ddPCR™ Microsatellite Instability (MSI) Kit

Catalog #12015172

User Guide

Test for the Detection of Microsatellite Instability by Droplet Digital™ PCR (ddPCR)

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Section 1
Introduction

Microsatellites are short tandem repeats of DNA, with repeat units that are typically 1 to 6 bp in length and repeated 5 to 50 times. They are distributed throughout the genome and are more susceptible to mutations. Microsatellite instability (MSI) arises when microsatellites undergo a length change as a result of an impaired mismatch repair (MMR) system. Approximately 15% of colorectal cancer patients exhibit an impaired MMR system and MSI, stemming from either somatic or germline mutations in MMR genes (Boland and Goel 2010). Studies have shown patients with MSI typically have a better prognosis and are more likely to respond to immunotherapy, underscoring the need for a test that can assess MSI (Dudley 2016, Vilar and Gruber 2010).

The ddPCR Microsatellite Instability Kit detects deletions and insertions in five mononucleotide microsatellite markers (BAT25, BAT26, NR21, NR24, and Mono27) using Droplet Digital PCR technology, in plasma or tumor tissue, including formalin-fixed paraffin-embedded (FFPE) tissue from colorectal cancer patients and other potentially MSI-relevant cancers. These five markers have been classified as sensitive markers to size alterations and shifts in microsatellites in tumors with defective MMR systems (Bacher, et al. 2004). The ddPCR MSI Kit consists of three assays: assay 1 (BAT25 and BAT26), assay 2 (NR21 and NR24), and assay 3 (Mono27). This test does not assess the mutational status of MMR genes or whether any such mutations are somatic or germline.

The ddPCR Microsatellite Instability Kit includes enough reagents for 200 reactions to test up to 56 samples including controls. The kit is intended for use on the Bio-Rad™ QX200™ Droplet Digital PCR System or the QX200 AutoDG™ Droplet Digital PCR System.

Section 2
Kit Contents

The ddPCR Microsatellite Instability Kit contents are listed in Table 1. The kit contains sufficient reagents for 200 reactions. Upon receipt, store the kit in a constant temperature freezer at -15°C to -25°C. The kit reagents can be frozen and thawed up to three times. Repeated freezing and thawing of the kit reagents more than three times is not recommended. Reagents can be used until the expiration date indicated on the tube when stored properly.

Table 1. Kit contents (catalog #12015172).

Reagent	Material Number	Quantity
ddPCR Multiplex Supermix (4x)	12005915	2
ddPCR MSI Assay 1 (BAT25/BAT26) (20x)	16009019	1
ddPCR MSI Assay 2 (NR21/NR24) (20x)	16009020	1
ddPCR MSI Assay 3 (Mono27) (20x)	16009029	1
ddPCR Positive Control	16009021	1
Uracil-DNA Glycosylase (UDG)	16009039 or 12017702	1
Nuclease-Free Water (no template control)	8006877	2

Section 3

Required Equipment, Reagents, and Consumables

This kit is designed for use on the Bio-Rad QX200 AutoDG Droplet Digital PCR System or the QX200 Droplet Digital PCR System. Materials and instruments required but not supplied for use with the QX200 AutoDG Droplet Digital PCR System are listed in Table 2. Materials and instruments required but not supplied for use with the QX200 Droplet Digital PCR System are listed in Table 3. Adjustable Rainin and Eppendorf pipets can be used with the materials listed in Table 2 and Table 3.

Table 2. List of materials and instruments needed but not supplied with the ddPCR Microsatellite Instability Kit for use on the QX200 AutoDG Droplet Digital PCR System.

Description	Vendor	Catalog Number
Automated Droplet Generator	Bio-Rad	1864101
DG32 Automated Droplet Generator Cartridges	Bio-Rad	1864108
Pipet Tips for the AutoDG System	Bio-Rad	1864120
Pipet Tip Waste Bins for the AutoDG System	Bio-Rad	1864125
Automated Droplet Generation Oil for Probes	Bio-Rad	1864110
C1000 Touch Thermal Cycler with 96–Deep Well Reaction Module	Bio-Rad	1851197
PX1 PCR Plate Sealer	Bio-Rad	1814000
PCR Plate Heat Seal	Bio-Rad	1814040
ddPCR 96-Well Plates	Bio-Rad	12001925
QX200 Droplet Reader	Bio-Rad	1864003
ddPCR Droplet Reader Oil	Bio-Rad	1863004
ddPCR Buffer Control for Probes (2x)	Bio-Rad	1863052
QX Manager Software Premium Edition, v2.1 or higher*	Bio-Rad	12018108
MSI Assay Protocol File, MSI10_FFPE_QR.apfpack**	Bio-Rad	N/A
MSI Assay Protocol File, MSI10_FFPE.apfpack**	Bio-Rad	N/A
MSI Assay Protocol File, MSI10_Plasma_QR.apfpack**	Bio-Rad	N/A
MSI Assay Protocol File, MSI10_Plasma.apfpack**	Bio-Rad	N/A
Vortexer	Any	N/A
Microcentrifuge	Any	N/A
Low TE Buffer (10mM Tris-HCl, 0.1mM EDTA, pH 8.0)	Any	N/A

* If not already installed, go to **bio-rad.com/QXSoftware** to download and install the latest QX Manager Software.

** Go to **bio-rad.com/ddPCR-MSI** to download the ddPCR MSI Assay Protocol Files.

Table 3. List of materials and instruments needed but not supplied with the ddPCR Microsatellite Instability Kit for use on the QX200 Droplet Digital PCR System.

Description	Vendor	Catalog Number
QX200 Droplet Generator	Bio-Rad	1864002
DG8 Cartridges for QX200 Droplet Generator	Bio-Rad	1864008
DG8 Gaskets for QX200 Droplet Generator	Bio-Rad	1863009
DG8 Cartridge Holder	Bio-Rad	1863051
Droplet Generation Oil for Probes	Bio-Rad	1863005
C1000 Touch Thermal Cycler with 96–Deep Well Reaction Module	Bio-Rad	1851197
PX1 PCR Plate Sealer	Bio-Rad	1814000
PCR Plate Heat Seal, foil, pierceable	Bio-Rad	1814040
QX200 Droplet Reader	Bio-Rad	1864003
ddPCR 96-Well Plates	Bio-Rad	12001925
ddPCR Droplet Reader Oil	Bio-Rad	1863004
ddPCR Buffer Control for Probes (2x)	Bio-Rad	1863052
QX Manager Software Premium Edition, v2.1 or higher*	Bio-Rad	12018108
MSI Assay Protocol File, MSI10_FFPE_QR.apfpack **	Bio-Rad	N/A
MSI Assay Protocol File, MSI10_FFPE.apfpack**	Bio-Rad	N/A
MSI Assay Protocol File, MSI10_Plasma_QR.apfpack**	Bio-Rad	N/A
MSI Assay Protocol File, MSI10_Plasma.apfpack**	Bio-Rad	N/A
Vortexer	Any	N/A
Microcentrifuge	Any	N/A
Low TE Buffer (10mM Tris-HCl, 0.1mM EDTA, pH 8.0)	Any	N/A

* If not already installed, go to **bio-rad.com/QXSoftware** to download and install the latest QX Manager Software.

** Go to **bio-rad.com/ddPCR-MSI** to download the ddPCR MSI Assay Protocol Files.

The Assay Protocol File contains pre-loaded assay specifications for all three MSI assays and an automated analysis procedure, including auto-thresholding. Only one APF is required to run the ddPCR MSI test. There are four MSI APFs to support different sample types and analysis options. The four MSI APFs are summarized in Table 4. Two of the APFs are compatible with FFPE or Fresh Frozen (FF) tumor samples. The other two APFs are compatible with plasma or Fresh Frozen (FF) tumor samples. If the DNA extracted from FF tumor is suspected of being damaged, then it's recommended to use only the APFs that are also compatible with FFPE tumor samples. System requirements to run the ddPCR MSI test are listed in

Table ***5***.

Table 4. Summary of MSI Assay Protocol Files.

File Name	Display Name in QX Manager	Sample Type*	Auto-thresholding	Auto-calling of Markers	Quality Rules
MSI10_FFPE_QR.apfpack	MSI FFPE Quality Rules v1.0	FFPE or FF tumor	yes	yes	yes
MSI10_FFPE.apfpack	MSI FFPE v1.0	FFPE or FF tumor	yes	yes	no
MSI10_Plasma_QR.apfpack	MSI Plasma Quality Rules v1.0	Plasma or FF tumor	yes	yes	yes
MSI10_Plasma.apfpack	MSI Plasma v1.0	Plasma or FF tumor	yes	yes	no

*FF = fresh frozen. It is recommended to use the FFPE compatible APFs for fresh frozen tumor if the extracted DNA is damaged.

Table 5. System Requirements.

Specification	Minimum Requirement
Operating System	Windows 10 64-Bit
CPU	6th Generation Intel 2 Core Processor
Hard Drive	500GB
System Memory	16 GB
Display Resolution	1920 x 1080
Ports	1 USB

Section 4
Precautions and Recommendations

This test should only be performed by adequately trained personnel. All samples should be handled as biosafety level 2 (BSL-2) with appropriate precautions taken and correct personal protective equipment (PPE) used. Plasma samples should be handled in a biosafety cabinet by individuals trained for the appropriate equipment and samples. All waste should be placed in biohazard bags, sharps containers, or appropriate fluid waste containers, and then disposed of appropriately in accordance with local guidelines.

The quality of results depends on strict compliance with the following good laboratory practice (for example the EN ISO 7218 standard), especially regarding PCR:

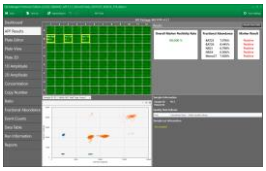
- Laboratory equipment (pipets, tubes, etc.) must not circulate between workstations
- It is essential to use a positive control and no template control (NTC) for PCR runs
- Do not use reagents after their expiration date
- Vortex reagents (with the exception of UDG) from the kit before use to ensure homogeneity

- The ddPCR Multiplex Supermix is especially viscous, it is recommended to fully thaw this reagent and vortex for 15 sec before use
- Thaw the positive control for a minimum of 30 min at room temperature and ensure thorough mixing before use
- Regularly calibrate pipets and instruments
- Change gloves often when changing environments or if you suspect your gloves are contaminated
- Clean workspaces, pipets, pipet tip boxes, and equipment that will interact with samples before and after use by wiping them down with 10% bleach followed by 70% ethanol to prevent contamination

Section 5
Protocol

It is strongly recommended to read the entire protocol before starting the test. The ddPCR MSI protocol consists of the following steps:

Step	Description	Instrument/Tool	Hands-on Time	Instrument Time
1	DNA extraction (FFPE or plasma)	Variable	Variable	Variable
2	Plate setup and droplet generation	 or 	<40 min	~45 min/plate
3	PCR amplification		<5 min	~150 min (~120 min for thermal cycling and ~30 min for cooling)
4	Droplet reading		<5 min	~120 min/plate

Step	Description	Instrument/Tool	Hands-on Time	Instrument Time
5	Analysis		None	~5 to 10 min

Sample Extraction

DNA Extraction - FFPE

Use any commercially available DNA FFPE extraction kit. The Promega ReliaPrep FFPE gDNA Miniprep System is recommended for genomic DNA extraction. Perform extraction with tissue containing a minimum of 10% tumor content and sufficient tissue to obtain a minimum yield of 10 ng. Quantitate the extracted DNA using fluorometry, quantitative PCR (qPCR), or ddPCR. Quantitation by ultraviolet (UV) absorbance is not recommended.

Note: Obtaining high-quality DNA from FFPE samples can be challenging. DNA degradation may result from a multitude of sources, such as poor formalin fixation, tissue age, and the extraction process. In the case of a highly degraded DNA sample, quantitation using an intercalating fluorescent dye may poorly correlate with the amount of amplifiable DNA, thus it is recommended to quantitate using qPCR or ddPCR.

