



TECHNICAL MANUAL

LMR MSI Analysis System

Instructions for Use of Product
MD2540

For Research Use Only. Not for use in diagnostic procedures.

LMR MSI Analysis System

All technical literature is available at: www.promega.com/protocols/
 Visit the web site to verify that you are using the most current version of this Technical Manual.
 E-mail Promega Technical Services if you have questions on use of this system: genetic@promega.com

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1. Description

Microsatellites are stretches of repeating DNA sequence that are distributed throughout the human genome and prone to insertion and deletion (indel) replication errors. Normally, the cellular DNA mismatch repair corrects errors. However, if the mismatch repair is dysfunctional (dMMR), errors within the microsatellites remain uncorrected, allowing the errors to be further propagated in subsequent replication. Indel changes at microsatellite markers are known as microsatellite instability (MSI).

The LMR (Long Mononucleotide Repeat) MSI Analysis System is a PCR-based DNA fragment sizing assay to detect microsatellite instability (MSI) in human cells. The analysis involves comparing allelic profiles of microsatellite markers generated by amplifying DNA from normal and tumor samples from the same individual. Fragment analysis of amplicons from a tumor sample that is DNA mismatch repair deficient (dMMR) will show fragment size changes in the allelic profile compared with the normal sample, indicating MSI.

The loci targeted by the LMR MSI Analysis System were specifically chosen to enable sensitive detection of mismatch repair deficiency. In addition to traditional MSI markers (NR-21, BAT-25, BAT-26 and MONO-27), the system targets LMR markers (BAT-52, BAT-56, BAT-59 and BAT-60). The LMR markers produce more pronounced fragment length changes, and thus improve detection of microsatellite instability that may not otherwise be detectable using traditional MSI markers alone. Furthermore, the PCR amplicon lengths were minimized to better detect MSI from degraded DNA, such as in formalin-fixed, paraffin-embedded (FFPE) tissue samples.

Table 1. LMR MSI Analysis System Locus Information.

Marker Name	Major Repeat Sequence	Size Range (bp) ¹	2800M Control DNA Alleles (bp) ²	Primer Dye
NR-21	(A) ₂₁	82–103	97	FL
BAT-60	(A) ₆₀	103–160	119, 125	FL
BAT-25	(A) ₂₅	72–101	88	JOE
BAT-59	(A) ₅₉	102–172	125, 157	JOE
BAT-26	(A) ₂₆	79–102	96	TMR-ET ^(a)
BAT-56	(A) ₅₆	114–189	160	TMR-ET
MONO-27	(A) ₂₇	99–126	117	CXR-ET ^(a)
BAT-52	(A) ₅₂	126–177	163	CXR-ET

¹Allele sizes were determined using the Applied Biosystem® 3500 Genetic Analyzer with POP-7® polymer and a 50cm capillary. The size ranges listed capture currently observed alleles; however, rare alleles outside of these size ranges may exist. Allele sizes may vary when using different polymers or instrument configurations.

²Instrument-to-instrument and day-to-day variability in the performance of capillary electrophoresis instruments can result in variations of ±1.5bp in the expected allele sizes for 2800M Control DNA.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT. #
LMR MSI Analysis System	100 reactions	MD2540

For Research Use Only. Not for use in diagnostic procedures. This system contains sufficient reagents for 100 reactions of 10µl each. Includes:

- 200µl LMR MSI 5X Master Mix
- 200µl LMR MSI 5X Primer Mix
- 25µl 2800M Control DNA, 10ng/µl
- 1,250µl Water, Amplification Grade
- 200µl WEN Internal Lane Standard 500

Storage Conditions: Upon receipt, store all components at -30°C to -10°C in a nonfrost-free freezer. Before the first use, store the 2800M Control DNA at $+2^{\circ}\text{C}$ to $+10^{\circ}\text{C}$ for at least 24 hours. After the first use, store WEN Internal Lane Standard (WEN ILS) 500 and the 2800M Control DNA at $+2^{\circ}\text{C}$ to $+10^{\circ}\text{C}$. Do not refreeze. The LMR MSI 5X Primer Mix and WEN ILS 500 are light-sensitive and must be stored in the dark.

Alternatively, store LMR MSI 5X Master Mix and LMR MSI 5X Primer Mix at $+2^{\circ}\text{C}$ to $+10^{\circ}\text{C}$ for up to 6 months without loss of activity.

3. Before You Begin

3.A. 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, provided by the manufacturer, 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: The LMR MSI Analysis System is intended for use with formalin-fixed paraffin-embedded tissues. Follow the guidelines established by your institution for the handling and disposal of these tissues.

PCR Precautions: 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 LMR MSI Analysis System requires strict adherence to the recommended procedures for amplification and fluorescence detection described in this manual.

Minimizing Contamination: 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 (LMR MSI 5X Master Mix, LMR MSI 5X Primer Mix, 2800M Control DNA and Water, Amplification Grade) in a separate room from those used following amplification (WEN ILS 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.B. Spectral Calibration

The *GenePrint*[®] 5C Matrix Standard (Cat.# B1930) is required for spectral calibration on the Applied Biosystems[®] 3500 Genetic Analyzers. For protocols and additional information about spectral calibration, see the *GenePrint*[®] 5C Matrix Standard Technical Manual #TM475, available at: www.promega.com/protocols/

4. Protocol for DNA Amplification Using the LMR MSI Analysis System

The LMR MSI Analysis System is designed for amplification of extracted DNA samples in a 10µl reaction volume.

The LMR MSI Analysis System was developed with the ProFlex® PCR System. This system is compatible with the Veriti® 96-Well Thermal Cycler, BioRad C1000 and Eppendorf Mastercycler x50s.

Note: Thermal cyclers other than those listed in this technical manual may be used, but will require optimization of cycling conditions, and may require additional validation in your lab.

The use of gloves and aerosol-resistant pipette tips is highly recommended to prevent cross-contamination. 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.

Take meticulous care to ensure successful amplification. A guide to amplification troubleshooting is provided in Section 8.

4.A. Amplification of Extracted DNA

We recommend using a high-quality extraction method such as the Maxwell® RSC DNA FFPE Kit (Cat.# AS1450) in order to maximize DNA fragment size and amplifiability. In addition, we recommend quantitating DNA using a fluorometric detection method such as QuantiFluor® dsDNA System (Cat.# E2670, E2671) for greater accuracy than that achieved using spectrophotometry.

If there are concerns regarding DNA fragmentation, we recommend using a qPCR-based method such as the ProNex® DNA QC Assay (Cat.# NG1002, NG1003). Instruments such as the Agilent Bioanalyzer or TapeStation can provide some information about DNA fragmentation, but unlike the ProNex® DNA QC Assay, these instruments are not able to predict how well samples will perform in amplification-based downstream applications.

Materials to Be Supplied By the User

- ProFlex® PCR System (e.g., Applied Biosystems® Cat.# 4484075 or equivalent thermal cycler)
- centrifuge compatible with 96-well plates or reaction tubes
- MicroAmp® optical 96-well reaction plate or 0.2ml MicroAmp® reaction tubes
- aerosol-resistant pipette tips
- 1.5ml microcentrifuge tubes

The LMR MSI Analysis System performs reliably when using 2.0ng of template DNA in a 10µl reaction and following the protocol detailed below.

Amplification Setup

Note: Thaw and centrifuge tubes briefly to move contents to the tube bottom, and then vortex reagents for 15 seconds before each use. Do not centrifuge the LMR MSI 5X Master Mix or LMR MSI 5X Primer Mix after vortexing because this may cause the reagents to be concentrated at the bottom of the tube.

4.A. Amplification of Extracted DNA (continued)

1. Determine the number of reactions to be set up. This should include positive and negative control reactions. Add 1 or 2 reactions to this number to compensate for pipetting error. While this approach does consume a small amount of each reagent, it ensures that you will have enough PCR amplification mix for all samples and that each reaction contains the same PCR amplification mix.
2. Use a clean plate for reaction assembly, and label it appropriately. Alternatively, determine the number of clean, 0.2ml reaction tubes required, and label them appropriately.
3. Add the final volume of each reagent listed in Table 2 to a clean tube.

Table 2. PCR Amplification Mix for Amplification of Extracted DNA.

PCR Mix Component ¹	Volume per Reaction	Number of Reactions	Final Volume
Water, Amplification Grade	To a final volume of 10.0µl	x	=
LMR MSI 5X Master Mix	2.0µl	x	=
LMR MSI 5X Primer Mix	2.0µl	x	=
Template DNA (2ng) ^{2,3,4}	Up to 6.0µl		
Total reaction volume	10.0µl		

¹Add Water, Amplification Grade, to the tube first, and then add LMR MSI 5X Master Mix and LMR MSI 5X Primer Mix. The template DNA will be added at Step 6.

²We recommend storing DNA templates in TE⁻⁴ buffer (10mM Tris-HCl [pH 8.0], 0.1mM EDTA) or TE⁻⁴ buffer with 20µg/ml glycogen. If the DNA template is stored in water, the DNA can be stored for up to 1 week at +2°C to +10°C or at -30°C to -10°C for longer-term storage. If the DNA template is stored in TE buffer that is not pH 8.0 or contains a higher EDTA concentration, the volume of DNA added should not exceed 20% of the final reaction volume. PCR amplification efficiency and quality can be greatly altered by changes in pH (due to added Tris-HCl), available magnesium concentration (due to chelation by EDTA) or other PCR inhibitors, which may be present at low concentrations, depending on the source of the template DNA and the extraction procedure used.

³Apparent DNA concentrations can differ depending on the DNA quantification method used. We strongly recommend that you perform experiments to determine the optimal DNA amount based on your DNA quantification method.

