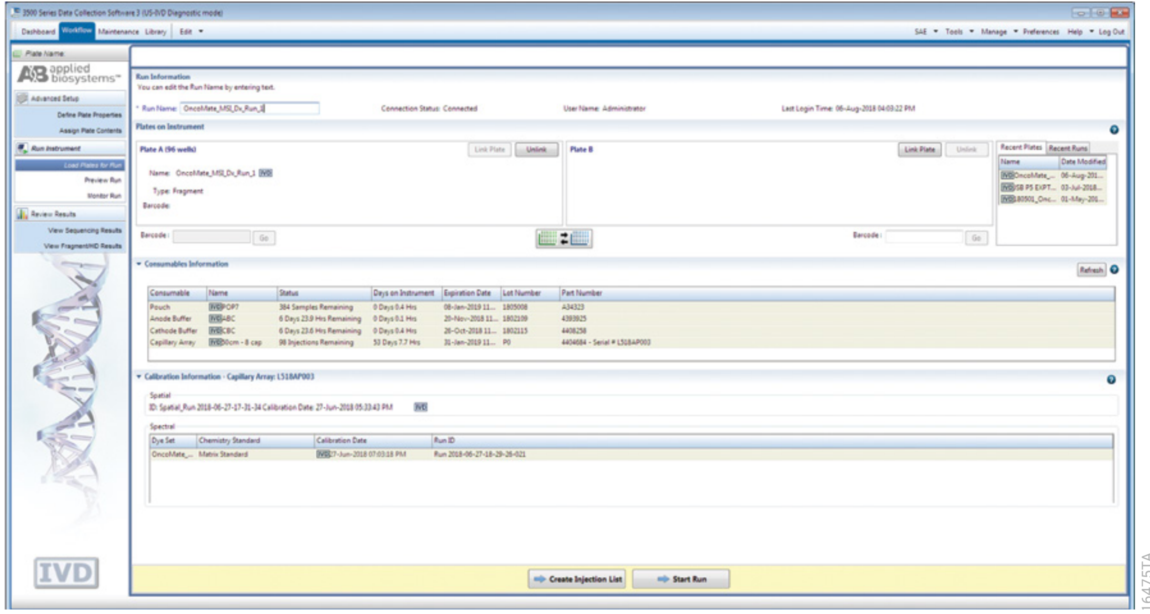


Figure 10. Run Information screen.

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 non-unique sample names, they are excluded during batch import by the interpretive software.

If it is desirable to analyze the original and reinjected 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.

5. Data Analysis using the OncoMate™ MSI Dx Interpretive Software

5.1 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 6.

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 11) 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.

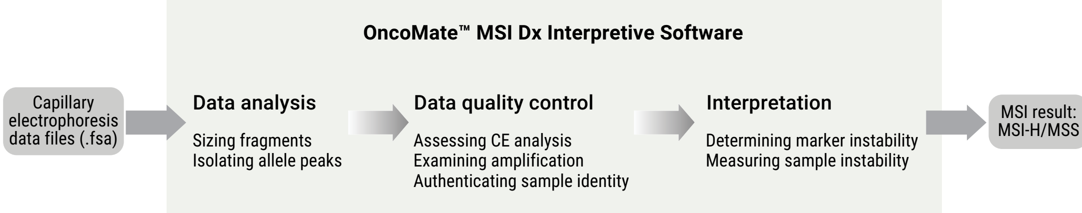


Figure 11. The OncoMate™ MSI Dx Interpretive Software automated workflow.

5.2 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 12). 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.

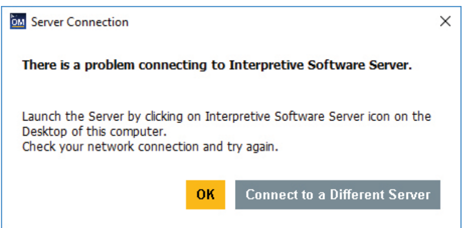


Figure 12. Server connection problem.

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**.

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

1. From the 'Home' screen of the OncoMate™ MSI Dx Interpretive Software Client (Figure 13), click **Import Samples** to open the 'Import Samples' file browser.