DNA Extraction - Plasma

Use any commercially available plasma cell-free DNA (cfDNA) extraction kit. The QIAGEN QIAamp Circulating Nucleic Acid Kit is recommended for cfDNA extraction. Perform extractions using a minimum of 1 ml of plasma. The cfDNA yield can be low in some samples. To ensure enough DNA is obtained, a higher input volume of 4 ml is recommended. Quantitate extracted cfDNA using fluorometry, qPCR, or ddPCR. Quantitation by UV-absorbance is not recommended.

Note: If carrier RNA is required in the isolation process, yeast tRNA is recommended. Other carrier RNAs may interfere with the assay. Additionally, if any carrier RNA is used, quantitate using qPCR or ddPCR rather than fluorometry since some dsDNA-intercalating fluorescent dyes cross-stain RNA.

Reaction Setup

1. For each of the three assays in the ddPCR Microsatellite Instability Kit, two controls are required, a no template control (NTC) and a positive control (PC). The NTC and PC are run as duplicate wells in the plate. Samples may be run as single well or as duplicate wells. Figures 1 and 2 show suggested plate layouts for running samples in single and duplicate wells per assay, respectively.
2. Wells A1 to A6 and B1 to B6 are fixed for NTC and PC, respectively, as shown in Figures 1 and 2.

Note: The number of NTCs and PCs and their location on the plate are fixed in the APF.

Figure. 1. Suggested sample plate layout for running samples in single wells per assay.

	1	2	3	4	5	6	7	8	9	10	11	12	
A	NTC	NTC	NTC	NTC	NTC	NTC	Sample 13	Sample 13	Sample 13	Sample 21	Sample 21	Sample 21	Assay 1
B	PC	PC	PC	PC	PC	PC	Sample 14	Sample 14	Sample 14	Sample 22	Sample 22	Sample 22	Assay 2
C	Sample 1	Sample 1	Sample 1	Sample 7	Sample 7	Sample 7	Sample 15	Sample 15	Sample 15	Sample 23	Sample 23	Sample 23	Assay 3
D	Sample 2	Sample 2	Sample 2	Sample 8	Sample 8	Sample 8	Sample 16	Sample 16	Sample 16	Sample 24	Sample 24	Sample 24	
E	Sample 3	Sample 3	Sample 3	Sample 9	Sample 9	Sample 9	Sample 17	Sample 17	Sample 17	Sample 25	Sample 25	Sample 25	
F	Sample 4	Sample 4	Sample 4	Sample 10	Sample 10	Sample 10	Sample 18	Sample 18	Sample 18	Sample 26	Sample 26	Sample 26	
G	Sample 5	Sample 5	Sample 5	Sample 11	Sample 11	Sample 11	Sample 19	Sample 19	Sample 19	Sample 27	Sample 27	Sample 27	
H	Sample 6	Sample 6	Sample 6	Sample 12	Sample 12	Sample 12	Sample 20	Sample 20	Sample 20	Sample 28	Sample 28	Sample 28	

Figure. 2. Suggested sample plate layout for running samples in duplicate wells per assay.

	1	2	3	4	5	6	7	8	9	10	11	12	
A	NTC	NTC	NTC	NTC	NTC	NTC	Sample 7	Sample 7	Sample 7	Sample 7	Sample 7	Sample 7	Assay 1
B	PC	PC	PC	PC	PC	PC	Sample 8	Sample 8	Sample 8	Sample 8	Sample 8	Sample 8	Assay 2
C	Sample 1	Sample 1	Sample 1	Sample 1	Sample 1	Sample 1	Sample 9	Sample 9	Sample 9	Sample 9	Sample 9	Sample 9	Assay 3
D	Sample 2	Sample 2	Sample 2	Sample 2	Sample 2	Sample 2	Sample 10	Sample 10	Sample 10	Sample 10	Sample 10	Sample 10	
E	Sample 3	Sample 3	Sample 3	Sample 3	Sample 3	Sample 3	Sample 11	Sample 11	Sample 11	Sample 11	Sample 11	Sample 11	
F	Sample 4	Sample 4	Sample 4	Sample 4	Sample 4	Sample 4	Sample 12	Sample 12	Sample 12	Sample 12	Sample 12	Sample 12	
G	Sample 5	Sample 5	Sample 5	Sample 5	Sample 5	Sample 5	Sample 13	Sample 13	Sample 13	Sample 13	Sample 13	Sample 13	
H	Sample 6	Sample 6	Sample 6	Sample 6	Sample 6	Sample 6	Sample 14	Sample 14	Sample 14	Sample 14	Sample 14	Sample 14	

3. **FFPE DNA and cfDNA must be run on separate plates.** Samples are analyzed using metrics specific for each sample type in the APF. More details about these metrics are described in Section 6 Data Analysis and Result Interpretation.

4. The input range for FFPE DNA and cfDNA samples is ~2 to 60 ng per reaction. The recommended input is 5 ng per reaction. Less than 2 ng can be run but this input may fall below the assay sensitivity depending on the tumor content. Dilute FFPE DNA and cfDNA samples to ~0.33 to 10 ng/µl (~0.8 ng/µl is recommended) in low TE buffer (10mM Tris-HCl, 0.1mM EDTA, pH 8.0).

   Note: The yield for most cfDNA extractions will be lower than the recommended input and will not require dilution.

5. When preparing the reaction mix, keep the UDG (required for FFPE samples only) on ice and thaw all other required reagents, including all three assays, nuclease-free water, and the PC at room temperature. The ddPCR Multiplex Supermix must be thawed at room temperature for a minimum of 30 min.

   Note: While UDG is only required for FFPE samples it can be used for FF and cfDNA samples that have cytosine deamination.

6. Vortex all components of the kit for 15 sec to mix, except UDG.

   Note: The ddPCR Multiplex Supermix is highly viscous and, when vortexed, bubbles may form. If bubbles form, allow them to rise and avoid them when aspirating. If 15 sec is not sufficient for the ddPCR Multiplex Supermix to be homogeneous, vortex again until it is homogeneous.

7. Do not vortex the UDG. Mix the UDG by gently inverting the tube five times. Spin down all the tubes briefly before using.

8. Label three 1.5 mL or 2.0 ml Eppendorf DNA LoBind Tubes, one for each assay. Prepare the reaction mixes as outlined in Table 6 (FFPE DNA samples) or Table 7 (cfDNA samples) for all three assays.

9. Combine the nuclease-free water, ddPCR Multiplex Supermix, assay, and UDG (FFPE DNA samples only) in the 1.5 ml or 2.0 ml tubes. **Note:** The ddPCR Multiplex Supermix is viscous, pipet slowly.

10. Vortex the three reaction mixtures for 10 sec and spin down briefly.

11. Aliquot 15.4 µl of each reaction mixture into the designated wells of a 96-well plate, according to Figure 1 or 2.

12. Add 6.6 µl of nuclease-free water (NTC), PC and sample DNA to the appropriate wells, as outlined in Figure 1 or 2.

    Note: If desired, it's possible to add more than 6.6 µl of sample per well. This can be done by reducing the amount of nuclease-free water in the reaction mix by the same volume of additional sample added.

13. If there are any unused wells in a column, add 11 µl of water + 11 µl of ddPCR Buffer Control for Probes.

Table 6. Reaction Mix for FFPE DNA.

	Samples and Controls (reactions + overage) Volume (µl)				
Plates		**0.5**	**1**	**1.5**	**2**
Control Reactions		**4**	**4**	**8**	**8**
Sample Reactions		**12**	**28**	**40**	**56**
Total Reactions	**1**	**16**	**32**	**48**	**64**
Component	**1x**	**(16 + 2)x**	**(32 + 3)x**	**(48 + 4)x**	**(64 + 4)x**
Nuclease-free water	8.58	154.4	300.3	446.2	583.4
ddPCR Multiplex Supermix	5.5	99.0	192.5	286.0	374.0
ddPCR MSI Assay 1, 2 or 3	1.1	19.8	38.5	57.2	74.8
UDG*	0.22	4.0	7.7	11.4	15.0
Sample	6.6				
Total	22	277.2	539.0	800.8	1047.2

*UDG is needed for FFPE DNA samples only.

Table 7. Reaction Mix for cfDNA.

	Samples and Controls (reactions + overage) Volume (µl)				
Plates		**0.5**	**1**	**1.5**	**2**
Control Reactions		**4**	**4**	**8**	**8**
Sample Reactions		**12**	**28**	**40**	**56**
Total Reactions	**1**	**16**	**32**	**48**	**64**
Component	**1x**	**(16 + 2)x**	**(32 + 3)x**	**(48 + 4)x**	**(64 + 4)x**
Nuclease-free water	8.8	158.4	308.0	457.6	598.4
ddPCR Multiplex Supermix	5.5	99.0	192.5	286.0	374.0
ddPCR MSI Assay 1, 2 or 3	1.1	19.8	38.5	57.2	74.8
Sample	6.6				
Total	22	277.2	539.0	800.8	1047.2

14. Cover the plate with a foil heat seal ensuring that the red line is up and visible.
15. Heat seal the plate using the PX1 PCR Plate Sealer set to 180°C and 5 sec.
16. Vortex each corner of the sealed plate for 5 sec to ensure the samples are well mixed.
17. Centrifuge the 96-well plate for 1 min at 1,150 rcf.
18. Perform droplet generation using either the QX200 Droplet Generator or the Automated Droplet Generator (AutoDG).
19. If using the QX200 Droplet Generator, refer to the QX200 Droplet Generator Instruction Manual (10031907) for operation details.
20. If using the AutoDG, refer to the Automated Droplet Generator Instruction Manual (10043138) for operation details.
21. After all the samples and controls have gone through droplet generation, heat seal the plate using a foil heat seal as described in steps 14 and 15.

Thermal Cycling

1. Transfer the plate to a thermal cycler and begin PCR amplification within 30 minutes of sealing the plate, using the thermal cycling conditions in Table 8.

   Note: It is important to use a thermal cycler with an adjustable ramp rate. The ramp rate should be set to 2°C/sec.

Table 8. Thermal cycling conditions.

Temperature, °C	Time	Cycles	Ramp Rate, °C/s
95	10 min	1	2
94	30 sec	40	
55	1 min		
98	10 min	1	
4	Hold	1	

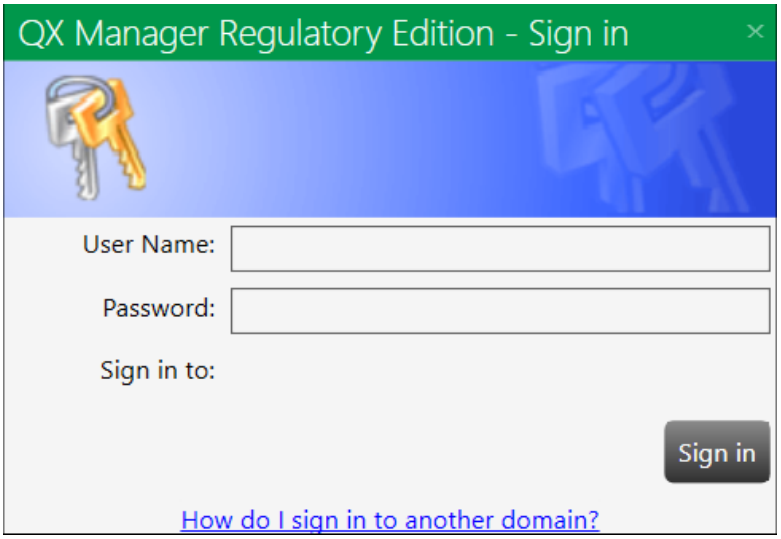
2. Upon completion of the thermal cycling run, let the plate hold at 4ºC for 30 min or until the lid temperature cools to 37ºC.

   Note: The plate may be stored at 2ºC to 8ºC overnight before analysis on the QX200 Droplet Reader.