⁴When using 2800M Control DNA, use 1ng of DNA per reaction.

4. Vortex the PCR amplification mix for 5–10 seconds, and then pipet PCR amplification mix into each reaction well. Failure to vortex the PCR amplification mix sufficiently can result in poor amplification or locus-to-locus imbalance.

Note: Do not store the PCR amplification mix. Add the mix to the wells of the reaction plate as soon as the mix is prepared. Add DNA as soon as possible to each well and follow immediately by thermal cycling.

5. Add the sample template DNA (2.0ng) for each sample to the respective well containing PCR amplification mix.
Note: The LMR MSI Analysis System was optimized using 2.0ng of DNA template. Further optimization of the template input quantity may be required to accommodate your laboratory's samples and chosen quantification method. Highly crosslinked or degraded samples may require higher template quantities.
6. For the positive amplification control, vortex the tube of 2800M Control DNA, then dilute an aliquot to an appropriate concentration to allow addition of 1.0ng to each amplification reaction in the desired template volume. To maximize accuracy, pipet volumes $\geq 1\mu\text{l}$ when diluting DNA. Add 1.0ng of diluted 2800M Control DNA to a reaction well containing PCR amplification mix.
7. For the negative amplification control, pipet Water, Amplification Grade, or TE⁻⁴ buffer instead of template DNA into a reaction well containing PCR amplification mix.
8. Seal or cap the plate or close the tubes.
Optional: Briefly centrifuge the plate to bring contents to the bottom of the wells and remove any air bubbles.

Thermal Cycling

Amplification and detection instrumentation may vary. You may need to optimize protocols including the amount of template DNA, cycle number, injection conditions and loading volume for your laboratory instrumentation. Testing at Promega shows that 29 cycles works well for 2.0ng of DNA templates.

Optional: Preheat the thermal cycler lid prior to loading the plate.

1. Place the reaction plate or tubes in the thermal cycler.
2. Select and run the recommended protocol, below. The total cycling time is approximately 1 hour.

Thermal Cycling Protocol

98°C for 3 minutes, then:

98°C for 10 seconds

61°C for 1 minute

72°C for 30 seconds

for 29 cycles, then:

60°C for 10 minutes

4°C soak

Note: When using the ProFlex® PCR System, the Veriti® 96-Well Thermal Cycler, Bio Rad C1000 and Eppendorf Mastercycler x50s, use the default ramping mode.

3. After completion of the thermal cycling protocol, proceed with fragment analysis or store amplified samples at -20°C protected from light.

4.A. Amplification of Extracted DNA (continued)

Notes:

- a. Long-term storage of amplified samples at 4°C or higher may produce artifacts.
- b. DNA quantity and quality affect PCR yield. Amplification and detection instrumentation may vary. You may need to optimize protocols including the amount of template DNA, cycle number, injection conditions and loading volume for your laboratory instrumentation.

Optional PCR Optimization

Cycle number should be optimized based on the results of an initial experiment to determine the sensitivity with your collection method, sample types and instrumentation.


1. Choose several samples that represent typical samples you encounter in the laboratory. Prepare them as you would using your normal workflow.
2. Prepare four identical reaction plates with these samples.
3. Amplify the samples using the thermal cycling protocol provided above, but subject each plate to a different number of cycles (27–30 cycles).
4. Following amplification, use your laboratory's validated separation and detection protocols to determine the optimal cycle number from these samples.


5. Instrument Setup and Sample Preparation

5.A. Detection of Amplified Fragments Using Applied Biosystems® 3500 or 3500xL Genetic Analyzers

Materials to Be Supplied By the User

- 95°C dry heating block, water bath or thermal cycler
- crushed ice, ice-water bath or a freezer plate block
- centrifuge compatible with 96-well plates
- aerosol-resistant pipette tips
- 3500/3500xL capillary array, 50cm
- plate retainer and base set (standard; e.g., Applied Biosystems® Cat.# 4410228)
- POP-7® polymer for the Applied Biosystems® 3500 or 3500xL Genetic Analyzer
- anode buffer container
- cathode buffer container
- MicroAmp® optical 96-well plate and septa, or equivalent (Applied Biosystems®)
- Hi-Di™ formamide (Applied Biosystems® Cat.# 4311320)

 The quality of formamide is critical. Use only the recommended formamide. Freeze formamide in aliquots at –20°C. Multiple freeze-thaw cycles or long-term storage at 4°C can cause breakdown of formamide. Poor-quality formamide may contain ions that compete with DNA during injection, which results in lower peak heights and reduced sensitivity. A longer injection time may not increase the signal.

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

Sample Preparation

1. Prepare a loading cocktail by combining and mixing internal lane standard (ILS) and formamide as follows:

$$[(0.5\mu\text{l ILS}) \times (\# \text{ samples})] + [(9.5\mu\text{l formamide}) \times (\# \text{ samples})]$$

Notes:

- a. The volume of internal lane standard used in the loading cocktail can be adjusted to change the intensity of the size standard peaks based on laboratory preferences.
- b. We do not recommend storing samples in loading cocktail in the injection plate for more than 12 hours prior to injection. Storing samples in loading cocktails for prolonged periods prior to injection may result in loss of signal in the amplified samples and internal lane standard. Consider using Stabilizer Reagent (see Section 8, Troubleshooting).

2. Vortex for 10–15 seconds to mix.
3. Pipet 10 μ l of formamide/internal lane standard mix into each well.
4. Add 1 μ l of amplified sample to each well. Cover the wells with appropriate septa.

Note: Instrument detection limits vary; therefore, injection time or the amount of sample mixed with loading cocktail may need to be increased or decreased. To modify the injection time in the run module, select **Instrument Protocol** from the Library menu in the data collection software. If peak heights are higher than desired, use less DNA template in the amplification reactions or reduce the number of cycles in the amplification program to achieve the desired signal intensity.

5. Centrifuge the plate briefly to remove air bubbles from the wells.
6. Denature samples at 95°C for 3 minutes, and then immediately chill on crushed ice or a freezer plate block or in an ice-water bath for 3 minutes. Denature samples just prior to loading the instrument.
7. Place the plate in the 3500 series 96-well standard plate base and cover with the plate retainer.

Note: The LMR MSI Analysis System was developed on the Applied Biosystems® 3500 instrument. This system is also compatible with the Promega Spectrum Compact CE System and Applied Biosystems® SeqStudio Genetic Analyzer.

5.A. Detection of Amplified Fragments Using the Applied Biosystems® 3500 or 3500xL Genetic Analyzers (continued)

Instrument Preparation

Refer to the *Applied Biosystems® 3500/3500xL Genetic Analyzer User Guide* for the instrument maintenance schedule and instructions on how to install the capillary array, buffers and polymer pouch and perform a spatial calibration. Samples may be analyzed as described in the *Applied Biosystems® 3500/3500xL Genetic Analyzer User Guide*.

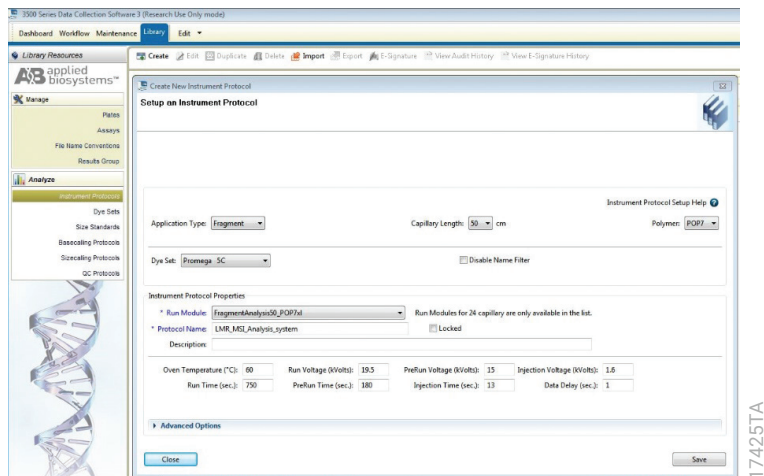
1. Open the 3500 Data Collection Software. The Dashboard screen will launch (Figure 1). To ensure that you are viewing the most up-to-date information, press the **Refresh** button. Ensure that the Consumables Information and Maintenance Notifications are acceptable.

Set the oven temperature to 60°C, and then select **Start Pre-Heat**. When the Oven Temperature and Detection Cell Temperature turn green, proceed with the first injection.



Figure 1. The Dashboard.

2. Prior to the first analysis using the LMR MSI Analysis System, you must create an Instrument Protocol, Size Standard, Sizecalling Protocol, Assay, File Name Convention and Results Group.
 - 2.a. To create a new Instrument Protocol, navigate to the Library, select **Instrument Protocols**, and then select **Create**. Alternatively, a previously created Instrument Protocol can be used.



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Figure 2. The 'Create New Instrument Protocol' window.

The recommended settings for the Applied Biosystems® 3500 Genetic Analyzer are:

Application Type	Fragment
Capillary Length	50cm
Polymer	POP-7®
Dye Set	Promega 5C
Run Module	FragmentAnalysis50_POP7
Injection Time ¹	13 seconds
Injection Voltage	1.6kV
Run Voltage	19.5kV
Run Time	750 seconds

¹Injection time may be modified to increase or decrease peak heights.

Injection time and voltage, run time and voltage are specific for 3500 Genetic Analyzer and may have to be adjusted for your equipment.

When creating an Instrument Protocol, select the same dye set that was used to perform the Promega 5C spectral calibration. Run time and other instrument settings should be optimized and validated in your laboratory.