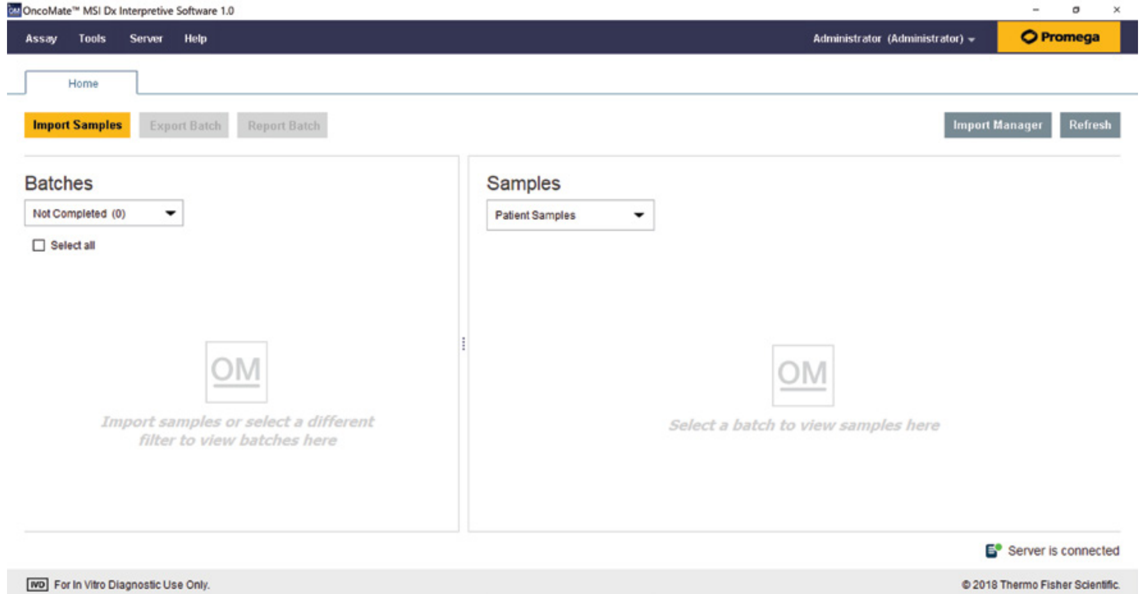


Figure 13. The OncoMate™ MSI Dx Interpretive Software 'Home' screen.

2. Browse to the folder containing the sample files (or injection folders containing sample files) that you wish to import (Figure 14). 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 4.9 for instructions on sample labeling using UDF1). Import will fail for sample files that do not conform to these requirements.

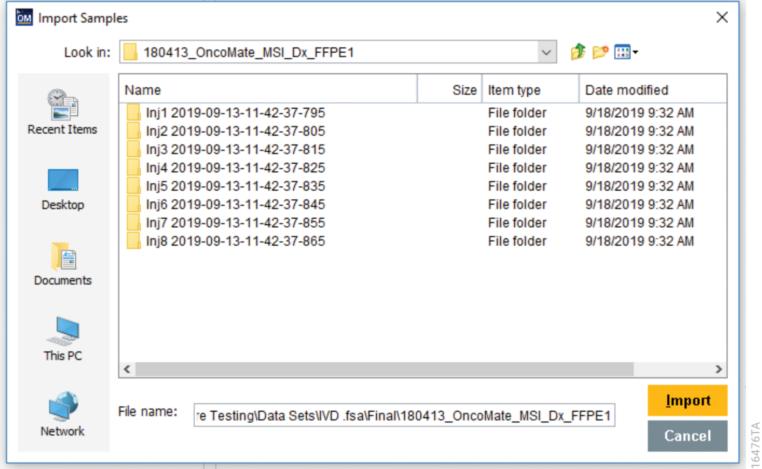


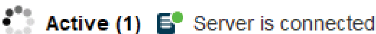
Figure 14. The OncoMate™ MSI Dx Interpretive Software 'Import Samples' screen.

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.

5.4 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 15) 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 5.6 for instructions to approve a QC Failed batch.

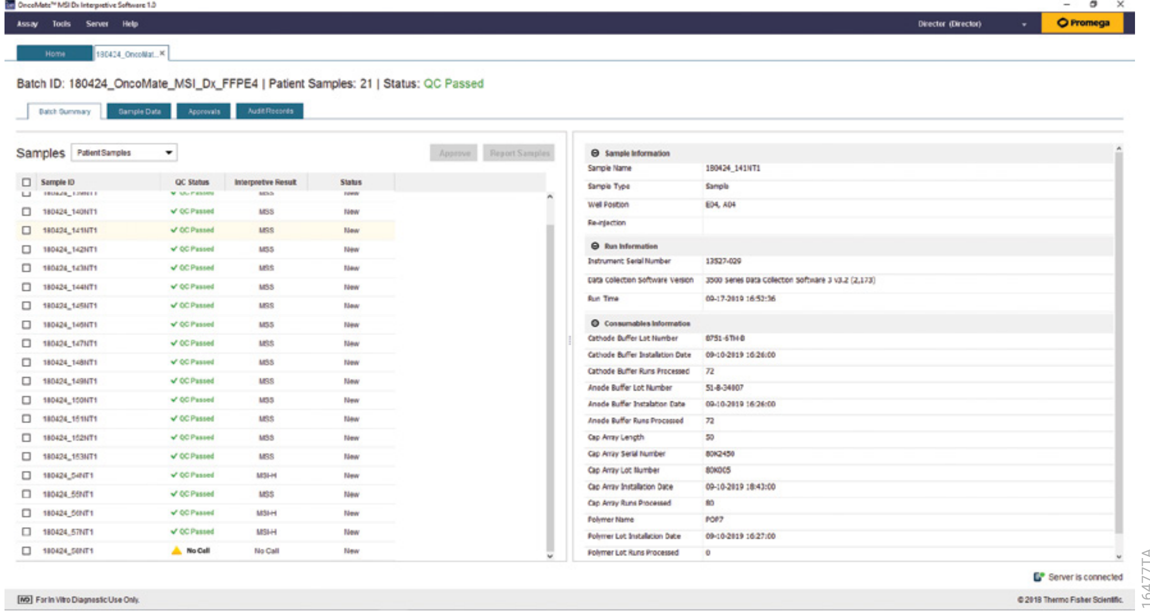


Figure 15. The batch screen opens to the 'Batch Summary' tab and presents information associated with the selected batch of samples, including electropherograms, approvals and audit records.