Data Acquisition

1. Launch QX Manager Software Premium Edition. A sign-in window will pop up (see Figure 3). Enter either local or network login credentials.

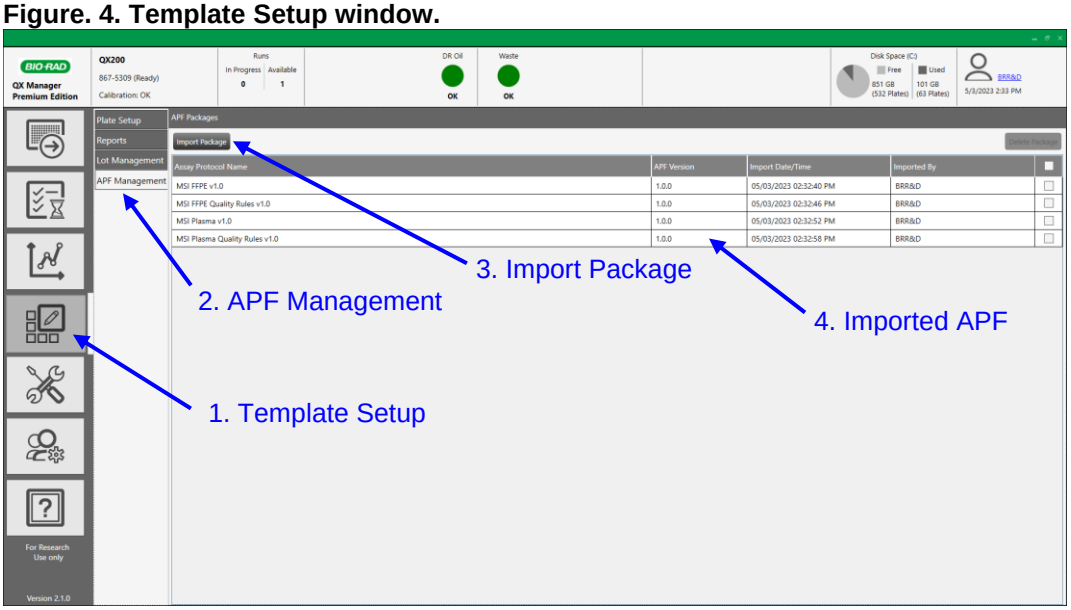
   Figure. 3. Sign-in window.



2. Import the MSI Assay Protocol Files (APFs) into QX Manager Software Premium Edition, if not done previously. APFs can be imported by selecting **Template Setup** > **APF Management** > **Import Package** (see Figure 4). A File Explorer window will appear. Select the desired APF and then click **Open**. Afterward the selected APF will appear in the list of APFs. There are four options of APFs (see Table 4 for more details):

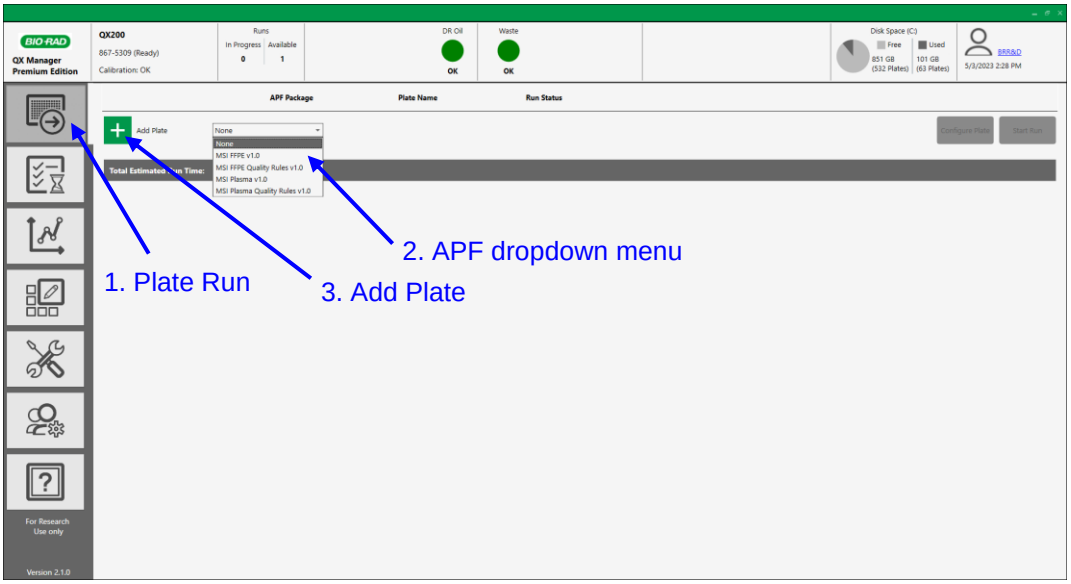
- MSI10_FFPE_QR.apfpack
- MSI10_FFPE.apfpack
- MSI10_Plasma_QR.apfpack
- MSI10_Plasma.apfpack

Figure. 4. Template Setup window.



3. In the left-hand navigation bar select the **Plate Run** tab (see Figure 5).

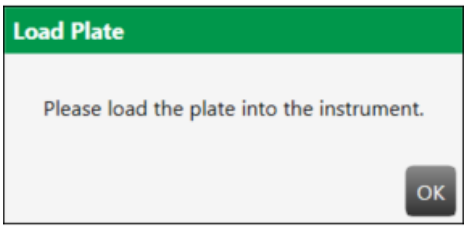
Figure. 5. Plate run tab.



4. In the **APF** field, select the APF file from the dropdown menu corresponding to the sample type to be run and the analysis desired, either MSI FFPE v1.0, MSI FFPE Quality Rules v1.0, MSI Plasma v1.0, or MSI Plasma Quality Rules v1.0 (see Figure 5).

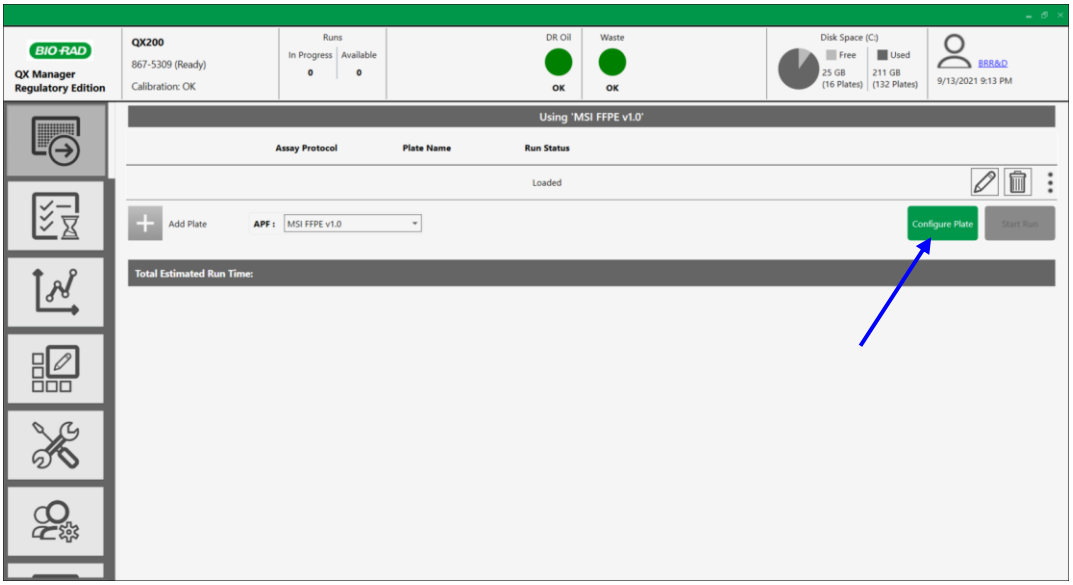
5. Then select the **Add Plate** button (see Figure 5). A pop-up window will then appear instructing to load the plate into the instrument (see Figure 6). Click **OK**. Then insert the 96-well plate into the QX200 Droplet Reader.

Figure. 6. Load Plate window.



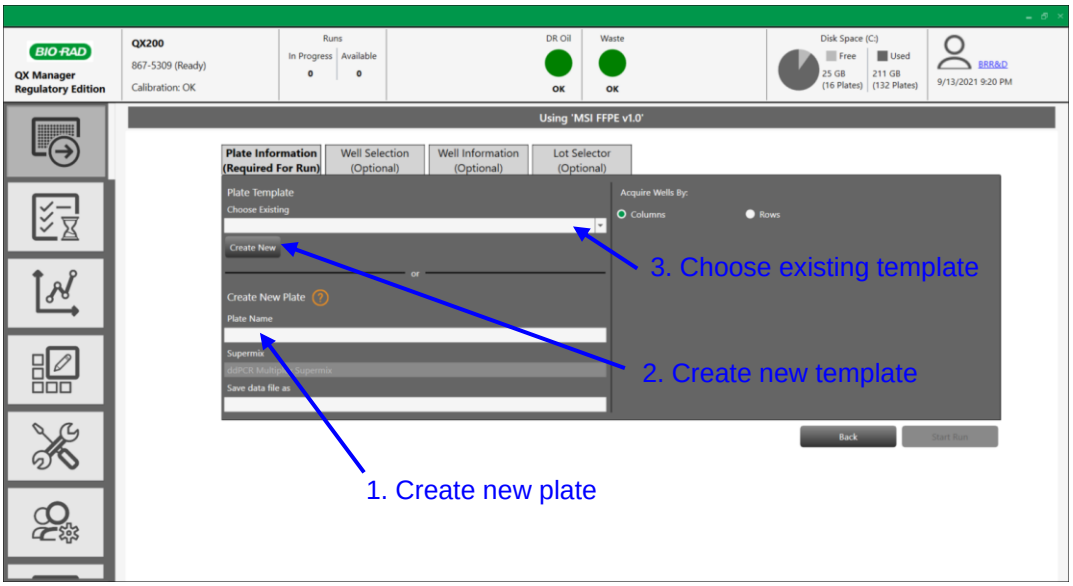
6. Add plate definitions by selecting the **Configure Plate** button (see Figure 7).

Figure. 7. Configure Plate button.



7. Apply plate definitions by creating a new plate, creating a new template, or choosing an existing template (see Figure 8).

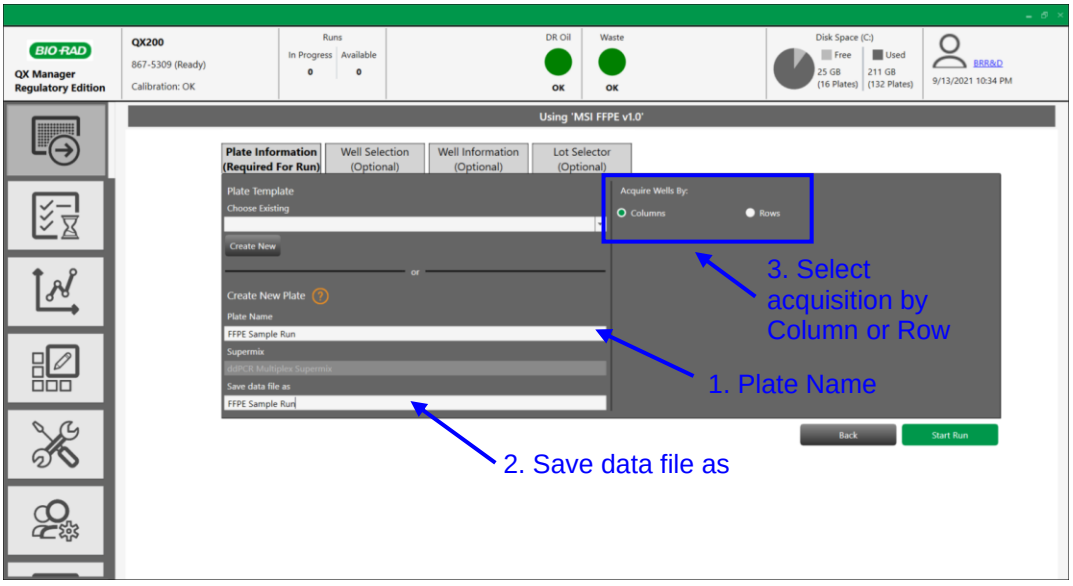
Figure. 8. Plate Definitions window.