When optimizing injection conditions in your laboratory, you can choose to create specific Instrument Protocols for each condition tested. If a single Instrument Protocol is used, follow the instructions in the *Applied Biosystems® 3500/3500xL Genetic Analyzers User Guide* to edit a library entry.

Assign a descriptive protocol name.

Note: For more detailed information refer to the *Applied Biosystems® 3500/3500xL Genetic Analyzers User Guide*.

5.A. Detection of Amplified Fragments Using the Applied Biosystems® 3500 or 3500xL Genetic Analyzers (continued)

- 2.b. To create a new Size Standard for the Sizecalling protocol, navigate to the Library. Select **Size Standards**, and then select **Create**. Alternatively, a previously created Size Standard can be used.

Assign the Size Standard a descriptive name such as “WEN_ILS_300”. Choose **Orange** as the Dye Color. The fragments in the size standard are 60, 65, 80, 100, 120, 140, 160, 180, 200, 225, 250, 275 and 300 bases. See Figure 3.

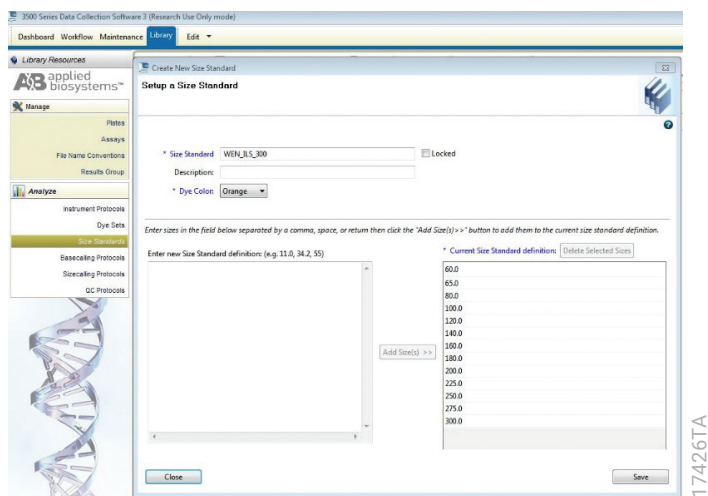


Figure 3. The 'Create New Size Standard' window.

- 2.c. To create a new Sizecalling Protocol, navigate to the Library. Select **Sizecalling Protocols**, and then select **Create**. Alternatively, a previously created Sizecalling Protocol can be used.

Assign a descriptive protocol name. Select the size standard created in Step 2.b. The settings for the Sizecalling protocol should be based on the internally-validated conditions for the LMR MSI Analysis System on the Applied Biosystems® 3500 or 3500xL Genetic Analyzer. Figure 4 shows one option for these settings.

Edit Sizecalling Protocol LMR_MSI_Fragment_Analysis

Setup a Sizecalling Protocol

* Protocol Name: LMR_MSI_Fragment_Analysis Locked

Description: LMR MSI Fragment Sizing, WEN ILS 300

Size Standard: WEN_ILS_300

Sizecaller: SizeCaller v1.1.0

Analysis Settings: QC Settings

Analysis Range: Full Sizing Range: Partial Size Calling Method: Local Southern

Analysis Start Point: 0 Sizing Start Size: 60 Primer Peak: Present

Analysis Stop Point: 1000000 Sizing Stop Size: 300

	<input checked="" type="checkbox"/> Blue	<input checked="" type="checkbox"/> Green	<input checked="" type="checkbox"/> Yellow	<input checked="" type="checkbox"/> Red	<input type="checkbox"/> Purple	<input checked="" type="checkbox"/> Orange
Minimum Peak Height	175	175	175	175	175	175

Common Settings

Use Smoothing: None

Use Baseline (Baseline Window (Pts)): 51

Minimum Peak Half Width: 2

Peak Window Size: 11

Polynomial Degree: 3

Slope Threshold Peak Start: 0.0

Slope Threshold Peak End: 0.0

Close Save

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Figure 4. The 'Create New Sizecalling Protocol' window.

5.A. Detection of Amplified Fragments Using the Applied Biosystems® 3500 or 3500xL Genetic Analyzers (continued)

- 2.d. To create a new Assay, navigate to the Library. Select **Assays**, and then select **Create**. Alternatively, a previously created Assay can be used.

In the 'Create New Assay' window (Figure 5), select the **Instrument Protocol** created in Step 2.a and the **Sizecalling Protocol** created in Step 2.c. Assign a descriptive assay name. Select the application type **Fragment**. An Assay is required for all named samples on a plate.

Note: If autoanalysis of sample data is desired, refer to the instrument user's manual for instructions.

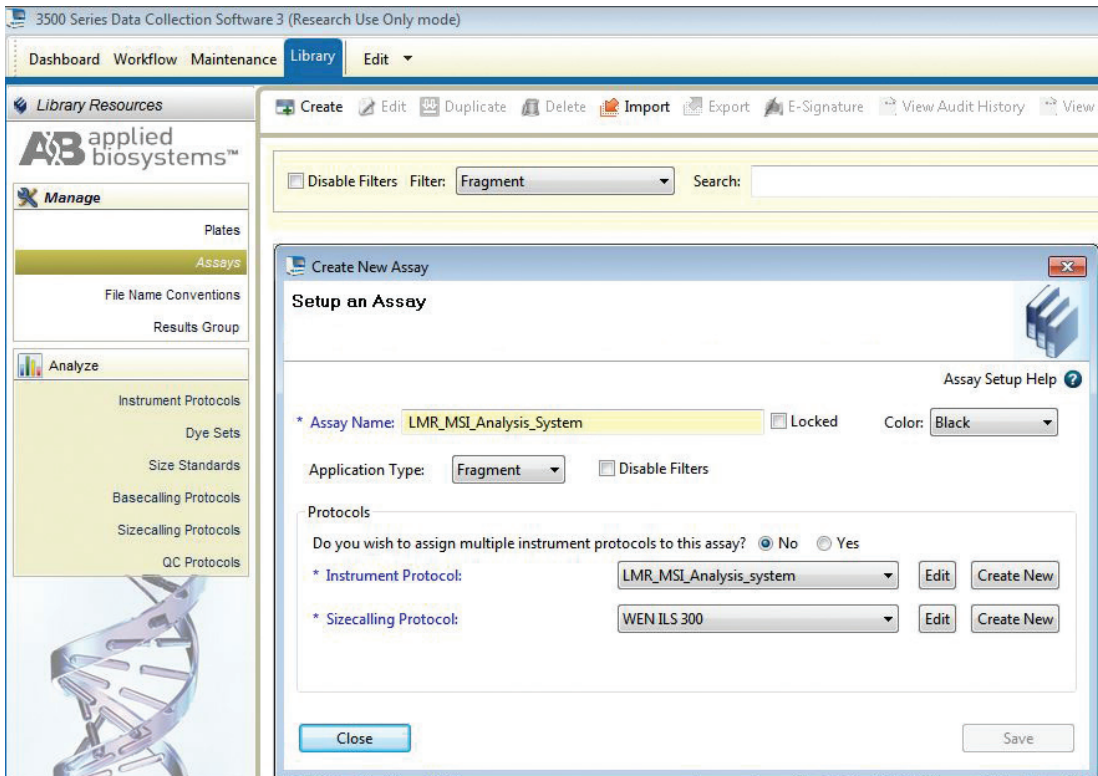


Figure 5. The 'Create New Assay' window.

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- 2.e. To create a new File Name Convention (Figure 6), navigate to the Library. Select **File Name Conventions**, and then select **Create**. Alternatively, a previously created File Name Convention can be used.
- Select the File Name Attributes according to your laboratory practices, and save with a descriptive name.

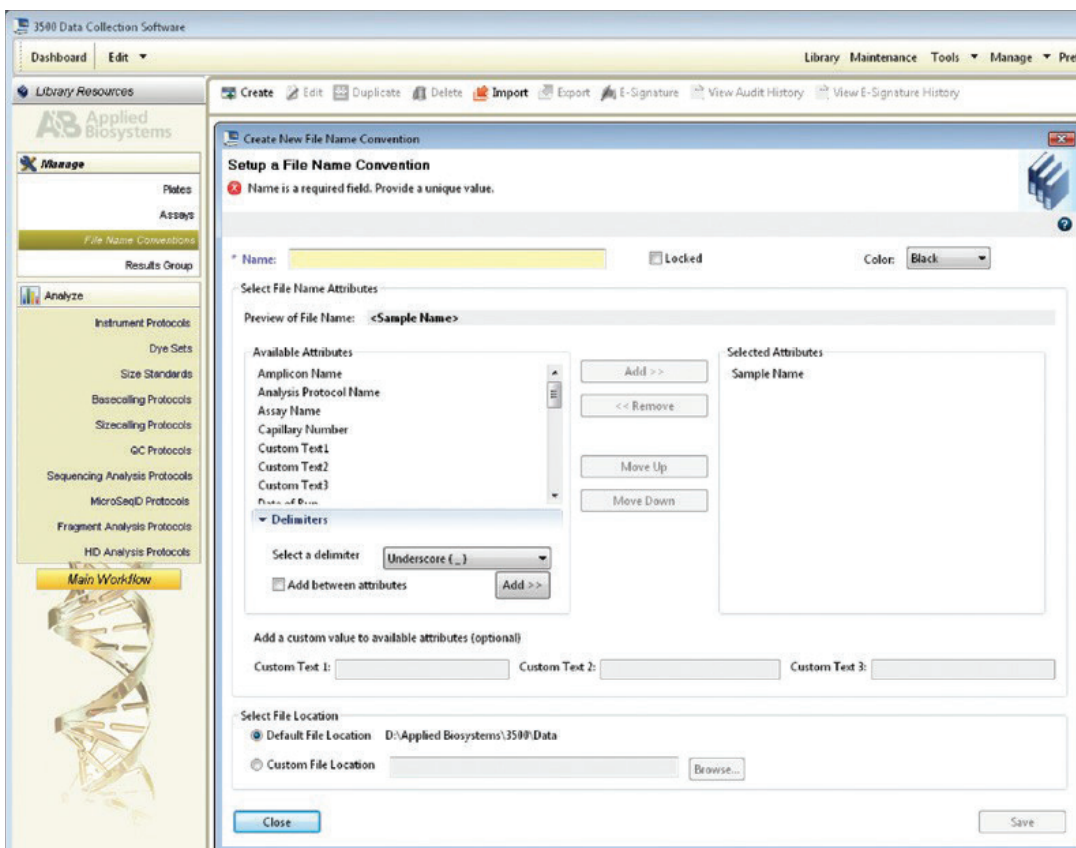


Figure 6. The 'Create New File Name Convention' window.

