

ddPLEX *EGFR/KRAS/BRAF* Mutation Detection Kit

Catalog #	Description
17010491	ddPLEX <i>EGFR/KRAS/BRAF</i> Mutation Detection Kit, includes ddPLEX <i>EGFR/KRAS/BRAF</i> Mutation Detection Assays, ddPLEX <i>EGFR/KRAS/BRAF</i> Positive Control, ddPCR™ Multiplex Supermix, and dithiothreitol (DTT)

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

Product Description

Biomarker testing for actionable oncogenic driver mutations is recommended in national and international guidelines based on the improved outcomes observed with use of targeted therapies in eligible patients with metastatic non-small-cell lung cancer (NSCLC). The ddPLEX *EGFR/KRAS/BRAF* Mutation Detection Kit, used on the Bio-Rad QX600™ Droplet Digital™ PCR (ddPCR) System, is a research use only (RUO) test for detecting and/or discriminating 37 mutations with approved targeted therapies in the *EGFR*, *KRAS*, and *BRAF* genes.

The ddPLEX *EGFR/KRAS/BRAF* Mutation Detection Kit is composed of two assays. The Mutant Assay tests for the presence of variants in the *EGFR*, *KRAS*, and *BRAF* genes and includes an internal control to verify assay performance. The Total Quant Assay measures the total copies in the *EGFR*, *KRAS*, and *BRAF* genes, which allows for calculation of variant

allele frequencies. A complete list of targets is provided in Table 1. The kit can be used to test DNA extracted from plasma and tumor tissues, including formalin-fixed paraffin-embedded (FFPE) tissue samples.

The ddPLEX *EGFR/KRAS/BRAF* Mutation Detection Kit includes sufficient reagents for a total of 120 reactions: 60 reactions of the ddPLEX *EGFR/KRAS/BRAF* Mutant Assay and 60 reactions of the ddPLEX *EGFR/KRAS/BRAF* Total Quant Assay. The kit can accommodate up to 42 samples per run in singlet utilizing a 96-well plate with all the required controls. The kit is designed for use with the Bio-Rad QX600 Droplet Digital PCR System or QX600 AutoDG™ Droplet Digital PCR System. The ddPLEX *EGFR/KRAS/BRAF* Mutation Detection Kit is compatible with QX Manager Software, Standard Edition, v2.1 and later.

Table 1. Targets of the ddPLEX *EGFR/KRAS/BRAF* Mutation Detection Kit.

Gene	Exon	Variants Detected (amino acid change / coding sequence)	Mutant Assay Cluster Assignment
<i>EGFR</i>	18	<i>p.G719A / c.2156 G>C</i>	All 3 variants are detected in 1 distinct cluster with no distinction between them
		<i>p.G719C / c.2155 G>T</i>	
		<i>p.G719S / c.2155 G>A</i>	
<i>EGFR</i>	19	<i>p.E746_A750del / c.2235_2249del</i>	All 19 variants are detected in 1 distinct cluster. No distinction between the 19 deletion types
		<i>p.E746_A750del / c.2236_2250del</i>	
		<i>p.L747_P753delinsS / c.2240_2257del</i>	
		<i>p.L747_T751del / c.2240_2254del</i>	
		<i>p.L747_A750delinsP / c.2239_2248delinsC</i>	
		<i>p.E746_S752delinsV / c.2237_2255delinsT</i>	
		<i>p.E746_T751delinsA / c.2237_2251del</i>	
		<i>p.L747_S752del / c.2239_2256del</i>	
		<i>p.L747_T751delinsP / c.2239_2251delinsC</i>	
		<i>p.L747_E749del / c.2239_2247del</i>	
		<i>p.E746_S752delinsA / c.2237_2254del</i>	
		<i>p.L747_P753delinsQ / c.2239_2258delinsCA</i>	
		<i>p.L747_T751delinsS / c.2240_2251del</i>	
		<i>p.L747_A750delinsP / c.2238_2248delinsGC</i>	
		<i>p.E746_T751del / c.2236_2253del</i>	
<i>p.E746_S752delinsD / c.2238_2255del</i>			
<i>p.E746_T751delinsI / c.2235_2252delinsAAT</i>			
<i>p.L747_T751delinsQ / c.2238_2252delinsGCA</i>			
<i>p.E746_E749del / c.2235_2246del</i>			



Visit [bio-rad.com/DropletDigitalPCRAssays](https://www.bio-rad.com/DropletDigitalPCRAssays) for more information.