Create New Plate

8. If creating a new plate, begin by entering a file name in the **Plate Name** and **Save data file as** fields (see Figure 9).

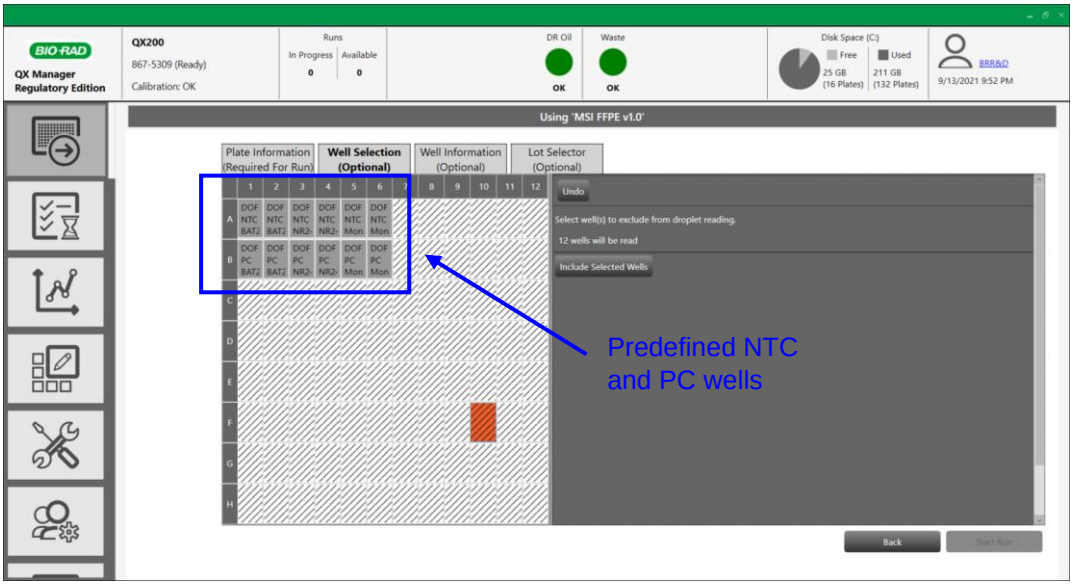
Figure. 9. New Plate Name setup.



9. Choose whether to acquire wells by **Column** or **Row** by selecting the appropriate radio button (see Figure 9).

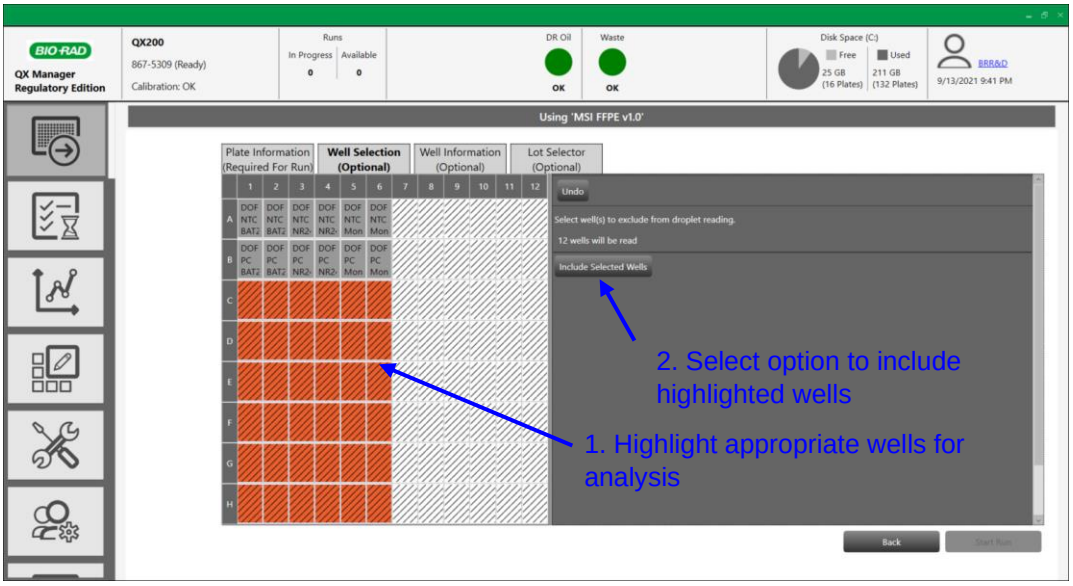
10. Begin defining the plate by choosing the wells to be analyzed in the **Well Selection** tab. Well definitions for all NTC and PC wells (A1 to A6 and B1 to B6, respectively) are predefined in all four MSI APFs (see Figure 10).

Figure. 10. Predefined NTC and PC wells.



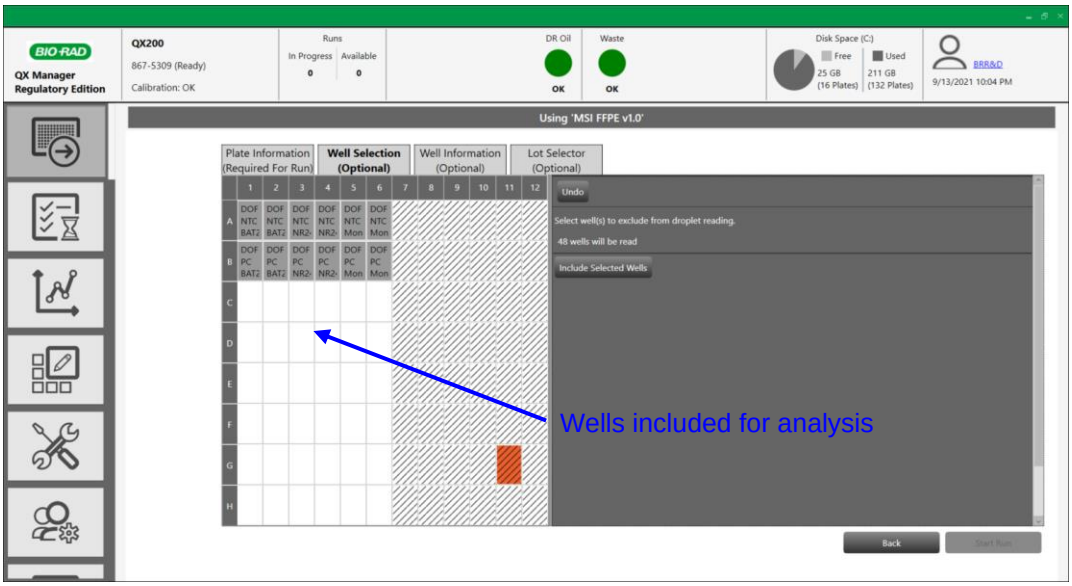
11. By default, all sample wells are excluded from analysis (marked by gray diagonal lines). To include wells, highlight all wells containing samples and then select option to **Include Selected Wells** (see Figure 11).

Figure. 11. Including wells for analysis.



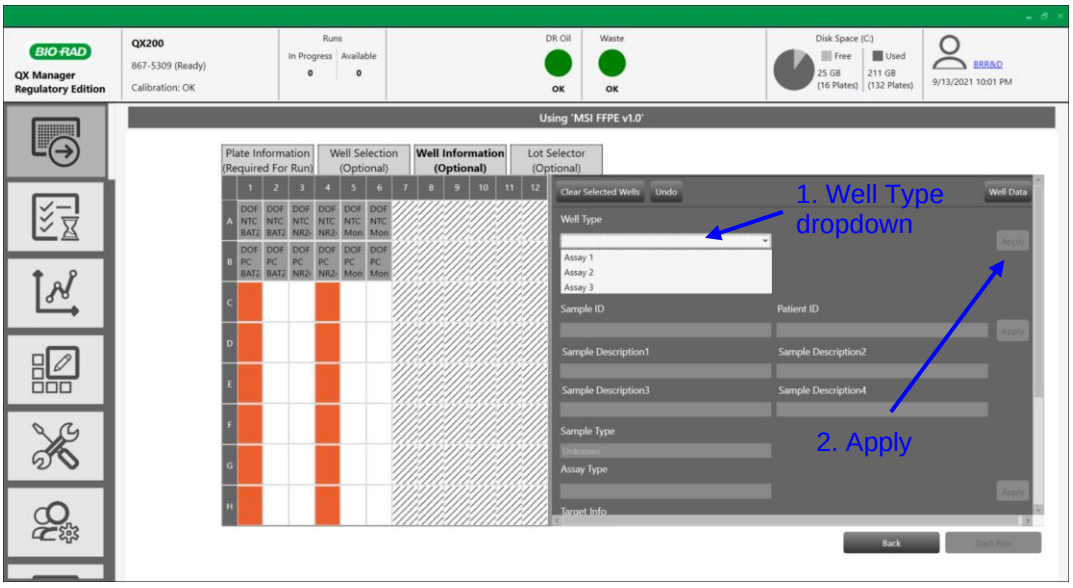
12. Disappearance of the gray diagonal lines indicate the appropriate wells are included in the analysis (see Figure 12).

Figure. 12. Included wells for analysis.



13. Next, go to the **Well Information** tab. Highlight all the wells containing Assay 1, and under the Well Type dropdown select “Assay 1”, and click **Apply** (see Figure 13). Applying Well Type will populate the appropriate **Experiment Type**, **Assay Type,** and **Target Info**.

Figure. 13. Applying Well Type.

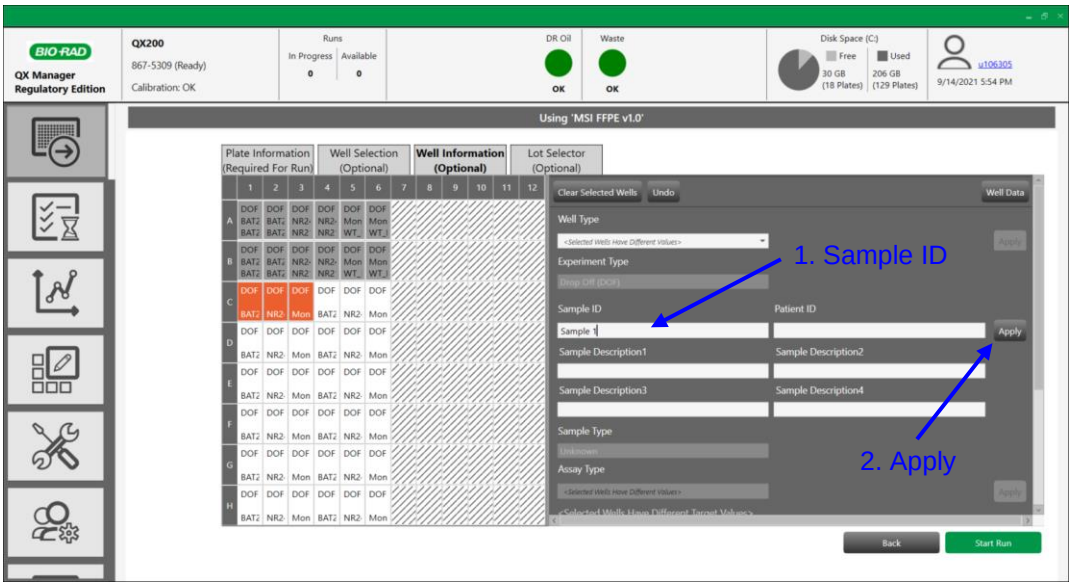


14. Repeat step 13 for wells containing **Assay 2** and **Assay 3**, and apply the appropriate Well Type.

15. Highlight all wells with the same sample, enter the name in the Sample ID field, and then click **Apply** (see Figure 14). Highlight all the NTC wells, enter “NTC” for the Sample ID, and then click **Apply**. Highlight all the PC wells, enter “PC” for the Sample ID, and then click **Apply**.