2. Select the 'Sample Data' tab (Figure 16) 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.

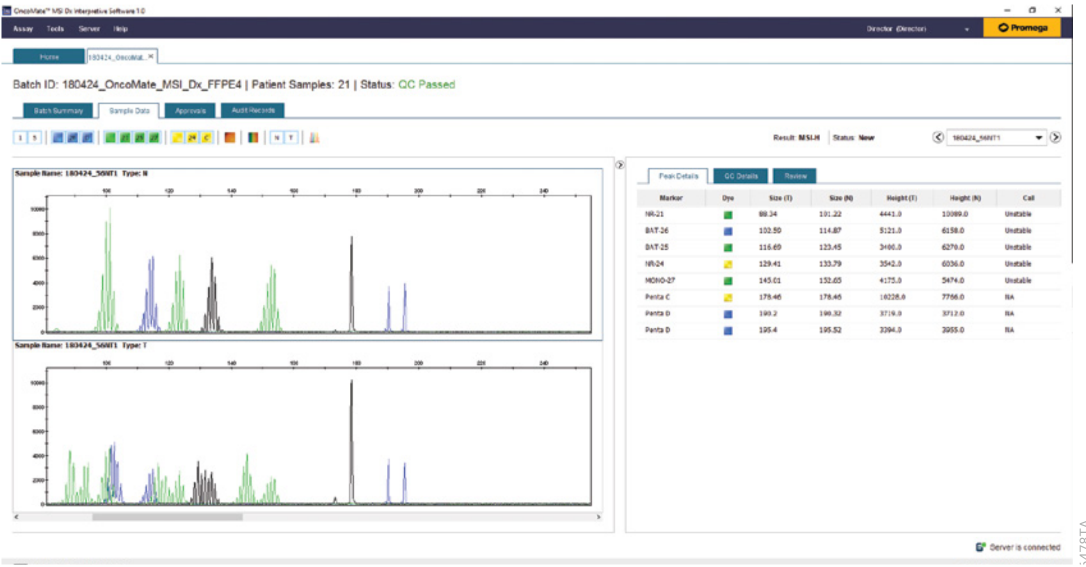


Figure 16. The 'Sample Data' tab displays sample electropherograms and provides Peak Details.

3. Review the sample data. If the MSI interpretive result is Invalid or No Call, select the 'QC Details' tab (Figure 17) to view any quality control flags that apply to the sample.

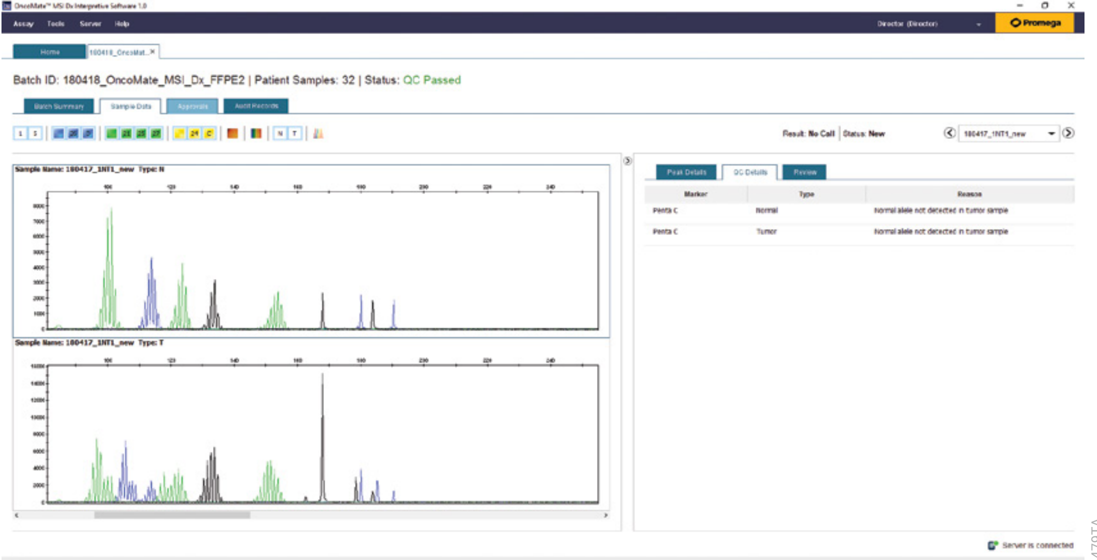


Figure 17. The 'QC Details' tab.