Gene	Exon	Variants Detected (amino acid change / coding sequence)	Mutant Assay Cluster Assignment
EGFR	20	<i>p.S768I / c.2303 G>T</i>	1 distinct cluster
		<i>p.V769-D770insASV / c.2303_2311dup</i>	All 7 insertion variants are detected in 1 distinct cluster. No distinction between the 7 insertion types
		<i>p.S768_D770dup (same as 770_771insSVD) / c.2307_2308insGCCAGCGTG</i>	
		<i>p.D770_N771insG / c.2310_2311insGGT</i>	
		<i>p.N771_H773dup / c.2311_2319dup</i>	
		<i>p.P772_H773dup / c.2314_2319dup</i>	
		<i>p.H773-V774insNPH / c.2319-2320insAAACCCAC</i>	
		<i>p.H773dup / c.2317_2319dup</i>	
		<i>p.T790M / c.2369 C>T</i>	1 distinct cluster
		<i>p.C797S / c.2390 G>C and c.2390 T>A</i>	Both variants are detected in 1 distinct cluster. No distinction between them
<i>p.T790M + p.C797S / c.2369 C>T and c.2390 G>C</i>	1 distinct cluster if both targets are in cis configuration and 2 clusters if in trans		
<i>p.T790M + p.C797S / c.2369 C>T and c.2390 T>A</i>	1 distinct cluster if both targets are in cis configuration and 2 clusters if in trans		
EGFR	21	<i>p.L858R / c.2573 T>G</i>	1 distinct cluster
		<i>p.L861Q / c.2582 T>A</i>	
KRAS	2	<i>p.G12C / c.34 G>T</i>	1 distinct cluster
BRAF	15	<i>p.V600E / c.1799 T>A</i>	1 distinct cluster

ddPLEX EGFR/KRAS/BRAF Mutation Detection Kit and Contents

The ddPLEX EGFR/KRAS/BRAF Mutation Detection Kit consists of the ddPLEX EGFR/KRAS/BRAF Mutation Detection Assays, ddPLEX EGFR/KRAS/BRAF Positive Control, ddPCR Multiplex Supermix, and DTT, each of which can be purchased separately (Table 2). All components of the ddPLEX EGFR/KRAS/BRAF Mutation Detection Assays are listed in Table 3. See Table 4 for ddPLEX Positive Control information.

Table 2. ddPLEX EGFR/KRAS/BRAF Mutation Detection Kit (#17010491) contents.

Catalog Number	Description	Quantity	Storage Conditions
12023484	ddPLEX EGFR/KRAS/BRAF Mutation Detection Assays	1	-20°C
12023522	ddPLEX EGFR/KRAS/BRAF Positive Control	1	-20°C*
12005909	ddPCR Multiplex Supermix, 1.2 mL (2 x 0.6 mL)	1	-20°C
12012171	Dithiothreitol (DTT), 2 mL (2 x 1 mL vials)	1	-20°C†

* It is recommended to store the ddPLEX Positive Control in a separate area from the other reaction components.

† After thawing DTT, store at 4°C for up to 2 weeks. Do not thaw and refreeze DTT.

Table 3. ddPLEX EGFR/KRAS/BRAF Mutation Detection Assays (#12023484) contents.

Description	Quantity	Volume, µL	Storage Conditions
ddPLEX EGFR/KRAS/BRAF Mutant Assay	1	170	-20°C, limit light exposure
ddPLEX EGFR/KRAS/BRAF Total Quant Assay	1	85	-20°C, limit light exposure
ddPLEX Enhancer	1	90	-20°C
ddPLEX Internal Control	1	500	-20°C*

*It is recommended to store the ddPLEX Internal Control in a separate area from the other reaction components.