Note: For correct analysis, the Sample ID for a sample must be identical for all three assays.

Figure. 14. Applying Sample IDs.

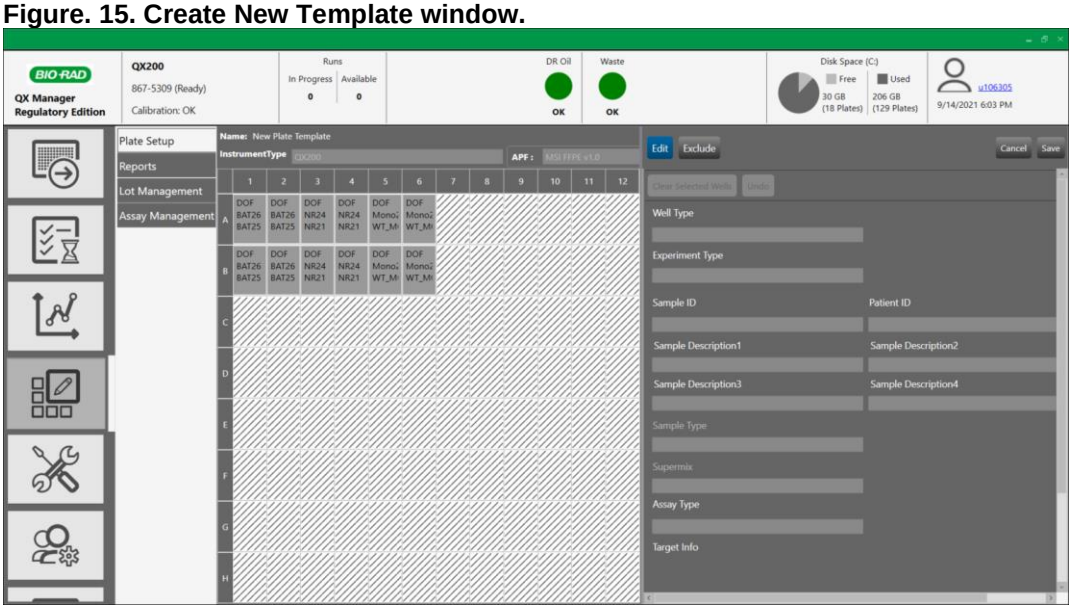


16. Click the **Start Run** button to initiate the run. A sign-in window will pop up (see Figure 3), enter either local or network login credentials and click **Sign in**.
17. Once the instrument initiates the run, a flashing green light indicates the run is in progress (fourth light under the droplet sign on QX200 Droplet Reader).

Create New Template

18. To create a new template for a run, select the **Create New** button (see Figure 8). A plate template will appear (see Figure 15).

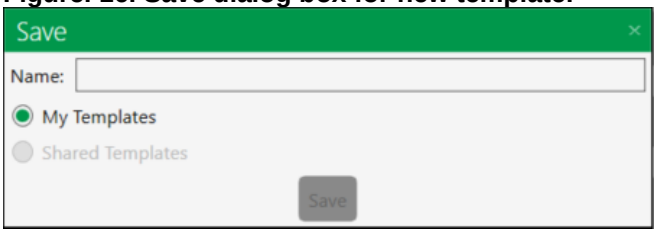
Figure. 15. Create New Template window.



19. If not done already, in the APF field, select the appropriate APF file from the dropdown menu corresponding to the sample type to be run and the desired analysis features (see Table 4 for more details). Then define the plate similarly as outlined for creating a new plate in steps 10 to 15.

20. Click **Save**. A Save dialog box will pop up (see Figure 16). Enter a name for the new template and then click **Save**. Choose whether to acquire wells by **Column** or **Row** by selecting the appropriate radio button (see Figure 9). Initiate the run by clicking **Start Run**.

Figure. 16. Save dialog box for new template.



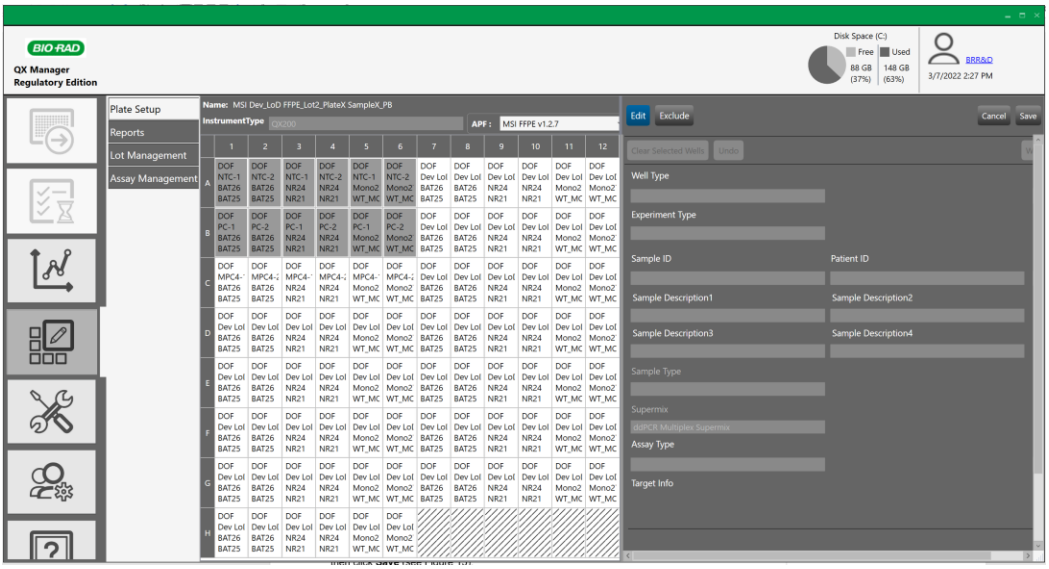
Choose Existing Template

21. Select an existing template for a run by selecting **Choose Existing Template** (see Figure 8).

22. A prefilled template will appear (see Figure 17 for an example). If needed, adjust well definitions, and then enter Sample IDs as described in step 15.

    Note: For correct analysis the Sample ID for a sample must be identical for all three assays.

Figure. 17. Choosing an existing template example.



23. Choose whether to acquire wells by **Column** or **Row** by selecting the appropriate radio button (see Figure 9). Initiate the run by clicking **Start Run**.

Section 6

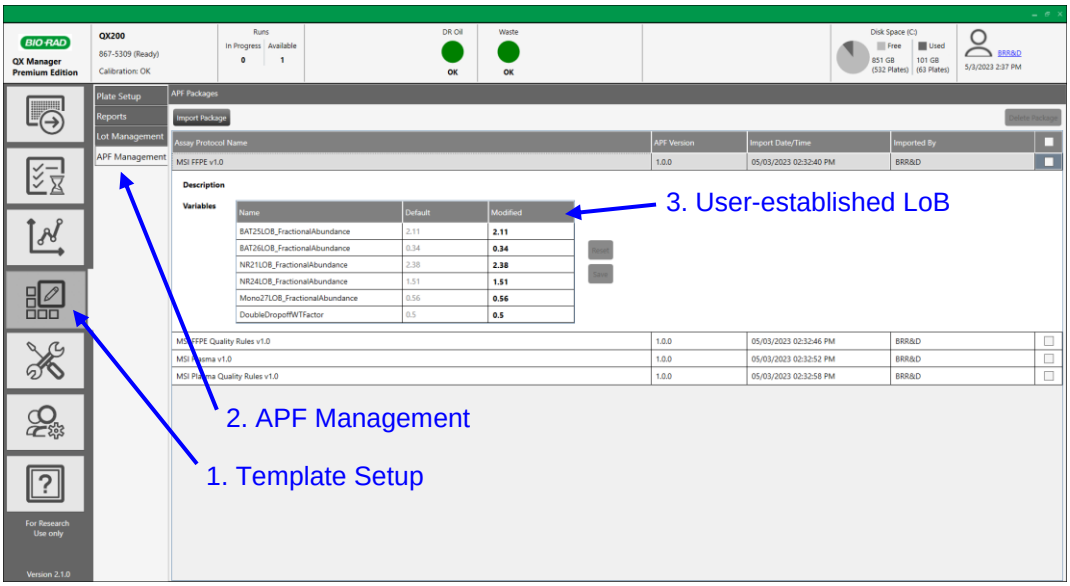
Data Analysis and Result Interpretation

After acquisition is complete, QX Manager Software Premium Edition automatically analyzes the run by setting the thresholds for all the controls and samples on the plate. Droplets are thresholded by an auto-thresholding algorithm that works in two steps: a pre-trained neural network generates initial droplet labels, then a droplet reclassification step occurs using the on-plate PC wells. The neural network was developed using a dataset of contrived and patient FF, FFPE and cfDNA samples (unpublished data). If needed, thresholds may be adjusted manually after the analysis is complete. **It is recommended for users to confirm the auto-threshold is adequate for their patient population.**

Note: The data analysis can take between 5 - 10 minutes. Do not turn off the machine or close the QX Manager Software Premium Edition window during the analysis period.

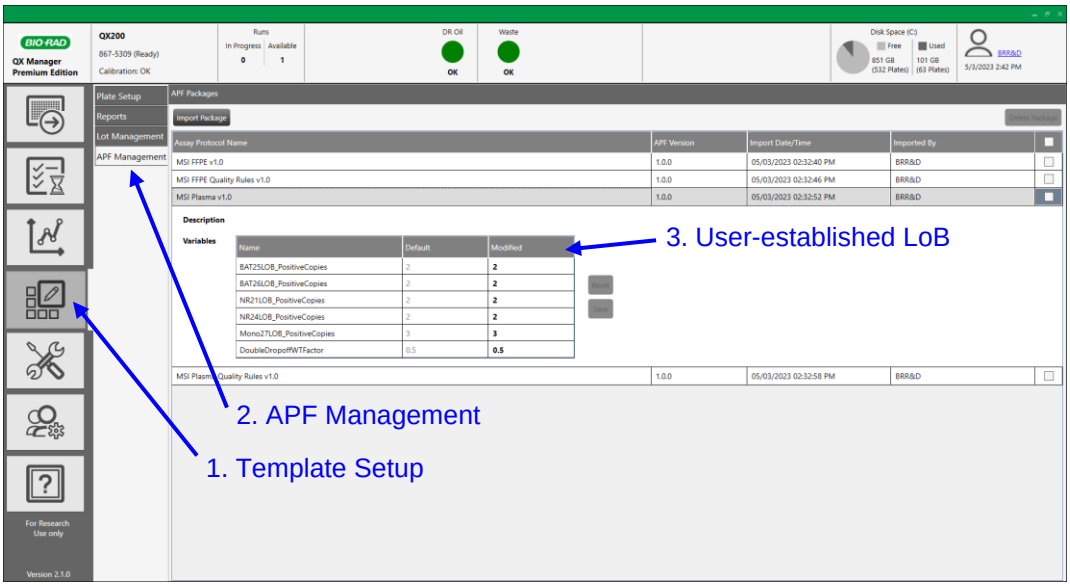
For FFPE samples, a marker is positive if the fractional abundance is greater than the limit of blank (LoB). The MSI FFPE APF includes a LoB based on an analytical LoB established by Bio-Rad (see Section 7 Appendix A for more details) and testing of clinical patient samples (data not shown). The LoB in the MSI10_FFPE_QR.apfpack and MSI10_FFPE.apfpack files are identical. The LoB values are 2.11%, 0.34%, 2.38%, 1.51%, and 0.56% fractional abundance for BAT25, BAT26, NR21, NR24 and Mono27, respectively (see Figure 18). The user can overwrite these LoB values by going to **Template Setup** > **APF Management** > selecting the appropriate APF > entering new LoB values in the Modified column (Figure 18). Values in the Modified column will overwrite values in the Default column. **It is recommended for users to determine the LoB for their patient population.**

Figure. 18. APF Management window for FFPE APF.