4. Select the ‘Review’ tab (Figure 18). To add comments to the review for the displayed sample, press **Add Comment**. Refer to Section 6 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 5.4, Steps 3–6, to continue the sample review process.

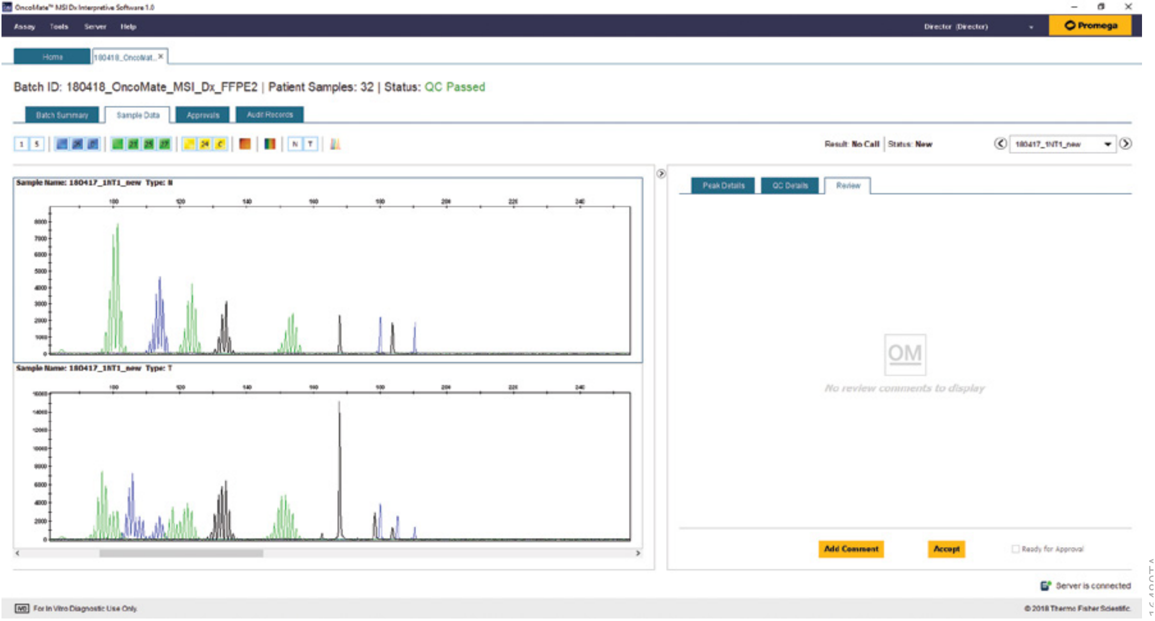


Figure 18. The ‘Review’ tab in the Details pane.

5.5 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 15).
2. Select the ‘Sample Data’ tab (Figure 16) 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 17) 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 15), 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.

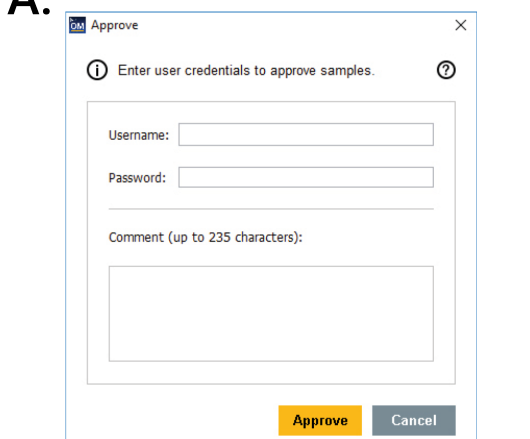
4. Select the 'Review' tab (Figure 18).
5. Complete this step if different users perform the reviews described in Sections 5.4 and 5.5: Press **Accept** to record the final review of the displayed matched sample pair, and press **Add Comment** to record any notes related to the sample review.
6. Check 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 5.5, Steps 3 through 7, to complete the sample review process.

5.6 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 15). 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 19, 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.

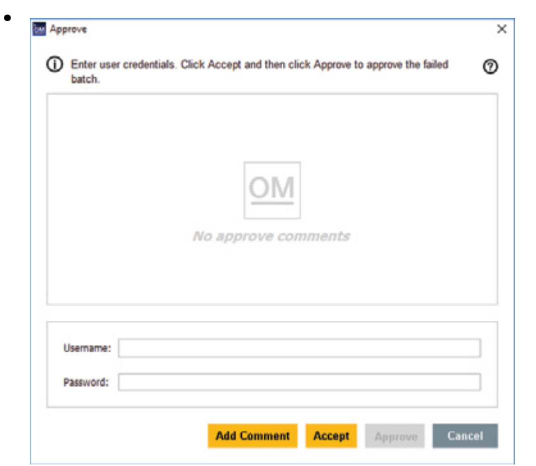


Figure 19. Panel A. 'Approve' screen shown for samples marked as Ready for Approval. **Panel B.** 'Approve' screen shown for batches marked as QC Failed.