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5.A. Detection of Amplified Fragments Using the Applied Biosystems® 3500 or 3500xL Genetic Analyzers (continued)

- 2.f. To create a new Results Group (Figure 7), navigate to the Library. Select **Results Group**, and then select **Create**. Alternatively, a previously created Results Group can be used.

Select the Results Group Attributes according to your laboratory practices. Save with a descriptive name.

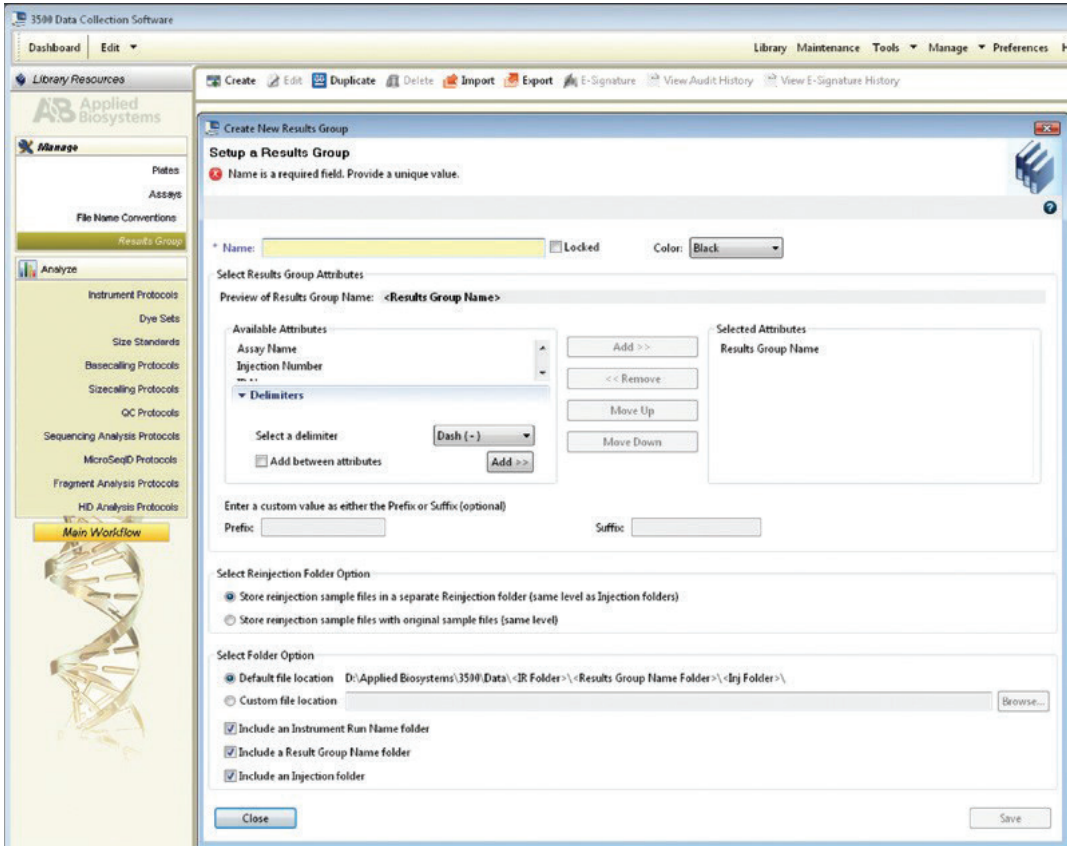


Figure 7. The 'Create New Results Group' window.

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3. To create a New Plate, navigate to the Library, and from the 'Manage' menu, select **Plates** and then **Create**.
4. Assign a descriptive plate name. Select the plate type Fragment from the drop-down menu (Figure 8).

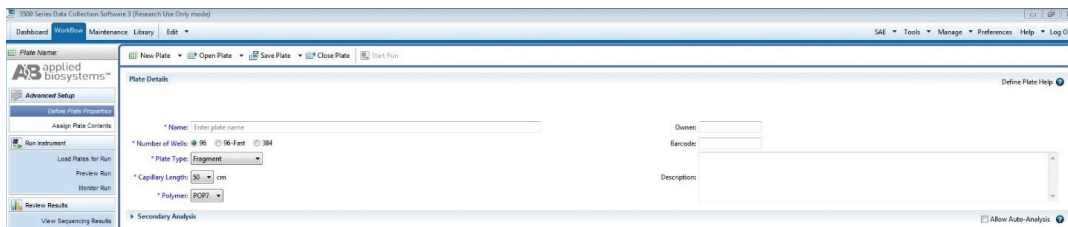


Figure 8. Defining plate properties.

5. Select **Assign Plate Contents** (Figure 9).

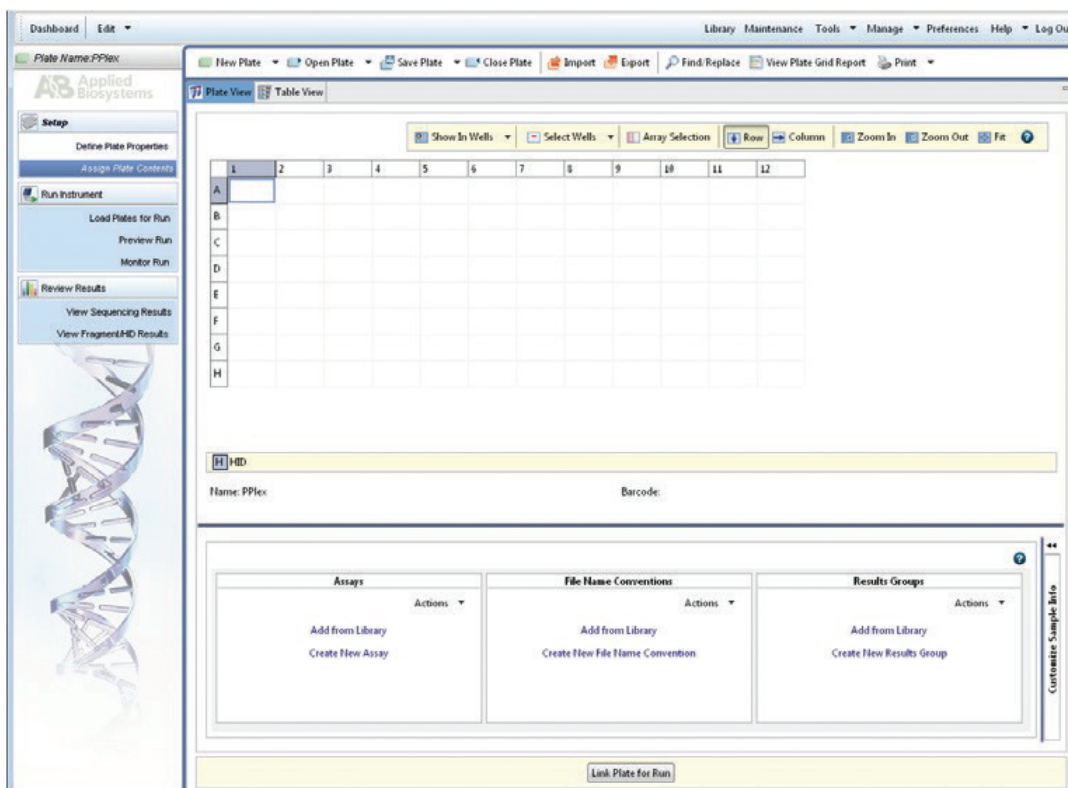
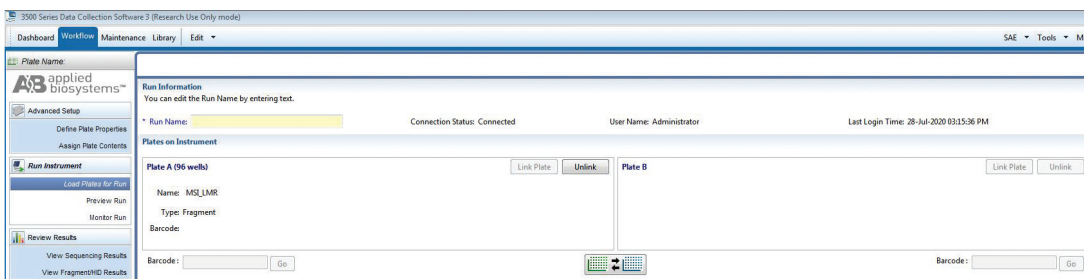


Figure 9. Assigning plate contents.