For Plasma samples, a marker is called positive if the observed mutant copies are greater than the LoB. The MSI Plasma APF includes a LoB based on an analytical LoB established by Bio-Rad (see Section 7 Appendix A for more details) and testing of clinical patient samples (data not shown). The LoB in the MSI10_Plasma_QR.apfpack and MSI10_Plasma.apfpack files are identical. The LoB values are 2, 2, 2, 2, and 3 copies for BAT25, BAT26, NR21, NR24 and Mono27, respectively (see Figure 19). The user can overwrite the LoB values by going to **Template Setup** > **APF Management** > selecting the appropriate APF > entering new LoB values in the Modified column. Values in the Modified column will overwrite values in the Default column (Figure 19). **It is recommended for users to determine the LoB for their patient population.**

Figure. 19. APF Management window for plasma APF



For each sample, all MSI APFs report the following results in the APF Results window (see Figures 20 and 21):

- Observed mutant copies per marker
- Mutant concentration (cp/µl) per marker
- Wildtype concentration per assay
- Mutant Fractional Abundance (%) per marker
- Marker Result (Negative, Positive, or Not Calculated)
- Marker Positivity Rate (% of positive markers of 5 total)

Mutant fractional abundance is calculated according to Equation 1. If no sample is detected, “Undefined” will be reported for fractional abundance. More details about the results reported by the APFs is described in Section 7 Appendix B.

Equation 1. Mutant Fractional Abundance

$$Mutant\ fractional\ abundance = \frac{Concentration\ of\ mutant}{Concentration\ of\ mutant + concentration\ of\ wildtype\ *}$$

** For Assay 1, the wildtype concentration of BAT25 and BAT26 are assumed to be equivalent. For Assay 2, the wildtype concentration of NR21 and NR24 are assumed to be equivalent.*

Figure. 20. APF Results window.

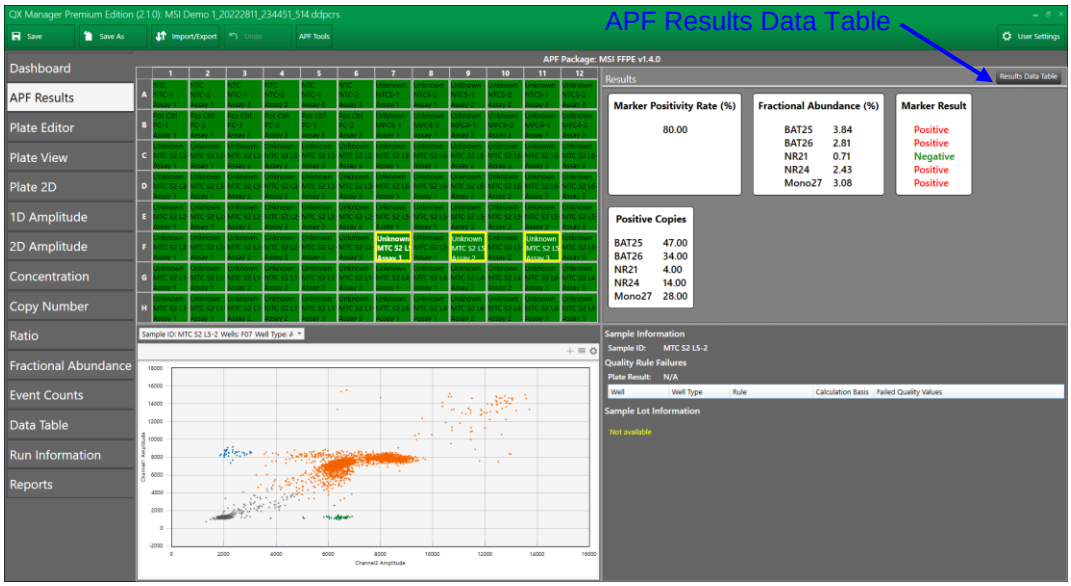
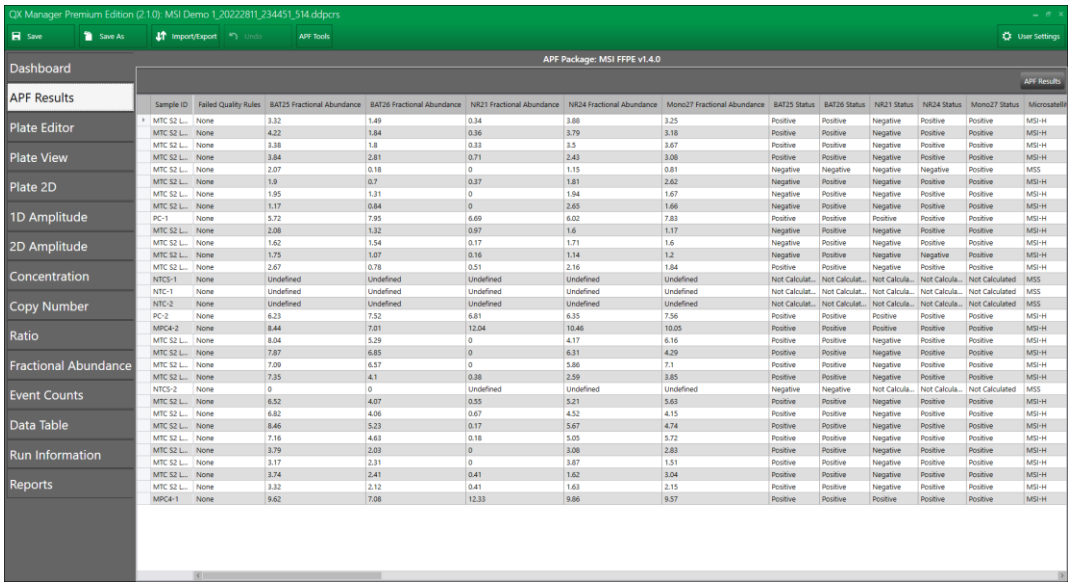


Figure. 21. APF Results Data Table.



The APFs with Quality Rules (MSI10_FFPE_QR.apfpack and MSI10_Plasma_QR.apfpack) contain the four rules listed in Table 9. These rules were determined by Bio-Rad using contrived and clinical samples (unpublished data). **It is recommended for users to verify these Quality Rules for their patient population.**

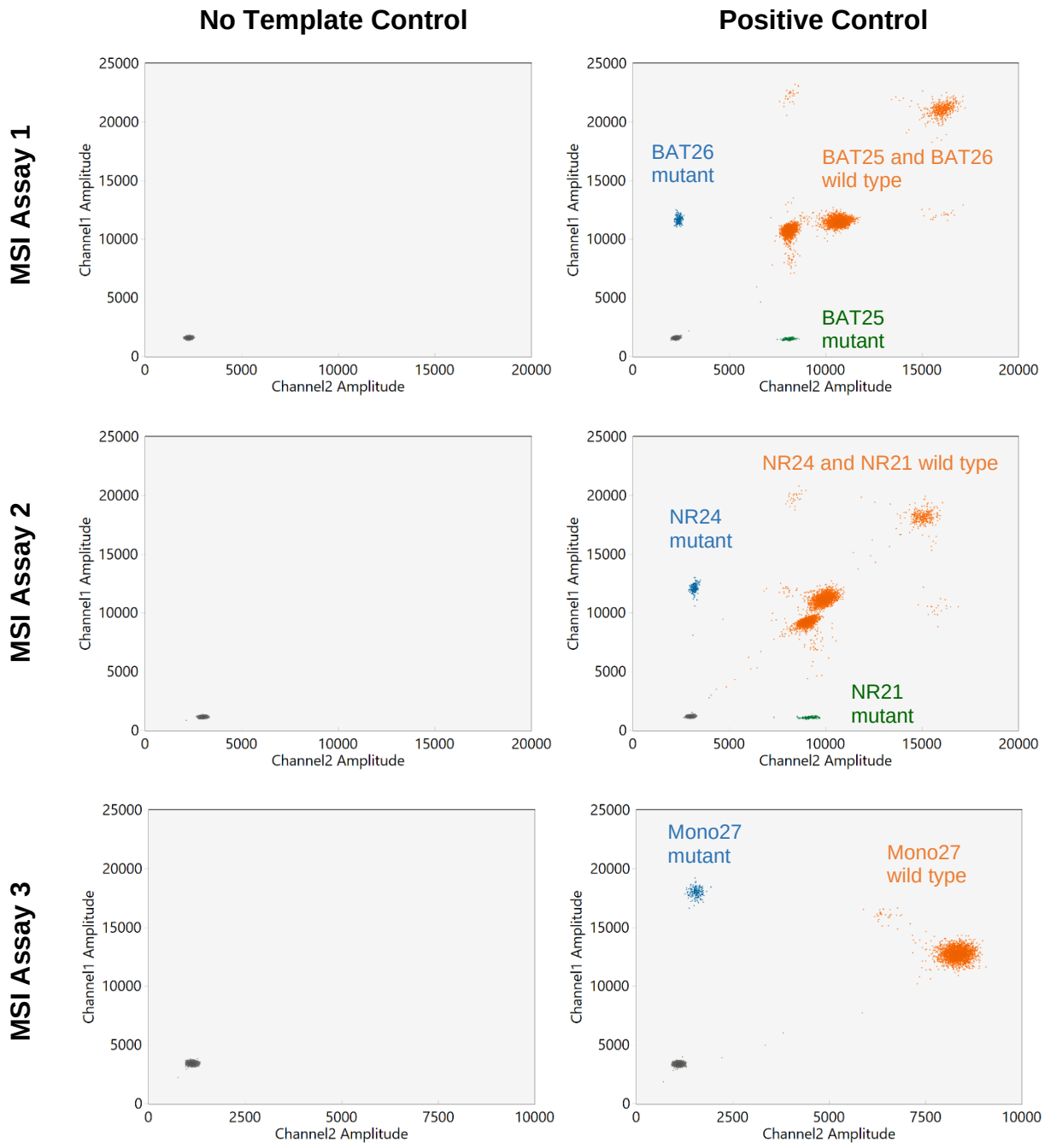
Table 9. Overview of Quality Rules.

Metric	Level*	Assay 1**	Assay 2**	Assay 3
Accepted Droplets	Well	10000 ≤ Droplets < 25000		
Dynamic Range	Well	Upper Limit Negative Droplets ≥ 25 and Wildtype Concentration ≤ 7500 cp/uL Lower Limit Wildtype ≥ 50 total observed copies		Upper Limit Negative Droplets ≥ 25 and Wildtype Concentration ≤ 3750 cp/uL Lower Limit Wildtype ≥ 50 total observed copies
NTC Quality	Plate	BAT25, BAT26, NR21, NR24, Mono27 observed copies ≤ 2.01 per reaction Wildtype observed copies ≤ 10.01 per reaction		
Positive Control Quality	Plate	Wildtype Concentration 100 - 800 cp/uL Fractional abundance 1.05 - 7.1% per marker***		Wildtype Concentration 50 - 400 cp/uL Fractional abundance 2.1 - 14.2%

*If a well-level rule fails then the well where the failure occurred is not analyzed. If a plate-level rule fails, including associated well-level rules, then all the wells of the same assay (e.g., Assay 1, 2 or 3) are not analyzed.
**Wildtype of both markers in Assay 1 and 2 are detected. Thus, the wildtype concentration ranges for Assay 1 and 2 are double compared to Assay 3.
***Wildtype concentration is not divided by 2 when calculating Fractional Abundance in the Quality Rules module, which results in a Fractional Abundance that will be half the calculated value in the APF Results summary reported in QX Manager Software Premium Edition.