Table 4. ddPLEX EGFR/KRAS/BRAF Positive Control (#12023522) contents.

Description	Quantity	Volume, µL	Storage Conditions
ddPLEX EGFR/KRAS/BRAF Positive Control	1	100	-20°C*

*It is recommended to store the ddPLEX Positive Control in a separate area from the other reaction components.

The ddPLEX EGFR/KRAS/BRAF Positive Control (#12023522) verifies the performance of the reaction and serves as a guide for data thresholding. It produces all intended clusters for both assays as it is a mixture of synthetic DNA consisting of EGFR, KRAS, and BRAF variants and three wild-type targets covered by the ddPLEX EGFR/KRAS/BRAF Mutation Detection Kit (Table 1). The three wild-type targets consist of wild-type EGFR exon 21, KRAS exon 2, and BRAF exon 15. The positive control is designed to resemble a sample with low input amount and low variant allele frequency. It has sufficient volume for 12 reactions of both the ddPLEX EGFR/KRAS/BRAF Mutant Assay and Total Quant Assay using the recommended input amounts.

The ddPLEX Internal Control is included as a component of the ddPLEX EGFR/KRAS/BRAF Mutation Detection Assays (#12023484). It consists of a synthetic, nonhuman DNA sequence in solution. The ddPLEX Internal Control serves as an exogenous control that is introduced into the Mutant Assay reaction mixture for monitoring ddPCR reaction performance.

All reagents should be stored in a constant temperature freezer at -15 to -25°C . All components of the ddPLEX EGFR/KRAS/BRAF Mutation Detection Assays and ddPLEX EGFR/KRAS/BRAF Positive Control can be frozen and thawed up to three times. Repeated freezing and thawing more than three times is not recommended. For DTT, thaw once and store at 4°C for up to 2 weeks. Do not thaw and refreeze DTT. For the ddPCR Multiplex Supermix, follow the storage instructions in the product insert. All reagents can be used until the expiration date indicated on the tube when stored properly.

Required Equipment, Reagents, and Consumables

The QX600 ddPCR System with QX Manager Software, Standard Edition, V2.1 or later, along with either the QX200™ Droplet Generator (manual) or Automated Droplet Generator, is required for use with the ddPLEX EGFR/KRAS/BRAF Mutation Detection Kit. The equipment, reagents, and consumables necessary for the QX600 ddPCR System are listed in Tables 5–7. These materials are not provided with the kit.

Table 5. Droplet generation and reader equipment, reagents, and consumables.

Description	Vendor	Catalog Number
QX600 Droplet Reader	Bio-Rad	12013328
Automated Droplet Generator or QX200 Droplet Generator	Bio-Rad	1864101 or 1864002
ddPCR Droplet Reader Oil	Bio-Rad	1863004
C1000 Touch Thermal Cycler with 96-Deep Well Reaction Module or PTC Tempo Deepwell Thermal Cycler	Bio-Rad	1851197 (discontinued) or 12015392
PCR Plate Heat Seal, foil, pierceable	Bio-Rad	1814040
PX1 PCR Plate Sealer	Bio-Rad	1814000
ddPCR 96-Well Plates, semi-skirted	Bio-Rad	12001925
Disposable powder-free gloves (latex or nitrile)	Any	–
Micropipets (1–10 μL , 2–20 μL , 20–200 μL , and 100–1,000 μL)	Any	–
Barrier pipet tips (sterile, RNase- and DNase-free)	Rainin or Eppendorf	Various
Microcentrifuge tubes, 1.5 mL, low-DNA binding	Any	–
Nuclease-free water	Any	–
HindIII restriction enzyme	Any	–
Uracil DNA glycosylase (UDG)*	Any	–
Vortexer	Any	–
Benchtup microcentrifuge	Any	–
Centrifuge for 96-well plates	Any	–

*Uracil DNA glycosylase (UDG) is required for FFPE samples that have not previously been treated.