5.A. Detection of Amplified Fragments Using the Applied Biosystems® 3500 or 3500xL Genetic Analyzers (continued)

6. Assign sample names to wells.
7. In the lower left portion of the screen, under Assays, use the 'Add from Library' option to select the Assay created in Step 2.d or one previously created. Click the **Add to Plate** button, and close the window.
8. Under File Name Conventions, use the 'Add from Library' option to select the File Name Convention created in Step 2.e or one previously created. Click the **Add to Plate** button, and close the window.
9. Under Results Groups, use the 'Add from Library' option to select the Results Group created in Step 2.f or one previously created. Click the **Add to Plate** button, and close the window.
10. Highlight the sample wells, and then select the boxes in the Assays, File Name Conventions and Results Groups that pertain to those samples.
11. Select **Link Plate for Run**.
12. The 'Load Plate' window will appear. Select **Yes**.
13. In the 'Run Information' window (Figure 10), assign a Run Name. Select **Start Run** (not shown).

Note: Each injection will take approximately 30 minutes.



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Figure 10. Assigning a Run Name.

6. Data Analysis Using GeneMapper® Software, Version 5 or Higher

The instructions in this section were written using GeneMapper® Software, version 5. Due to potential differences between individual software versions, some of the instructions may not apply to all software versions.

6.A. Importing LMR MSI Panels and Bins into GeneMapper® Software

To facilitate analysis of data generated with the LMR MSI Analysis System, we have created panels and bins text files to allow automatic assignment of genotypes using GeneMapper® software. We recommend that users receive training from Applied Biosystems® on the GeneMapper® software to familiarize themselves with proper operation of the software.

Getting Started

1. To obtain the proper panels and bins text files and WEN_ILS_300.xml file for the LMR MSI Analysis System go to: www.promega.com/resources/software-firmware/fragment-analysis/lmr-msi-analysis-system-panels-and-bins-request/
The WEN_ILS_300.xml file can be used for the WEN ILS 500 size standard supplied with the LMR MSI Analysis System. Only the 60–300 base internal lane standard fragments are required for analysis.
2. Select the **LMR MSI Analysis System** and select **GeneMapper**. Enter your contact information, and select **Submit**.
3. Save the LMR_MSI_Panels_vX.x.txt and LMR_MSI_Bins_vX.x. where “X.x” refers to the most recent version of the panels and bins text files, to a known location on your computer.
4. Save the WEN_ILS_300.xml file to a known location on your computer.

Importing Panels and Bins Text Files

1. Open the GeneMapper® software.
2. Select **Tools** and then **Panel Manager**.
3. Highlight the **Panel Manager** icon in the upper left navigation pane.
4. Select **File** and then **Import Panels**.
5. Navigate to the panels text file downloaded in the ‘Getting Started’ section. Select the file and then **Import**.
6. In the navigation pane, highlight the LMR MSI panels folder that you just imported in Step 5.
7. Select **File** and then **Import Bin Set**.
8. Navigate to the bins text file downloaded in the Getting Started section. Select the file and then **Import**.
9. In the navigation pane, highlight the ‘LMR MSI panels’ folder that you just imported in Step 5.
10. At the bottom of the ‘Panel Manager’ window, select **OK**. This will save the panels and bins text files and close the window automatically.

6.B. Importing the WEN ILS 500 Size Standard into GeneMapper® Software

There are two options when creating a size standard. Use this protocol or the alternative protocol in Section 6.C.

The WEN_ILS_300.xml file can be used for the WEN ILS 500 size standard supplied with the LMR MSI Analysis System. Only the 60–300 base internal lane standard fragments are required for analysis.

1. Select **Tools** and then **GeneMapper Manager**.
2. Select the 'Size Standard' tab.
3. Select **Import**.
4. Navigate to the WEN_ILS_300.xml file downloaded in Section 6.A.
5. Highlight the file, and then select **Import**.
6. Select **Done** to save changes and close the GeneMapper® Manager.

6.C. Creating a Size Standard with GeneMapper® Software

1. Select **Tools** and then **GeneMapper Manager**.
2. Select the 'Size Standard' tab.
3. Select **New**.

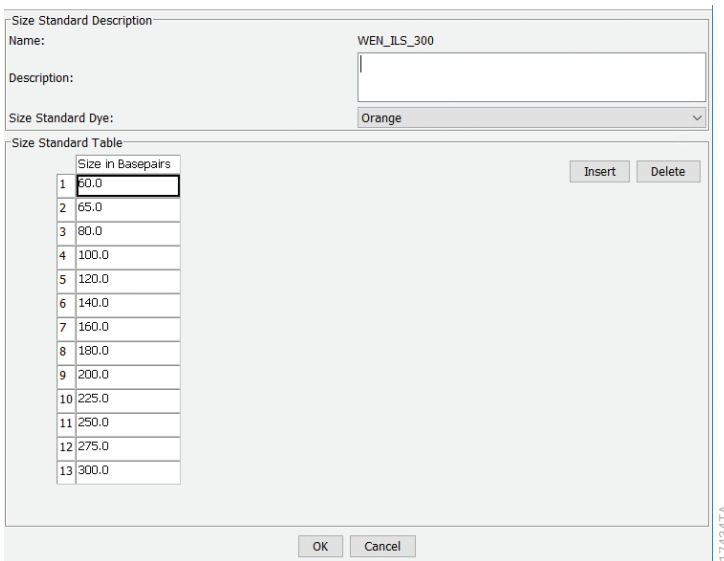


Figure 11. The GeneMapper® Size Standard Editor, version 5 or higher.

4. Enter a detailed name, such as "WEN_ILS_300".
5. Choose **Orange** for the Size Standard Dye.

6. Enter the sizes of the internal lane standard fragments (60, 65, 80, 100, 120, 140, 160, 180, 200, 225, 250, 275 and 300 bases).
7. Select **OK**.

6.D. Creating an Analysis Method with GeneMapper® Software

These instructions are intended as a guide to start analyzing data in GeneMapper® software. They are not intended as a comprehensive guide for using GeneMapper® software. We recommend that users contact Applied Biosystems® for training on the software.

Processing Data for LMR MSI Samples

1. Select **Tools** and then **GeneMapper® Manager**.
2. Select the 'Analysis Methods' tab.
3. Select **New**, and a new analysis method dialog box will open.
4. Enter a descriptive name for the analysis method, such as "LMR MSI".
5. Select the 'Allele' tab (Figure 12).

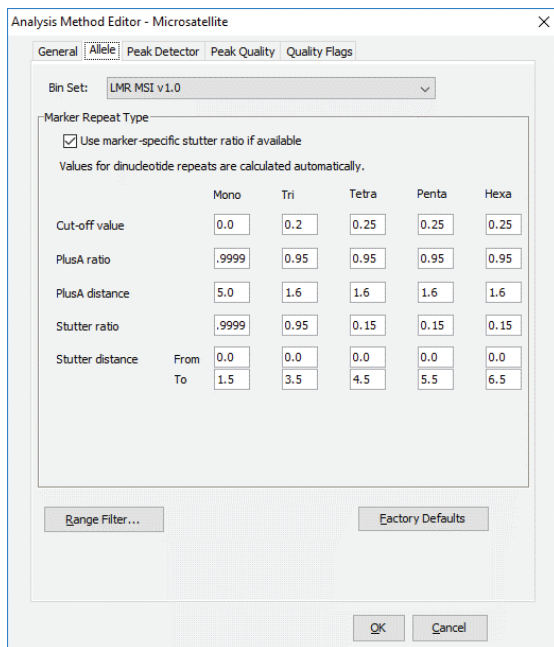


Figure 12. The GeneMapper® Software, version 5, 'Allele' tab.

6.D. Creating an Analysis Method with GeneMapper® Software (continued)

7. Select the bins text file that was imported in Section 6.A.
8. If you do not check the 'Use marker-specific stutter ratio and distance if available' box, you will need to optimize these settings. In-house validation should be performed. If in-house validation is not performed, ensure that the 'Use marker-specific stutter ratio and distance if available' box is checked. This will assign locus-specific stutter filters and distances from the imported stutter file. We recommend the settings shown in Figure 12 for proper filtering of stutter peaks when using the LMR MSI Analysis System.
9. Select the 'Peak Detector' tab (Figure 13). You may need to optimize these settings. In-house validation should be performed.

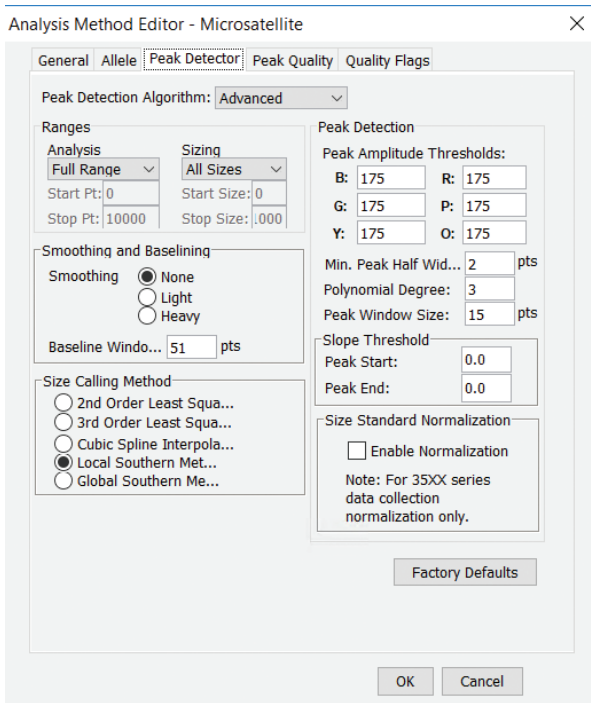


Figure 13. The GeneMapper® Software, version 5 or higher, 'Peak Detector' tab.