Typical 2-dimensional plots for the NTC and PC for the three ddPCR MSI assays are shown in Figure 22. For the PC, all markers should have a fractional abundance of 2.1% to 14.2%, the wildtype concentration for MSI Assays 1 and 2 should be 100 to 800 cp/µL, and the wildtype concentration for MSI Assay 3 should be 50 to 400 cp/µL. For MSI determination, a tumor is typically classified as microsatellite instability-high (MSI-H) if two or more markers are positive (≥ 40% positivity). If one or none of the markers are positive (or ≤ 20% positivity), the tumor is typically classified as microsatellite stable (MSS) (Vilar and Gruber 2010).

Figure. 22. 2-Dimensional plots of ddPCR MSI Controls



Section 7

Appendices

Appendix A: Performance Specifications

Limit of Blank

The LoB for FFPE samples was established by testing in quadruplicate 30 colorectal cancer FFPE samples that showed a deficient mismatch repair status by immunohistochemistry using one lot of the ddPCR MSI Kit. All FFPE samples were extracted using the Promega ReliaPrep FFPE DNA Kit and then 2 ng per reaction was tested. For each of the 5 markers (BAT25, BAT26, NR21, NR24, and Mono27) the mutant fractional abundance was rank ordered and the 95th percentile was determined to be the LoB (see Table 10).

Table 10. LoB for FFPE samples.

n	95th Rank	LoB (mutant fractional abundance, %)				
		BAT25	BAT26	NR21	NR24	Mono27
120	114	0.88	0.25	0.97	2.02	0.49

The LoB for plasma samples was established by testing in quadruplicate 30 normal whole blood and 6 normal plasma samples at an input of 2 ng per reaction using two ddPCR MSI Kit lots. The normal whole blood samples were extracted using the QIAGEN QIAamp DNA Mini Kit and the normal plasma samples were extracted using the QIAamp Circulating Nucleic Acid Kit. For each of the five markers (BAT25, BAT26, NR21, NR24, and Mono27) the observed mutant copies were rank ordered per lot and with combined lots, and the 95th percentile was determined to be the LoB (see Table 11).

Table 11. LoB for Plasma samples.

Lot	n	95th Rank	LoB (mutant copies)				
			BAT25	BAT26	NR21	NR24	Mono27
1	144	137	1	0	1	2	2
2	144	137	1	0	1	1	3
Combined	288	274	1	0	1	2	3

Limit of Detection

To establish the limit of detection (LoD) for FFPE samples, one lot of the ddPCR MSI Kit was tested. Since the LoD is dependent on both the total input and the percentage tumor content of the sample, two input levels were tested with a dilution range of mutant fractional abundance for all five markers. One set of samples was tested at 2 ng per reaction with a mutant fraction abundance of 1%, 2%, 4%, 6%, 8%, and 10%. A second set of samples was tested at 5 ng per reaction with a mutant fraction abundance of 0.5%, 1%, 2%, 6% and 8%. Each input and mutant fractional abundance level was tested in 40 replicates. All samples were contrived by spiking mutant plasmid DNA into a pool of MSS gDNA. Markers were called positive if the fractional abundance was greater than the LoB (see Tables 12 and 13). The lowest mutant fraction abundance level with a positivity rate of ≥95% was determined to be the LoD. The LoD for both input levels tested are summarized in Table 14.

Table 12. Positivity rate for FFPE samples tested at 2 ng per reaction.

Target Fractional Abundance	BAT25 Fractional Abundance			BAT26 Fractional Abundance		
	Mean	# Positives	Positivity, %	Mean	# Positives	Positivity, %
10	11.90	40/40	100	12.61	40/40	100
8	9.96	40/40	100	10.22	40/40	100
6	7.55	40/40	100	7.87	40/40	100
4	4.87	40/40	100	5.17	40/40	100
2	3.12	40/40	100	2.61	40/40	100
1	1.84*	38/40	95	1.51*	40/40	100
	NR21 Fractional Abundance			NR24 Fractional Abundance		
	Mean	# Positives	Positivity, %	Mean	# Positives	Positivity, %
10	11.21	40/40	100	10.88	40/40	100
8	9.11	40/40	100	8.93	40/40	100
6	7.09	40/40	100	6.40	40/40	100
4	4.48	40/40	100	4.69*	40/40	100
2	2.48*	40/40	100	2.47	29/40	72.5
1	1.32	29/40	72.5	1.19	2/40	5
	Mono27 Fractional Abundance					
	Mean	# Positives	Positivity, %			
10	10.64	40/40	100			
8	8.65	40/40	100			
6	6.45	40/40	100			
4	4.08	40/40	100			
2	2.34*	40/40	100			
1	1.04	36/40	90			

*LoD – lowest dilution level with at least 95% positivity.

Table 13. Positivity rate for FFPE samples tested at 5 ng per reaction.

Target Fractional Abundance	BAT25 Fractional Abundance			BAT26 Fractional Abundance		
	Average	# Positives	Positivity, %	Average	# Positives	Positivity, %
8	9.12	40/40	100	9.32	40/40	100
6	6.90	40/40	100	7.04	40/40	100
4	4.76	40/40	100	4.89	40/40	100
2	2.47*	40/40	100	2.63	40/40	100
1	1.45	37/40	92.5	1.41*	40/40	100
0.5	0.83	17/40	42.5	0.68	37/40	92.5
	NR21 Fractional Abundance			NR24 Fractional Abundance		
	Average	# Positives	Positivity, %	Average	# Positives	Positivity, %
8	7.76	40/40	100	8.59	40/40	100
6	5.71	40/40	100	6.36	40/40	100
4	4.00	40/40	100	4.55*	40/40	100
2	2.12*	40/40	100	2.44	35/40	87.5
1	1.23	28/40	70	1.27	1/40	2.5
0.5	0.71	5/40	12.5	0.67	0/40	0
	Mono27 Fractional Abundance					
	Average	# Positives	Positivity, %			
8	8.55	40/40	100			
6	6.20	40/40	100			
4	4.25	40/40	100			
2	2.21	40/40	100			
1	1.15*	40/40	100			
0.5	0.63	28/40	70			

*LoD – lowest dilution level with at least 95% positivity.

Table 14. LoD for FFPE samples.

Input per Reaction	n	% Fractional Abundance				
		BAT25	BAT26	NR21	NR24	Mono27
2 ng	40	1.84	1.51	2.48	4.69	2.34
5 ng	40	2.47	1.41	2.11	4.55	1.15

The LoD for plasma samples was established by testing six contrived samples using one lot of the ddPCR MSI kit. Five samples were tested at an input of 2 ng per reaction and a range of approximately 2, 5, 10, 20 and 50 mutant copies. A sixth sample was tested at an input of 5 ng per reaction with five mutant copies. All samples were contrived by spiking mutant plasmid DNA into a background of normal gDNA extracted from whole blood. All six samples were tested in 40 replicates. Markers were called positive if the copies detected were greater than the LoB of the combined lot (see Table 15). The lowest copy level with a positivity rate of ≥95% was determined to be the LoD. The LoD for BAT25, BAT26, NR21, NR24, and Mono27 was 4.7, 2.4, 5.7, 5.9, and 12.4 copies, respectively.

Table 15. Positivity rate for plasma samples tested.

Input Target, Mutant Copies	BAT25 Copies			BAT26 Copies		
	Mean	# Positives	Positivity, %	Mean	# Positives	Positivity, %
2 ng, 50 copies	51.8	40/40	100	55.7	40/40	100
2 ng, 20 copies	19.2	40/40	100	22.9	40/40	100
2 ng, 10 copies	10.5	40/40	100	11.9	40/40	100
2 ng, 5 copies	4.7*	39/40	97.5	5.4	40/40	100
2 ng, 2 copies	2.3	24/40	60	2.4*	40/40	95
5 ng, 5 copies	5.5	40/40	100	4.1	40/40	100
	NR21 Copies			NR24 Copies		
	Mean	# Positives	Positivity, %	Mean	# Positives	Positivity, %
2 ng, 50 copies	52.3	40/40	100	59.1	40/40	100
2 ng, 20 copies	21.6	40/40	100	23.8	40/40	100
2 ng, 10 copies	10.2	40/40	100	11.9	40/40	100
2 ng, 5 copies	5.7*	38/40	95	5.9*	39/40	97.5
2 ng, 2 copies	2.6	29/40	72.5	3.4	25/40	62.5
5 ng, 5 copies	4.5	37/40	92.5	5.8	37/40	92.5
	Mono27 Copies					
	Mean	# Positives	Positivity, %			
2 ng, 50 copies	60.9	40/40	100			
2 ng, 20 copies	24.3	39/39**	100			
2 ng, 10 copies	12.4*	40/40	100			
2 ng, 5 copies	6.9	37/40	92.5			
2 ng, 2 copies	3.9	21/40	52.5			
5 ng, 5 copies	5.6	35/39**	89.7			

*LoD – lowest copies with at least 95% positivity.
**1 replicate was lost due to bad droplet generation.

Reproducibility

The reproducibility of the ddPCR MSI Kit was established by testing six samples using two kit lots, two operators and two instrument systems. Each kit lot was tested over 3 days with each operator performing one run per instrument system per day. The six samples consisted of two input per reaction levels (2 ng and 5 ng) with three mutant fractional abundance levels (5%, 10%, and 20%). Four replicates of each sample were tested per run for a total of 96 replicates. The total CV measured for the wildtype concentration was <10% for all samples tested (Table 16). The total CV measured for the fractional abundance was <17% for all samples tested (Table 17).

Table 16. Coefficient of variation for wildtype concentration for variation sources.

ddPCR MSI Assay	Sample Input per Reaction	Fractional Abundance, %	n	% CV of Wildtype Concentration				
				Lot	Operator	System	Day	Total
1	2 ng	5	96	0.80	1.56	1.37	2.60	8.16
		10	96	1.91	0.51	1.99	1.32	6.51
		20	96	0.81	0.88	2.27	0.82	7.00
	5 ng	5	95*	1.21	1.21	3.93	2.83	6.23
		10	96	0.84	2.07	0.59	0.73	6.01
		20	96	0.53	0.69	0.60	1.06	5.00
2	2 ng	5	96	0.61	1.06	1.19	1.25	5.90
		10	96	0.56	1.03	1.36	0.65	5.15
		20	96	0.70	0.76	1.26	1.15	6.57
	5 ng	5	95*	0.49	1.33	0.96	0.50	4.25
		10	96	0.55	1.42	1.09	0.70	5.41
		20	96	0.50	0.66	0.53	0.82	4.53
3	2 ng	5	96	0.69	0.87	0.57	2.05	6.65
		10	96	0.85	0.66	0.97	0.83	6.13
		20	96	0.86	1.00	1.01	1.13	7.99
	5 ng	5	95*	0.58	0.72	1.39	0.70	5.53
		10	96	0.56	1.91	0.54	0.63	5.30
		20	96	0.67	0.62	0.83	0.74	5.61

*One replicate was lost due to bad droplet generation.

Table 17. Coefficient of variation for mutant fractional abundance for variation sources.