Table 6. Reagents and consumables for automated droplet generation.

Description	Vendor	Catalog Number
Automated Droplet Generation Oil for Probes	Bio-Rad	1864110
DG32 Automated Droplet Generator Cartridges	Bio-Rad	1864108 or 1864109
Pipet Tips for the AutoDG System	Bio-Rad	1864120 or 1864121
Pipet Tip Waste Bins for the AutoDG System	Bio-Rad	1864125

Table 7. Reagents and consumables for manual droplet generation.

Description	Vendor	Catalog Number
Droplet Generation Oil for Probes	Bio-Rad	1863005
DG8 Cartridge Holder	Bio-Rad	1863051
DG8 Cartridges for QX200/QX100 Droplet Generator	Bio-Rad	1864008
DG8 Gaskets for QX200/QX100 Droplet Generator	Bio-Rad	1863009

Safety Information

Personal protective equipment including a lab coat, gloves, and safety glasses should be worn when performing any of the following procedures. Refer to the safety data sheets available on the Bio-Rad website for more information.

Precautions and Recommendations

- 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
- The ddPCR Multiplex Supermix is especially viscous. It must be thawed at room temperature for 10 min and vortexed thoroughly for 30 sec before use
- After thawing DTT, store at 4°C for up to 2 weeks. Do not thaw and refreeze DTT
- ddPLEX Positive Control and Internal Control contain DNA templates and should not be used in any DNA template-free areas
- HindIII is recommended for optimal performance with nonfragmented DNA. For fragmented DNA, the assay may work without restriction enzyme. However, digestion is still recommended to increase the precision, reproducibility, and accuracy of the Droplet Digital PCR
- It is recommended to use a positive control, negative control, and no template control (NTC) for PCR runs
- Do not use reagents after their expiration date
- Use the latest version of the QX Manager Software, Standard Edition, for data acquisition and analysis

ddPLEX EGFR/KRAS/BRAF Mutation Detection Kit Workflow

It is recommended to read through the entire protocol prior to setting up the reactions. The reaction setup and data analysis steps used for the ddPLEX EGFR/KRAS/BRAF Mutant and Total Quant Assays vary slightly but have similar workflows. Both assays can be run with the same controls and on the same plate. See Figure 1 for an overview of the workflow.