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 19, 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 15), 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.

5.7 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 20), 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.

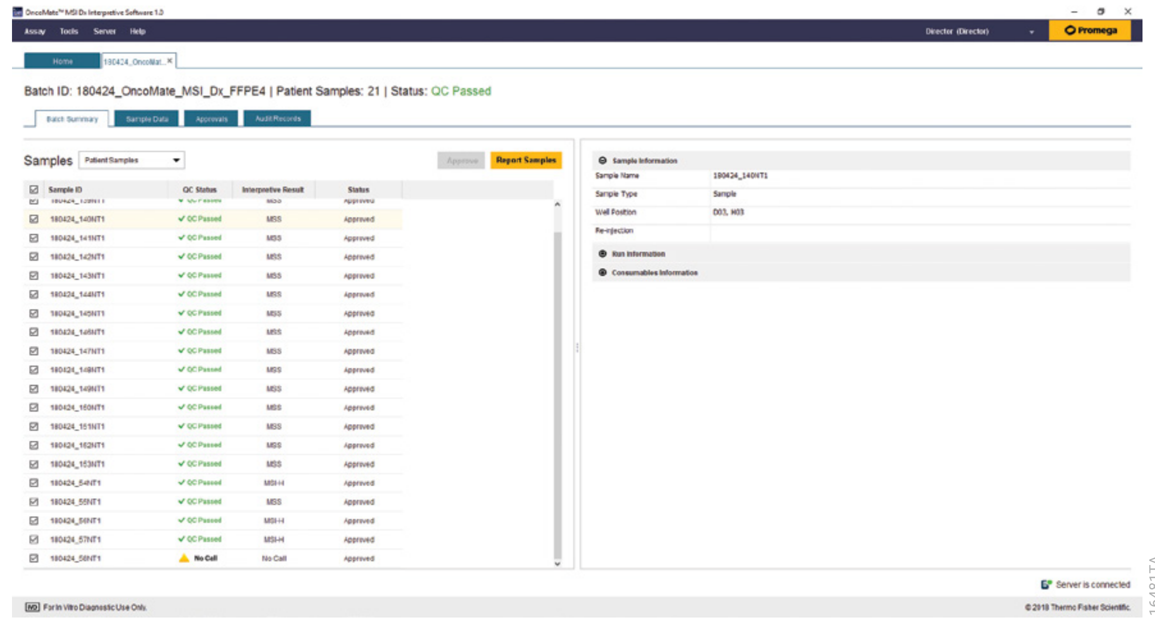


Figure 20. Sample reports are created by clicking Report Samples from the 'Batch Summary' tab.

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 21).

   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.

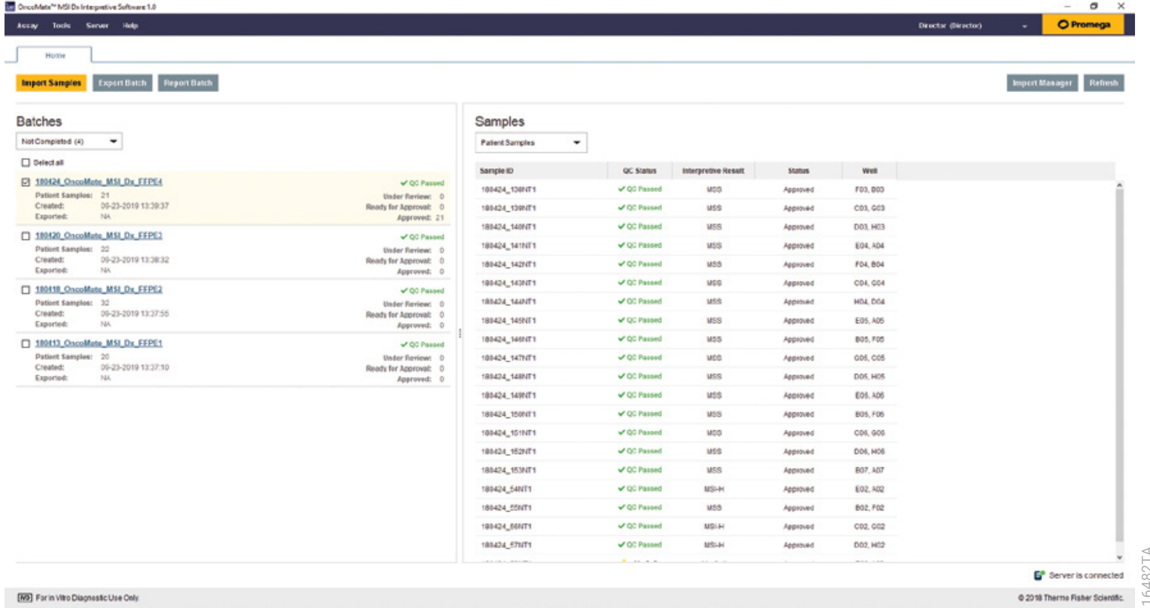


Figure 21. From the 'Home' screen, select one or more batches and press Report Batch to create batch summary reports.