Notes:

- a. Select full range or partial range for the analysis range. When using a partial range, choose an appropriate analysis range based on your data. Choose a start point after the primer peak and just before the first defined internal lane standard peak to help ensure proper sizing of the internal lane standard.
- b. The peak amplitude thresholds are the minimum peak heights at which the software will call a peak. Individual laboratories should determine their peak amplitude thresholds from internal validation studies.
- c. If normalization was not used during data collection, make sure the normalization box is unchecked.

10. Select the 'Peak Quality' tab. You can change the settings for peak quality.
Note: For Steps 10 and 11, see the GeneMapper® user's manual for more information.
11. Select the 'SQ & GQ Settings' tab. You can change these settings.
12. Select **Save** to save the new analysis method.
13. Select **Done** to exit the GeneMapper® Manager.

Processing Data for LMR MSI Samples

1. Select **File** and then **New Project**.
2. Select **Microsatellite** as project type
3. Select **File** and then **Add Samples to Project**.
4. Browse to the location of the run files. Highlight desired files, and then select **Add to list** followed by **Add**.
5. In the 'Sample Type' column, use the drop-down menu to select **Sample, Positive Control** or **Negative Control** as appropriate for the sample.
6. In the 'Analysis Method' column, select the analysis method created previously in this section.
7. In the 'Panel' column, select the panels text file that was imported in Section 6.A.
8. In the 'Size Standard' column, select the size standard that was imported in Section 6.B or created in Section 6.C.
9. Select **Analyze** (green arrow button) to start data analysis.
10. If all analysis requirements are met, the 'Save Project' window will open (Figure 14).

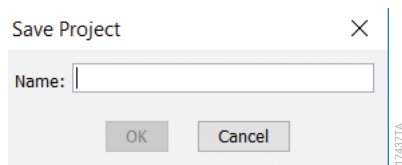


Figure 14. The 'Save Project' window.

11. Enter the project name.

When the analysis is finished we recommend that you review any yellow or red marker header bars in the plots view and handle them according to laboratory standard operating procedures.

The values displayed in the 'Analysis Method Peak Quality' and 'SQ & GQ Settings' tabs are defaults and will affect the quality values displayed in the plot settings. We recommend that you modify the values in these tabs to fit your laboratory's data analysis protocols.

6.E. Controls

1. Observe the results for the negative control. Using the protocols defined in this manual, the negative control should be devoid of amplification products.
2. Observe the results for the 2800M Control DNA. The expected 2800M Control DNA marker designations for each locus are listed in Table 1.

7. Results

Representative results for normal, non-tumor tissue using the LMR MSI Analysis System are shown in Figure 15.

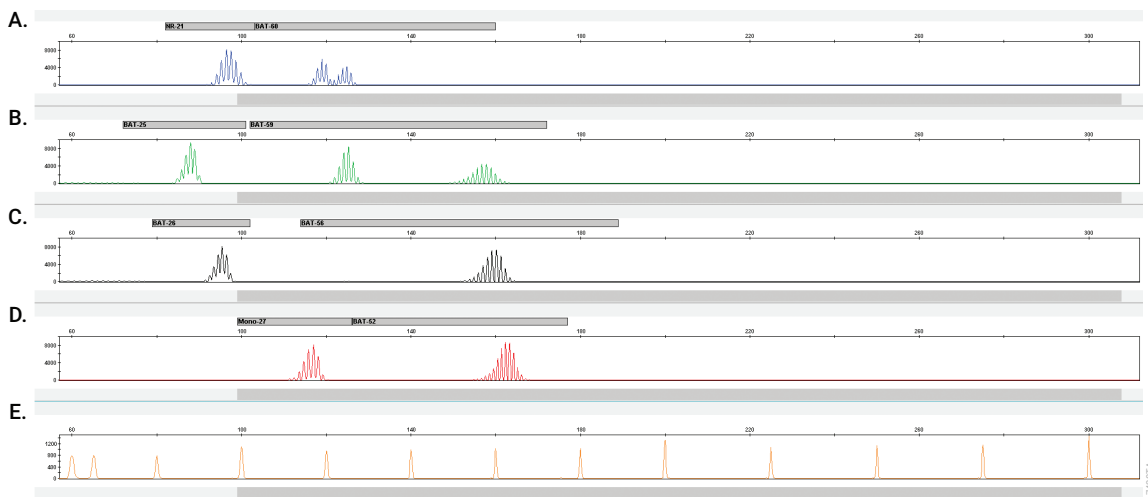


Figure 15. Representative results for the LMR MSI Analysis System. The 2800M Control DNA (1ng) was amplified using the LMR MSI Analysis System. Amplification products were mixed with WEN Internal Lane Standard 500 and analyzed using an Applied Biosystem® 3500 Genetic Analyzer with POP-7® polymer and a 50cm capillary. **Panel A.** An electropherogram showing peaks of the fluorescein-labeled loci: NR-21 and BAT-60. **Panel B.** An electropherogram showing the peaks of JOE-labeled loci: BAT-25 and BAT-59. **Panel C.** An electropherogram showing the peaks of the TMR-ET-labeled loci: BAT-26 and BAT-56. **Panel D.** An electropherogram showing the peaks of the CXR-ET-labeled loci: Mono-27 and BAT-52. **Panel E.** An electropherogram showing the 60bp to 300bp fragments of the WEN Internal Lane Standard 500.

MSI detection is based on comparing allelic profiles generated by amplifying matching normal and tumor sample DNA. The appearance of novel alleles in the tumor sample indicates microsatellite instability (Figure 16). The LMR MSI Analysis System co-amplifies and detects a panel of microsatellite markers that have been shown to be sensitive and specific for detection of MSI unstable or high (MSI-H) samples with mismatch repair deficiencies. Specific PCR product sizes and the respective fluorescent dyes used for labeling of each microsatellite marker are described in Table 1.

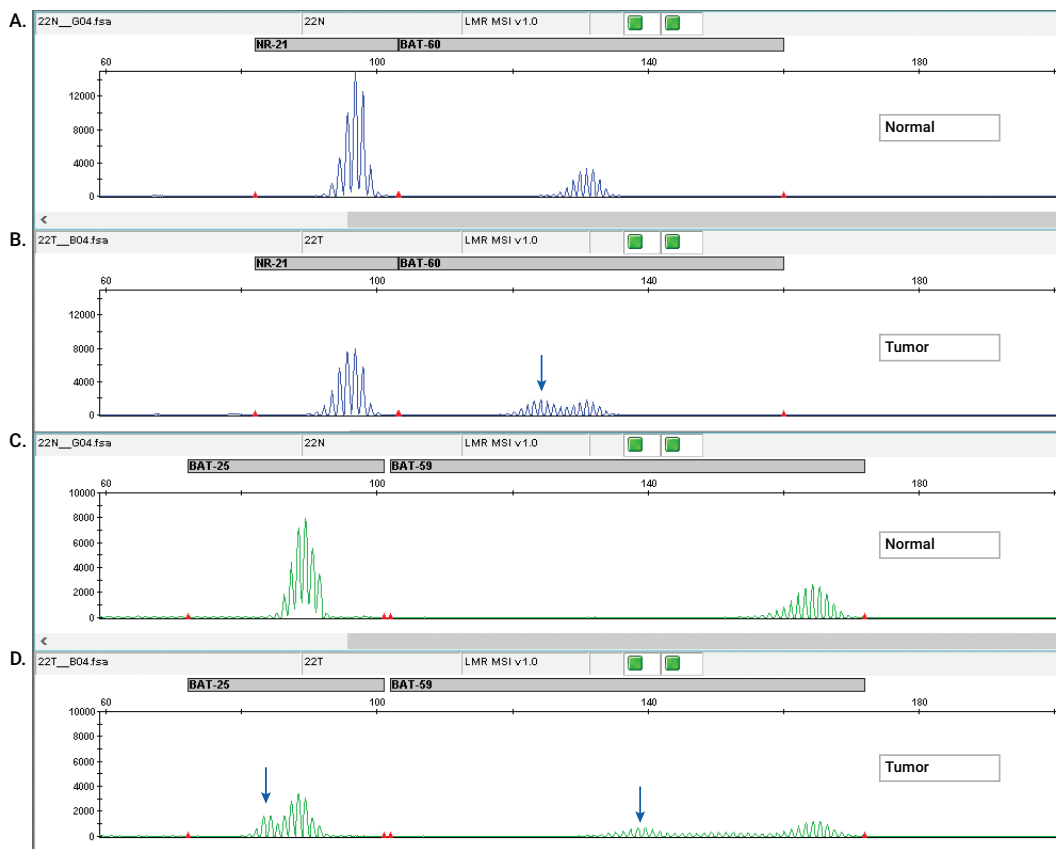


Figure 16. Microsatellite instability data using the LMR MSI Analysis System. Matching normal (Panels A, C, E, G) and tumor samples (Panels B, D, F, H) were analyzed using the LMR MSI Analysis System. Two nanograms of genomic DNA was amplified and analyzed using Applied Biosystem® 3500 Genetic Analyzer with POP-7® polymer and a 50cm capillary. The allelic patterns of the normal and tumor samples are shown. The presence of new alleles in the tumor sample (indicated by arrows) that were not present in the normal sample indicates MSI instability. PCR product sizes and respective fluorescent dyes for loci contained in the LMR MSI Analysis System are provided in Table 1 (figure continued, next page).