Marker	Sample Input per Reaction	Fractional Abundance, %	n	% CV of Fractional Abundance				
				Lot	Operator	System	Day	Total
BAT25	2 ng	5	96	1.71	2.18	2.41	2.00	15.76
		10	96	1.13	1.16	1.62	1.36	10.17
		20	96	0.80	0.86	1.53	0.87	7.41
	5 ng	5	95*	3.52	1.99	1.76	3.42	16.74
		10	96	1.95	3.81	3.40	1.80	12.12
		20	96	0.81	0.56	0.96	0.94	5.31
BAT26	2 ng	5	96	1.59	1.72	2.14	2.67	14.78
		10	96	1.18	0.97	2.03	1.29	9.19
		20	96	0.95	0.94	2.33	1.00	8.73
	5 ng	5	95*	2.49	2.87	1.63	3.13	15.80
		10	96	1.38	5.05	2.03	3.27	13.72
		20	96	0.59	0.59	0.64	0.72	5.02
NR21	2 ng	5	96	1.93	1.93	1.80	2.59	16.19
		10	96	1.11	1.04	1.39	1.11	9.22
		20	96	0.83	0.93	0.99	0.89	6.95
	5 ng	5	95*	1.06	1.03	1.06	1.75	9.29
		10	96	0.86	0.85	0.85	1.04	7.62
		20	96	0.64	0.67	0.87	0.70	5.65
NR24	2 ng	5	96	3.06	5.88	2.93	2.82	15.31
		10	96	1.13	1.11	1.78	1.28	10.17
		20	96	0.87	0.83	0.95	1.46	7.25
	5 ng	5	95*	1.21	1.14	1.13	1.40	10.17
		10	96	1.06	0.87	0.79	0.93	7.22
		20	96	0.52	0.52	0.71	0.75	4.80
Mono27	2 ng	5	96	1.92	1.63	1.67	1.83	14.71
		10	96	1.00	1.43	1.21	1.34	9.23
		20	96	0.77	0.78	0.75	1.04	6.79
	5 ng	5	95*	1.20	1.15	1.22	1.32	10.31
		10	96	0.80	0.99	0.80	0.96	7.08
		20	96	0.54	0.91	0.67	0.57	4.91

*One replicate was lost due to bad droplet generation.

Appendix B: APF Result Reporting

The result reporting for the following four APFs are summarized in Table 18, 19, 20 and 21, respectively.

- MSI10_FFPE_QR.apfpack
- MSI10_FFPE.apfpack
- MSI10_Plasma_QR.apfpack
- MSI10_Plasma.apfpack

For the APFs with Quality Rules, Table 18 and 20, show the expected results when all the rules pass or when one or more rules fail.

Table 18. APF custom results for MSI10_FFPE_QR.apfpack

Sample Type	Quality Rules	Fractional Abundance (%)*	Positive Copies*	Marker Result**	% Marker Positivity***
No Template Control (NTC)	All pass for one or both replicates	Undefined	0	Valid	0%
	All pass for one or both replicates	0 - 100	0, 1, or 2 copies	Valid	20, 40, 60, 80 or 100
	Both replicates fail for one assay	Undefined	Undefined	Invalid	Indeterminate
Positive Control (PC)	All pass	2.1 - 14.2	copies detected	Valid	20, 40, 60, 80 or 100
	Both replicates fail for one assay	Undefined	Undefined	Invalid	Indeterminate
Samples	All pass	> LoB cutoff	copies detected	Positive	0, 20, 40, 80 or 100 (if all 5 Marker Results are Positive/Negative) or Indeterminate
	All pass	≤ LoB cutoff	copies detected	Negative	
	Both replicates of NTC and/or PC of the same assay fails	Undefined	Undefined	Not Calculated	Indeterminate
	Any well-level rule fails	Undefined	Undefined	Not Calculated	Indeterminate
All	All replicates fail for all assays	Not Calculated	Not Calculated	Not Calculated	Not Calculated

*Undefined is reported if no sample is detected or when a Quality Rule fails. If mutant is detected and no wildtype is detected the fractional abundance will be 100%.

**If 1 or 2 mutant copies are detected and 0 to 10 wildtype copies are detected, the fractional abundance will be > 0% and the Marker Result will be Valid.

***Result is dependent on LoB cutoff.

Table 19. APF custom results for MSI10_FFPE.apfpack

Sample Type	Fractional Abundance (%)*	Positive Copies	Marker Result**	% Marker Positivity***
All	Undefined	0	Not Calculated	Indeterminate
	≤ LoB cutoff	copies detected	Negative	0, 20, 40, 80 or 100 (if all 5 Marker Results are Positive/Negative) or Indeterminate
	> LoB cutoff	copies detected	Positive	

*Undefined will be reported if no sample is detected or if sample is overloaded and there are no negative droplets. If mutant is detect and no wildtype, the fractional abundance will be 100%.

**For NTC, Marker Result is dependent on Positive Copies detected.

***Result is dependent on LoB cutoff.

Table 20. APF custom results for MSI10_Plasma_QR.apfpack

Sample Type	Quality Rules	Fractional Abundance (%)*	Positive Copies*	Marker Result	% Marker Positivity**
No Template Control (NTC)	All pass for one or both replicates	Undefined	0	Valid	0
	All pass for one or both replicates	0 - 100	0, 1 or 2 copies	Valid	0, 20, 40, 60, 80 or 100
	Both replicates fail for one assay	Undefined	Undefined	Invalid	Indeterminate
Positive Control (PC)	All pass for one or both replicates	2.1 - 14.2	copies detected	Valid	20, 40, 60, 80 or 100
	Both replicates fail for one assay	Undefined	Undefined	Invalid	Indeterminate
Samples	All pass	fractional abundance detected	> LoB cutoff	Positive	0, 20, 40, 80 or 100 (if all 5 Marker Results are Positive/Negative) or Indeterminate
	All pass	fractional abundance detected	≤ LoB cutoff	Negative	
	Both replicates of NTC and/or PC of the same assay fails	Undefined	Undefined	Not Calculated	Indeterminate
	Any well-level rule fails	Undefined	Undefined	Not Calculated	Indeterminate
All	All replicates fail for all assays	Not Calculated	Not Calculated	Not Calculated	Not Calculated

*Undefined is reported if no sample is detected or when a Quality Rule fails. If mutant is detected and no wildtype is detected, the fractional abundance will be 100%.

**Result is dependent on LoB cutoff.

Table 21. APF custom results for MSI10_Plasma.apfpack

Sample Type	Fractional Abundance (%)*	Positive Copies	Marker Result	% Marker Positivity
All	Undefined	0	Negative	0, 20, 40, 80 or 100
	0 - 100 or Undefined	≤ LoB cutoff	Negative	
	0 - 100 or Undefined	> LoB cutoff	Positive	

*Undefined will be reported if no sample is detected or if sample is overloaded and there are no negative droplets

Section 8
References

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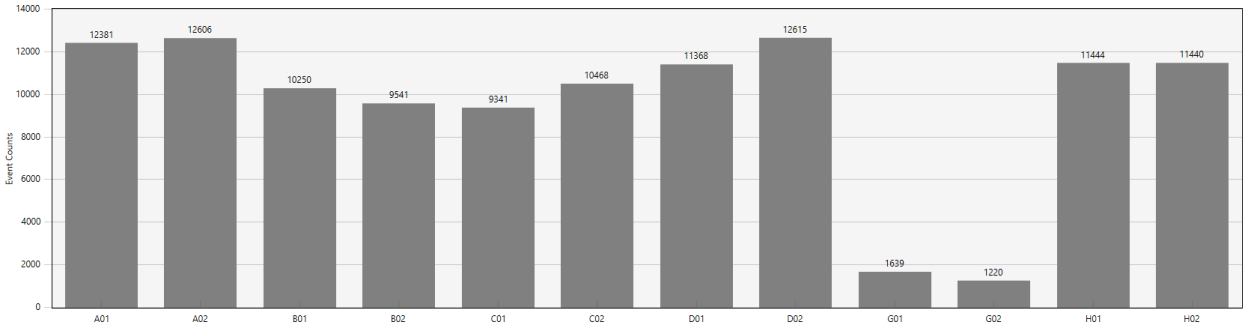
Section 9
Troubleshooting

No or Low Total Droplet Counts in Sample Wells

Problem: Droplet count is <10,000 (Figure 23). If control wells have droplet counts <10,000, the issue is likely caused by inhibitors carried over from the sample extraction.

Resolution: Exclude sample wells with low droplet counts from analysis. If possible, dilute the sample to decrease inhibitor concentration and repeat ddPCR. Alternatively, re-extract the sample to eliminate inhibitors. (Note: Quality Rules in the APF can be set to automatically exclude wells with low droplet counts.)

Figure. 23. Event count graph in which wells have a total droplet count <10,000.

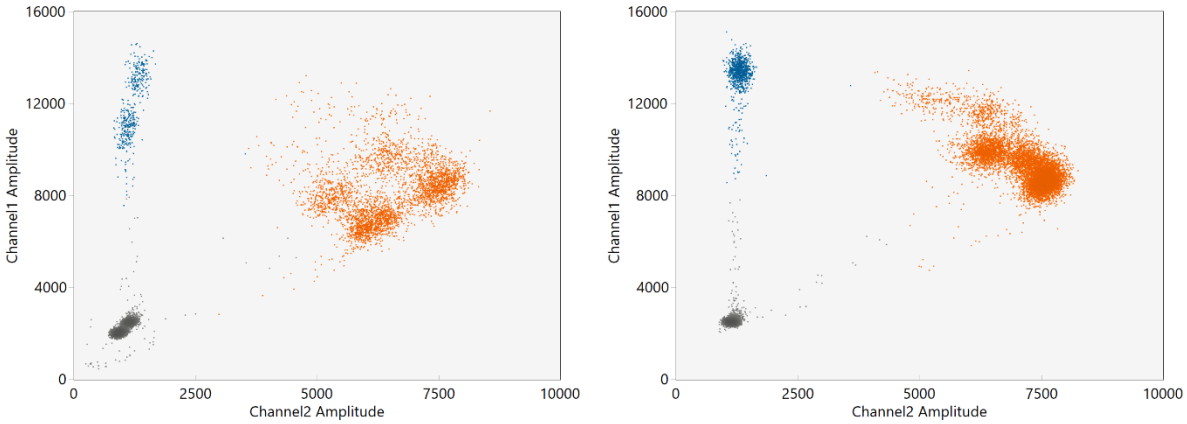


Mirroring

Problem: Droplets exhibit two distinct sizes (Figure 24), which indicates a potential consumable failure or particulates from samples, environment, tips, or reagents.

Resolution: Exclude the well from analysis and repeat ddPCR, preferably with a different lot of droplet generation consumables.

Figure. 24. Mirroring example of MSI Assay 3. Problem well (left) vs. normal well (right).

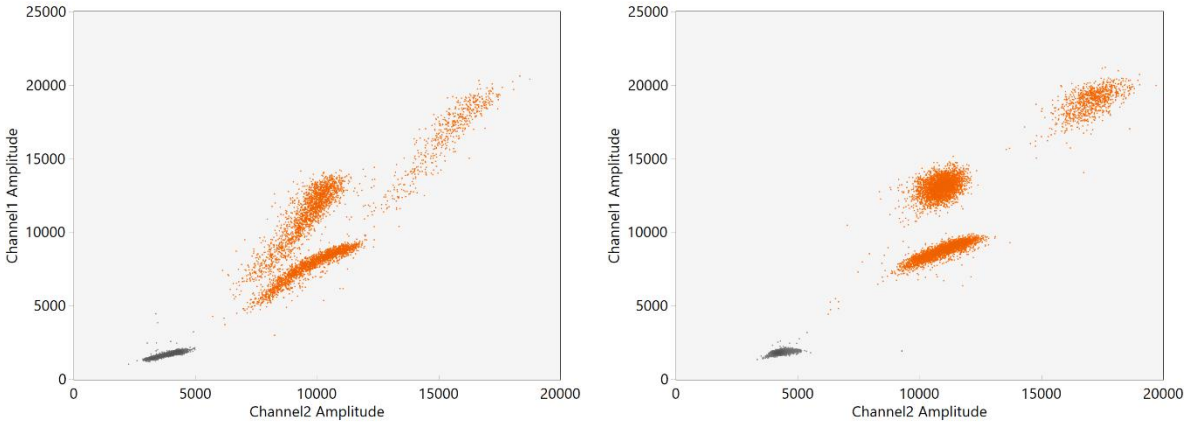


Droplet Shredding

Problem: Shredded droplets appear on the diagonal through the negative droplet cluster (Figure 25).

Resolution: Exclude well from analysis or repeat ddPCR. (Note: Droplet shredding typically results in droplet counts <10,000. Quality Rules can be set to automatically exclude wells with low droplet counts.)

Figure. 25. Shearing example of MSI Assay 2. Problem well (left) vs. normal well (right).



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