6. Interpretation of Results

6.1 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.

6.2 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.

6.3 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 6.6).

Tables 5 and 6 summarize the marker stability calls and sample interpretive results, respectively, returned by the interpretive software.

Table 5. Marker-Level Stability Calls Provided by the OncoMate™ MSI Dx Interpretive Software.

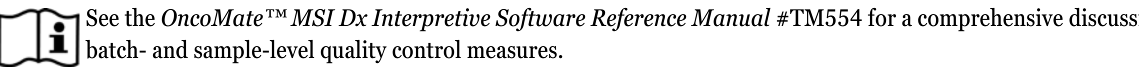
Value	Description
Stable	Microsatellite instability was not detected for the marker. The difference in allele size of the mononucleotide-repeat region analyzed was less than 2.75bp for the normal and tumor sample pair.
Unstable	Microsatellite instability was detected for the marker. The difference in 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 6.4). View the 'QC Details' tab for information about failed quality attributes for the sample. See Section 6.6 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 6.4) 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 6. 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 6.4). View the 'QC Details' tab for information about failed quality attributes for the sample. See Section 6.6 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 6.4). 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.

6.4 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 7.

Table 7. 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]	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.

6.5 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 6.5) 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.

6.6 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 10, Troubleshooting, for information on resolving Invalid results.

No Call:

A No Call Interpretive result is returned with certain data QC messages (see Section 6.4). The following subsections describe scenarios for which a manual MSI determination may be possible for No Call samples. See Section 10, 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 22).

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.

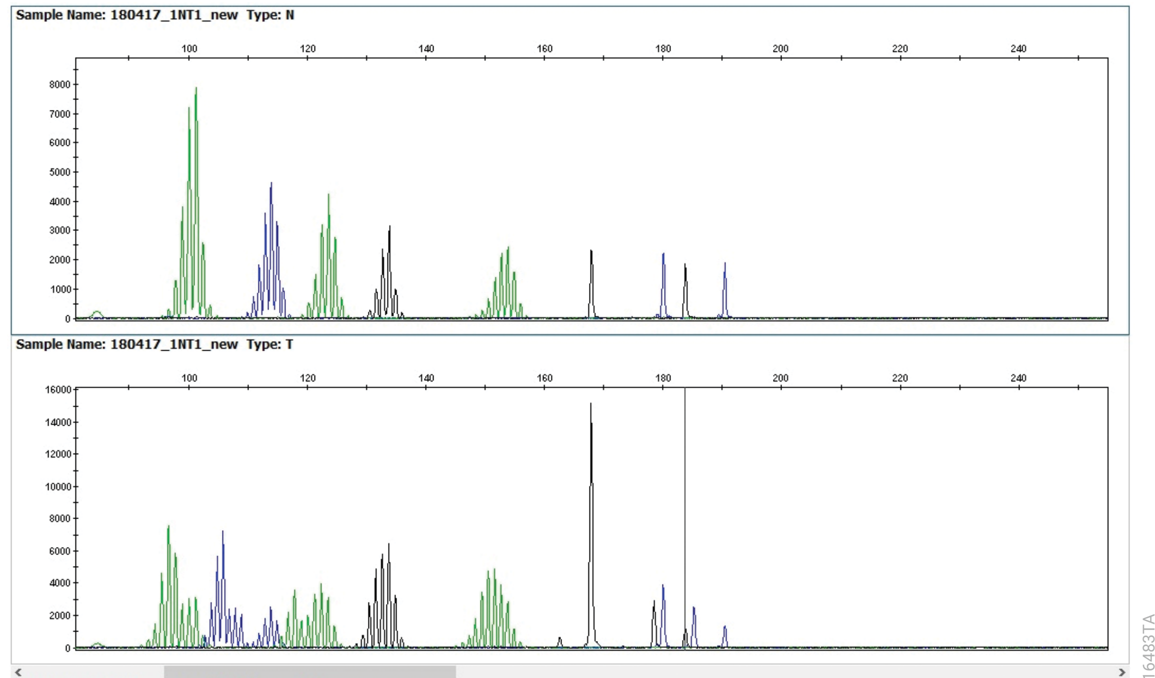


Figure 22. A peak that was detected but filtered by the interpretive software allele-calling algorithm. In cases of pronounced allelic imbalance in pentanucleotide-repeat loci, one of the normal alleles in the tumor sample may be filtered by the software as stutter. If this "lost" normal peak is present 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.

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 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.

7.3 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 8 for expected 2800M Control DNA results. No-template controls should not have amplified peaks above the 175RFU calling threshold.

Table 8. 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 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.

7.5 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 6.4, Table 7, 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.

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 '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.

9. 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.

9.1 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.