7. Results (continued)

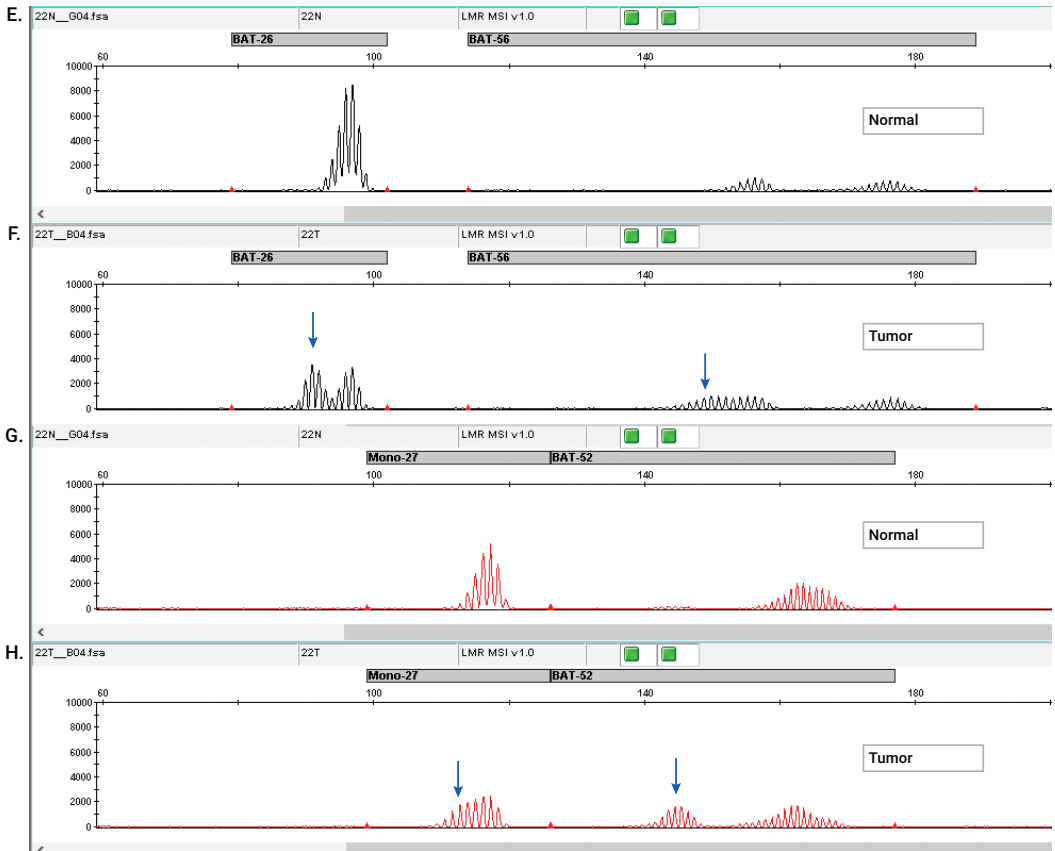


Figure 16. Microsatellite instability data using the LMR MSI Analysis System (continued). Samples with alterations in two or more of the eight microsatellite markers are conventionally classified as MSI-high (MSI-H), while samples bearing zero or one altered markers are commonly classified as microsatellite stable (MSS). Samples with a single altered marker are relatively uncommon. Such findings may need to be confirmed by repeating the LMR MSI Analysis System assay or using additional analysis methods.

Stutter

Amplification of microsatellite markers will yield one or two major allele peaks, depending on whether the individual is homozygous (one peak) or heterozygous (two peaks) for that marker. In addition, amplification of microsatellite markers often generates artifact peaks due to stutter. Electropherograms of mononucleotide markers show a number of less intense stutter peaks at 1bp intervals from the most prominent or true allele peak (Figure 17).

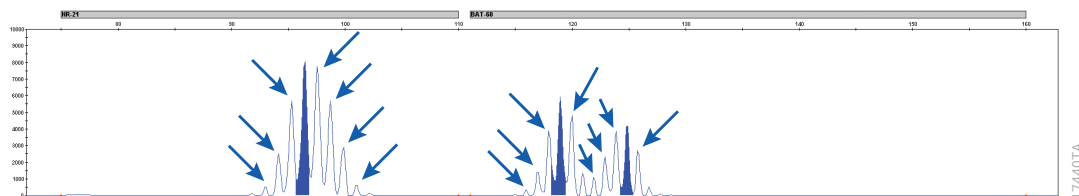


Figure 17. Stutter peaks. Stutter peaks (indicated by arrows), generated by polymerase slippage during PCR amplification of short tandem repeats, are clearly visible at the mononucleotide marker NR-21 (left) and BAT-60 (right) at 1bp intervals from the true or tallest allele peak (solid colored peak).

Instability of a given marker is recognized as an alteration in the length of the microsatellite due to deletion or insertion of a repeating unit, which produces a novel length allele in tumor DNA compared to matching normal DNA. The mononucleotide markers NR-21, BAT-25, BAT-26 and Mono-27 are nearly monomorphic so that most individuals are homozygous for the same common allele for a given marker. In contrast, the remaining mononucleotide LMR markers BAT-60, BAT-59, BAT-56 and BAT-52 are polymorphic. Heterozygosity (2 alleles) is commonly observed at these markers.

For data analysis, the GeneMapper® analysis software is recommended. The software allows manual and automated analysis of the raw data and generates electropherograms with accompanying data tables displaying PCR fragment lengths (in base pairs) and peak heights in relative fluorescent units (RFU). Results should be reviewed to eliminate artifact peaks caused by bleedthrough, stutter or CE spikes (Figures 17 and 18). Refer to the *GeneMapper® software user's manual* for detailed instructions on fragment analysis and genotyping. To simplify data analysis, we have created panels and bins text files to automatically assign genotypes using GeneMapper® software. The proper panels and bins text files for use with GeneMapper® software can be obtained from the Promega web site:

www.promega.com/resources/software-firmware/fragment-analysis/lmr-msi-analysis-system-panels-and-bins-request/

8. Results (continued)

Bleedthrough

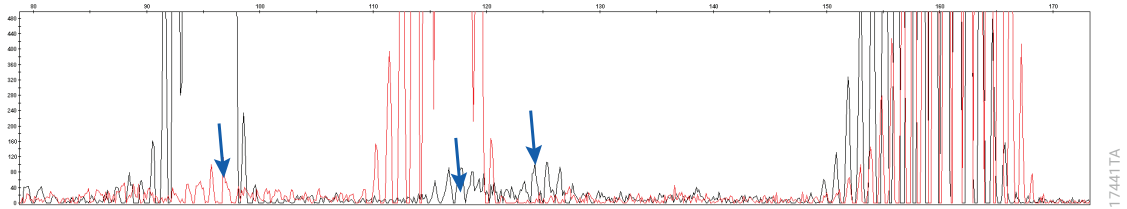


Figure 18. Bleedthrough or pull-up peaks. Small peaks (indicated by arrows) that overlap much higher peaks in another color channel are called bleedthrough or pull-up peaks. Bleedthrough can occur when peak heights are very high or saturated causing the dye to “bleed” into another dye channel. Alternatively, the analysis software may fail to distinguish between dye channels if a poor or incorrect matrix was applied to the samples.

Artifacts

In addition to stutter peaks, the following low-level artifact peaks may be observed with the LMR MSI Analysis System. The following artifacts have been observed when using POP-7[®] polymer and a 50cm capillary array for capillary electrophoresis: Artifacts within the NR-21 marker displaying as broad peaks in the size range of 83bp–90bp and 100bp–103bp. Known amplification artifacts are also observed within the BAT-25 marker as broad peaks in the size range of 86bp–89bp. These peaks should not be considered as an allele when determining the stability of the NR-21 or BAT-25 markers.

Other amplification artifacts may occur when too much DNA, or low quality DNA, is used as input to the LMR MSI Analysis System. The baseline signal in the NR-21, BAT-25 and BAT-26 markers may become elevated and resemble stutter peaks at 1bp intervals (Figure 19). This elevated baseline is DNA dependent and is not observed in every sample. Adopting the suggested analysis parameters for GeneMapper[®] analysis in Section 6 will help filter these artifacts out when identifying (i.e., calling) alleles for these markers.

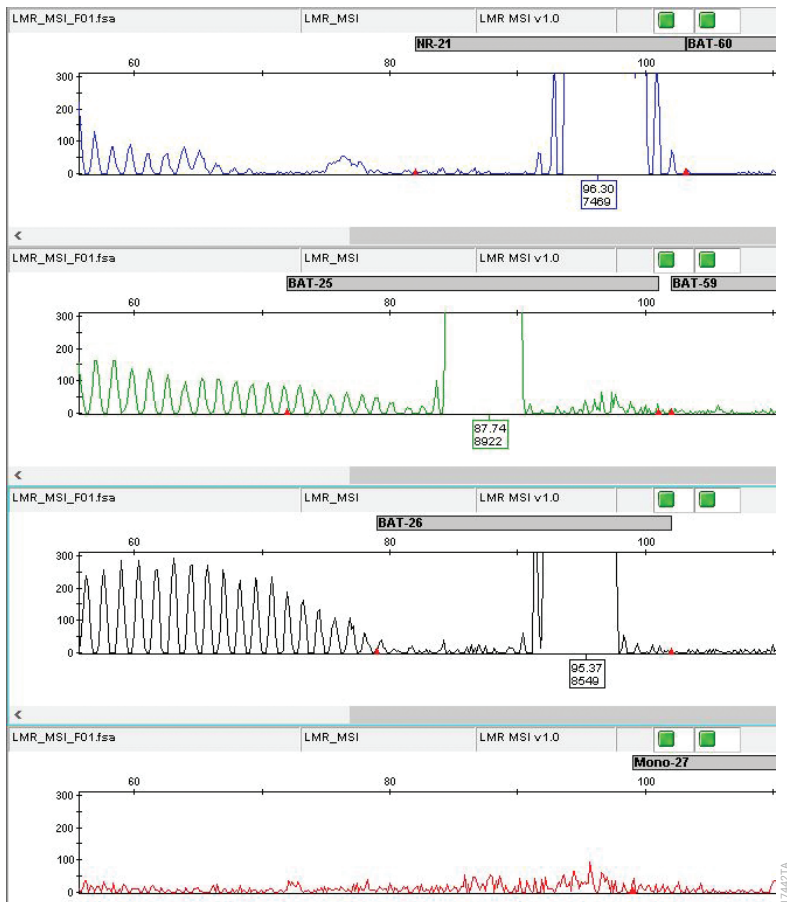


Figure 19. Elevated baseline. Example of elevated baseline in the NR-21, BAT-25 and BAT-26 markers. Mono-27 is an example of a typical baseline.



8. Troubleshooting

For questions not addressed here, consult the user guide for the applicable instrument or accessory or contact your local Promega Branch Office or Distributor. Contact information available at: www.promega.com. E-mail: genetic@promega.com.

For troubleshooting GeneMapper® software, version 5, refer to *GeneMapper® Software Version 5 Reference and Troubleshooting Guide*. If a different version of GeneMapper® software is used please refer to the appropriate software guide.

Amplification and Fragment Detection

Symptoms

Faint or absent allele peaks

Causes and Comments

Impure template DNA. Because a small amount of template is needed, this is rarely a problem. Depending on the DNA extraction procedure used and sample source, inhibitors might be present in the DNA sample. Faint or absent peaks may be seen more often when using the maximum template volume reaction.

Insufficient template DNA. Make sure DNA is accurately quantitated, and use the recommended amount of template DNA.

High salt concentration or altered pH. If the DNA template is stored in TE buffer that is not pH 8.0 or contains a higher EDTA concentration, the DNA volume should not exceed 20% of the total reaction volume. Carryover of K⁺, Na⁺, Mg²⁺ or EDTA from the DNA sample can negatively affect PCR. A change in pH also may affect PCR. Store DNA in TE⁻⁴ buffer (10mM Tris-HCl [pH 8.0], 0.1mM EDTA), TE⁻⁴ buffer with 20µg/ml glycogen or nuclease-free water. Faint or absent peaks may be seen more often when using the maximum template volume or reduced amplification reaction volume. We recommend storing DNA in TE⁻⁴ buffer; storing DNA in water is also acceptable. When storing DNA in water, -20°C is recommended for long-term storage.

Degraded DNA. Prepare new genomic DNA. If problem persists, FFPE tissue sample quality may be too low for successful analysis. Consider performing a DNA size analysis (by qPCR, such as ProNex® DNA QC Assay, or capillary electrophoresis instrument, such as BioAnalyzer) if problem persists.

Poor capillary electrophoresis injection (WEN ILS 500 peaks also affected). Re-inject the sample. Check the laser power.

Symptoms

Faint or absent allele peaks (continued)

Causes and Comments

Poor-quality DNA. Improper or prolonged fixation of paraffin-embedded samples can result in low DNA yields and poor quality DNA. Repeat DNA preparation. See Section 4 for DNA purification product recommendations. Alternatively, increase the amount of input DNA during PCR and/or increase the CE injection/voltage.

Poor-quality formamide was used. Use only Hi-Di™ formamide when analyzing samples.

Thermal cycler, plate or tube problems. Review the thermal cycling protocol in Section 4. Confirm PCR program is correct. Use recommended thermal cycler with appropriate plates or tubes. We have not tested other reaction tubes, plates or thermal cyclers. Calibrate the thermal cycler heating block if necessary.

Samples were not denatured completely. Heat-denature sample for the recommended time, and then cool on crushed ice or a freezer plate block or in an ice-water bath immediately prior to capillary electrophoresis. Do not cool samples in a thermal cycler set at 4°C, because this may lead to artifacts due to DNA re-annealing.

LMR MSI 5X Master Mix was not vortexed well before use. Vortex the 5X Master Mix for 15 seconds before dispensing into the PCR amplification mix.

An air bubble formed at the bottom of the reaction tube. Use a pipette to remove the air bubble, or centrifuge the reactions briefly before thermal cycling.

Amplification reaction components were not added to the bottom of the tube or well. Gently tap the tube or plate to move the liquid to the bottom or centrifuge briefly.

Primer concentration was too low. Use the recommended primer concentration. Vortex LMR MSI 5X Primer Pair Mix for 15 seconds before use.

Improper storage of the 2800M Control DNA. Store the 2800M Control DNA at +2°C to +10°C. Make sure that the 2800M Control DNA is stored at +2°C to +10°C for at least 24 hours before use; do not refreeze. Vortex before use.

The 2800M Control DNA dilution was prepared previously and reused. Prepare a fresh dilution of 2800M Control DNA, and repeat amplification reactions and capillary electrophoresis analysis, using a freshly prepared positive control sample and a negative control.

7. Troubleshooting (continued)

Symptoms	Causes and Comments
Excessive fluorescent signal for allele peaks	Too much template DNA. Make sure DNA was accurately quantitated, and use 2ng of sample DNA and 1ng of 2800M Control DNA. Dilute PCR product 1:5 to 1:10 in deionized water or lower injection time on the CE instrument.
Extra peaks visible in one or all dye channels	<p>Stutter peaks were mistaken for microsatellite alleles.</p> <p>Amplification of microsatellite markers generates artifacts that appear as smaller peaks 1bp above or below the prominent mononucleotide-repeat allele(s). Stutter product peak heights will be higher if too much template DNA was used.</p> <p>Excess amount of DNA. We recommend 2ng of sample DNA and 1ng of 2800M Control DNA. Amplification of greater amounts of DNA template may result in a higher number of stutter products. Use less DNA template, or reduce the number of cycles in the amplification program.</p> <p><u>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. Confirm that correct spectral calibration was used. Repeat spectral calibration on capillary electrophoresis instrument. Make sure DNA is accurately quantified and diluted. Repeat amplification using 1.0ng of sample DNA. If problem persists, repeat the amplification with less DNA.</u></p> <p>Samples were not properly denatured prior to loading. Heat-denature samples for the recommended time, and cool on crushed ice or in an ice-water bath immediately prior to loading the capillary.</p> <p>Capillary electrophoresis-related artifacts. Aberrant peaks may be observed during capillary electrophoresis. Ensure that polymer is at room temperature before installation and use. Repeat injections. Repeat amplification and capillary electrophoresis if problem persists.</p> <p>Contamination with another template DNA or previously amplified DNA. Use aerosol-resistant pipette tips, and change gloves regularly.</p> <p>An unstable tumor sample was mistakenly analyzed as a normal reference sample. Verify that samples were identified and labeled correctly. Repeat amplification and capillary electrophoresis analysis to confirm root cause.</p>

Symptoms

Preferential amplification of smaller markers

Causes and Comments

DNA was cross-linked. DNA prepared from formalin-fixed, paraffin-embedded samples often is cross-linked with other DNA or protein molecules, preventing amplification of longer DNA fragments. Repeat amplification using more DNA. If saturation of smaller markers is observed with more DNA, repeat DNA extraction and sample analysis using a different tissue section. If problem persists, FFPE tissue sample quality may be too low for successful analysis.

Degraded DNA. DNA template was degraded into smaller fragments, with the larger markers showing diminished yield. Verify correct storage conditions for 2800M Control DNA and FFPE DNA samples. Repeat amplification reaction using more DNA. If saturation of smaller markers is observed with more DNA, repeat DNA extraction and sample analysis using a different tissue section. If problem persists, FFPE tissue sample quality may be too low for successful analysis.

Insufficient template DNA. Make sure DNA is accurately quantified and diluted, and repeat amplification using 2ng of template DNA if a quantification error is detected. If problem persists, repeat the amplification reaction with more DNA, or repeat DNA preparation using a different tissue section.

If decreased peak heights are observed in both your amplification products and internal lane standard consider using Stabilizer Reagent.

9. Composition of Buffers and Solutions

TE⁻⁴ buffer (10mM Tris-HCl, 0.1mM EDTA)

1.21g Tris base

0.037g EDTA (Na²⁺ EDTA • 2H₂O)

Dissolve Tris base and EDTA in 900ml of deionized water. Adjust to pH 8.0 with HCl. Bring the final volume to 1 liter with deionized water.

TE⁻⁴ buffer with 20µg/ml glycogen

1.21g Tris base

0.037g EDTA (Na²⁺ EDTA • 2H₂O)

20µg/ml glycogen

Dissolve Tris base and EDTA in 900ml of deionized water. Adjust to pH 8.0 with HCl. Add glycogen. Bring the final volume to 1 liter with deionized water.

10. Related Products

Product	Size	Cat.#
Maxwell [®] RSC DNA FFPE Kit	48 preps	AS1450
ReliaPrep [™] FFPE gDNA Miniprep System	10 reactions	A2351
GenePrint [®] 5C Matrix Standard	5 preps	B1930
2800M Control DNA	25µl	DD7101
Water, Amplification Grade	5 × 1,250µl	DW0991
WEN Internal Lane Standard 500	200µl	DG5001
Spectrum Compact CE System, 4-Capillary	please enquire	CE1304
Stabilizer Reagent	500 preps	DM6571
ProNex [®] DNA QC Assay BioRad CFX96 [™]	200 reaction	NG1004
	800 reactions	NG1005
ProNex [®] DNA QC Assay ABI 7500/7500FAST	200 reaction	NG1002
	800 reactions	NG1003
QuantiFluor [®] ONE dsDNA System	100 reactions	E4871
	500 reactions	E4870
QuantiFluor [®] dsDNA System	1ml	E2670
	100µl	E2671

11. Summary of Changes

The following changes were made to the 1/24 revision of this document:

1. Updated link in Sections 6 and 7.
2. Changed font and cover image.
3. Made minor text edits.

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