9.2 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 9), 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 reinjection or reamplification 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 9. 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

9.3 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.

9.4 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 10 for the DNA input study, and for MSI-H cases in the tumor content study in Table 11. Table 12 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 13 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 13.

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 10. 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 11. 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 12. 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 13. 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)

9.5 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.

9.6 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 14).

Table 14. 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.

9.7 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.

9.8 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 15) 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 16 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 17.

A total of 13 results were initially Invalid during testing. All 13 samples were reinjected, resulting in eight samples that resolved and five samples that did not resolve. Upon reamplification, three of the five unresolved samples were resolved. A total of two samples remained Invalid after reamplification 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 15. 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 16. 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 17. 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)

9.9 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 reinjection.

9.10 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 18. 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 Tables 18 and 19.

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 19 and Table 20. The OncoMate™ MSI Dx Analysis System effectively identified tumors with MMR deficiency and shows strong agreement with the VENTANA MMR IHC panel.

Table 18. 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 19. 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 20. 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

9.11 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 21. Tables 22 and 23 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 21. Comparison and Agreement of the OncoMate™ MSI Dx Analysis System Interpretative Results vs. NGS MMR (All Samples).

OncoMate™ MSI Dx Analysis System	DNA Sequencing Results			
	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 22. 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 23. 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

10. Troubleshooting

For questions not addressed here, consult the other technical manuals associated with the OncoMate™ MSI Dx Analysis System (listed in Section 1.1). 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 7, Assay Quality Controls). See Table 7 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 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 instrument 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 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 if 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 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 Software 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 CE run were not imported into the OncoMate™ MSI Dx Interpretive Software OncoMate™ MSI Dx Interpretive Software observation: Notification displayed in the import manager	The Sample Name entered into the Applied Biosystems® 3500 Dx Genetic Analyzer DCS was not identical for the matched tumor and normal sample pair. Sample files with mismatched sample names will not be identified as a pair by the OncoMate™ 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 CE run were not imported into the OncoMate™ MSI Dx Interpretive Software (continued) OncoMate™ MSI Dx Interpretive Software observation: Notification displayed in the import manager	The required 'N' or 'T' designations were not entered (or 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 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 DNA amplification reaction (samples also may be affected) OncoMate™ MSI Dx Interpretive Software QC details: "No allele detected" "Marker peak height too high to evaluate"	**Recommended Actions:** 1. Repeat amplification for all samples. Ensure that control DNA dilutions, reagents and mixtures are vortexed according to the instructions for use.
	The 2800M Control DNA stock solution or 2800M Control DNA 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 4.5 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-repeat 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. 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 intensity less than 700RFU for a mononucleotide-repeat marker interpreted as stable (continued) OncoMate™ MSI Dx Interpretive Software QC details: “No allele detected” “Low allele peak height detected”	Thermal cycler or reaction plate problems (positive control reaction also was affected). Confirm that the PCR program is correct and that the selected thermal cycler meets the requirements for the OncoMate™ MSI Dx Analysis System (see Section 3.4). Calibration of heat block may be required. The assay was developed using the materials listed in Section 2.3; the use of other materials is not supported. Repeat 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 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 CE 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 4 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 4 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 QC details: "Poor Sizing Quality" "Marker peak height too high to evaluate"	CE-related artifacts. Aberrant peaks may be observed during 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 QC 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 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 OncoMate™ MSI Dx Interpretive Software QC details: “Broad peak shape detected” 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, 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 pentanucleotide alleles present in the normal sample are absent from the tumor sample OncoMate™ MSI Dx Interpretive Software QC details: “Normal sample not detected in tumor sample”	Normal and tumor DNA samples from different individuals were 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 sample 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 6.6 (“No Call due to sample authentication”) and Figure 22 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 6.5), 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 7), 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 4.4).

11. References

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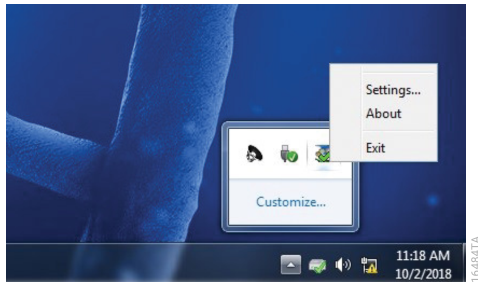
12. Additional Information

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

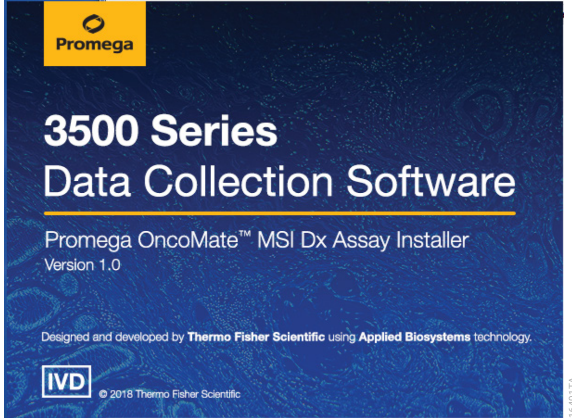
13. Appendix

13.1 OncoMate™ MSI Dx Assay Installation

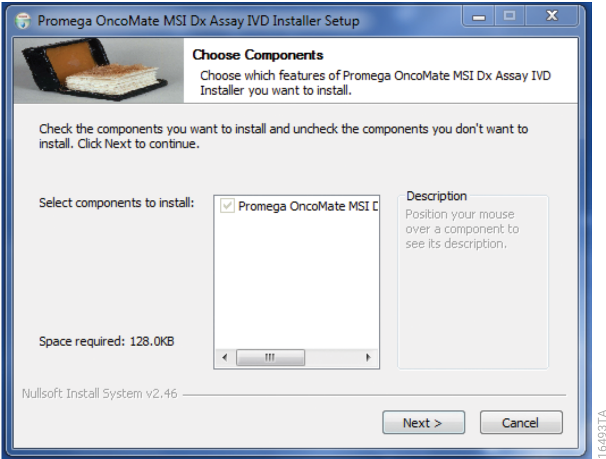
1. Copy the OncoMate_MSI_Dx_Assay_IVD_Installer.exe file to a folder on the target computer system or a memory stick.
2. Exit the Applied Biosystems® 3500 Series Data Collection Software.
3. Exit the services started by the data collection software by opening the system tray, right clicking the '3500 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.



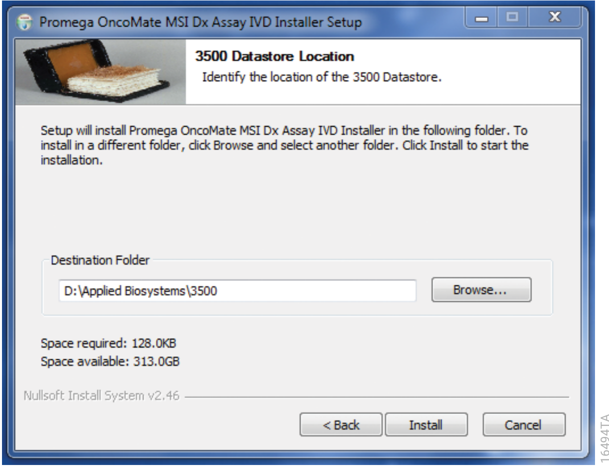
4. Double click the OncoMate_MSI_Dx_Assay_IVD_Installer.exe file to begin assay installation.
5. You will see the following splash screen:



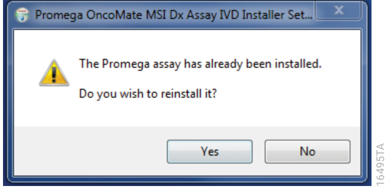
6. When the following window appears, click **Next >**:



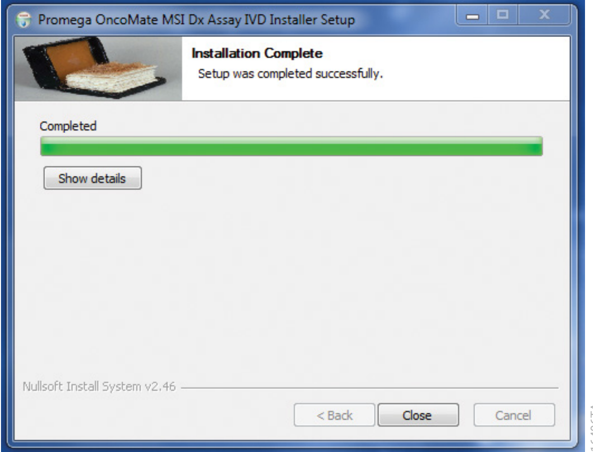
7. The destination folder for the Assay will be displayed. Click **Install**:



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

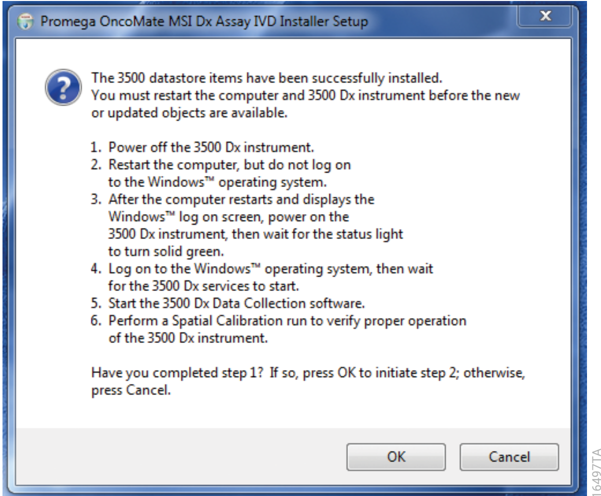


8. When the installation is completed, the following window will be displayed. Click **Close**:



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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[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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