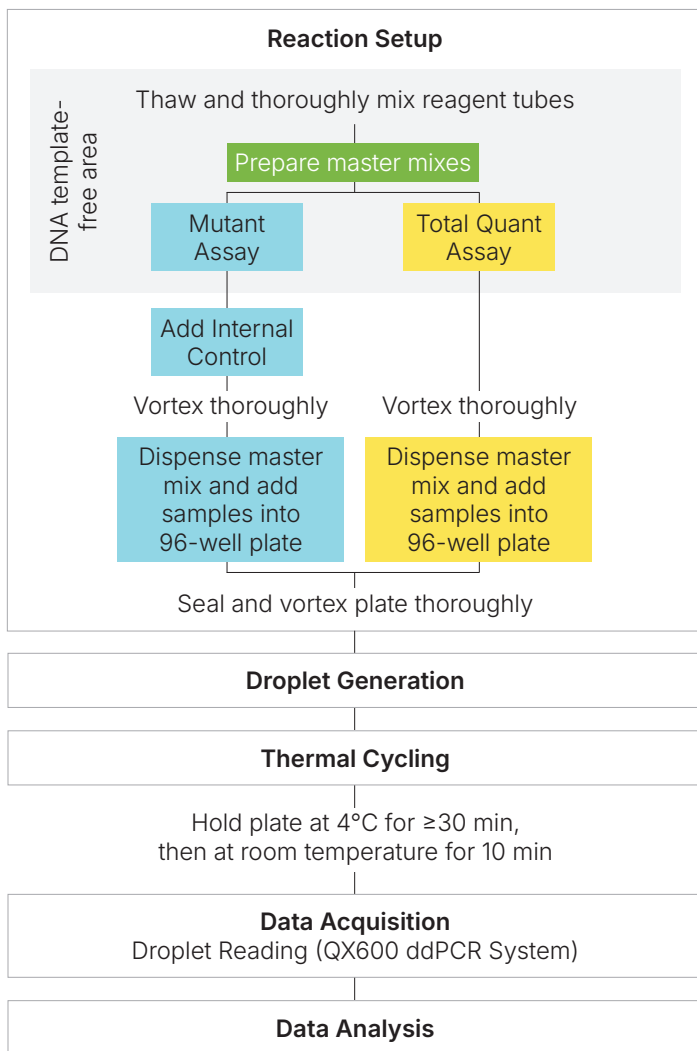


Fig. 1. Overview of ddPLEX EGFR/KRAS/BRAF Mutation Detection Kit workflow.

Protocol

Sample Considerations

The input amount into the Mutant Assay may be 2–10 μL and up to 132 ng of human genomic DNA. Please note that subsampling error may significantly affect the accuracy of the experiment at very low input DNA amounts. Prior quantification of samples by quantitative PCR or Droplet Digital PCR is recommended. Note that higher sensitivity may be obtained with input amounts at the higher end of the range, but greater than 132 ng will increase the risk of false positives. The input volume into the Total Quant Assay is minimized to conserve sample, however at least 2 μL is recommended for pipetting accuracy. DNA (1–53 ng) should be used in the Total Quant Assay. Purified DNA is recommended for optimal assay performance. Refer to Table 8 for a summary of the input amounts for each assay.

Table 8. Input amounts for the ddPLEX EGFR/KRAS/BRAF Mutation Detection Assays.

Assay	Input Volume, μL	Input Amount, ng
Mutant	2–10	Up to 132
Total Quant	2–10	1–53

Reaction Setup

- Determine the number of controls and samples to be tested prior to setting up the reaction mixes. At least two replicates of each of the following controls are recommended: positive control (PC); no template control (NTC) consisting of nuclease-free water, sample elution buffer, or similar; and negative control (NC), consisting of a known negative DNA sample (no EGFR, KRAS, or BRAF mutations).

Note: Steps 2–5 should be carried out in a DNA template-free area.
- Thaw all assay reagents and equilibrate to room temperature except for HindIII (and UDG if applicable), which should be left on ice or in a cold block during preparation. Note the following:
 - HindIII is recommended for optimal performance with nonfragmented DNA
 - The 4x ddPCR Multiplex Supermix must be thawed at room temperature for 10 min and vortexed thoroughly for at least 30 sec
 - Mix all other tubes (except for HindIII and UDG) thoroughly by vortexing at maximum speed for 10–30 sec to ensure homogeneity because a concentration gradient may form during -20°C storage. For HindIII and UDG, flick or invert the tube several times to mix. Centrifuge all tubes briefly to collect the contents at the bottom of each tube.
 - Thorough mixing of each component at this step is critical.**

3. The reagents and volumes for one reaction of the Mutant Assay are shown in Tables 9–10 for non-FFPE and FFPE samples, respectively. Calculate the volumes required for a master mix based on the total number of controls and samples to be tested. Tables 9–10 also show an example of calculated volumes for 24 reactions with 15% overage, which is recommended to account for liquid loss during pipetting. Refer to Table 9 or 10 as appropriate for the sample type to be tested, and assemble the mutant master mix in a DNA template-free area, except for the internal control DNA and samples. These templates will be added in subsequent steps.

Table 9. Preparation of the Mutant Assay master mix, for non-FFPE samples.

Component	Volume per Reaction, μL 1 Well	Volume per 24 Reactions + Overage,* μL
Nuclease-free water	6.41 (variable for total reaction volume = 22 μL)	176.9 (adjust based on sample volume)
ddPCR Multiplex Supermix	5.5	151.8
ddPLEX EGFR/KRAS/BRAF Mutant Assay	2.2	60.7
ddPLEX Enhancer	0.32	8.8
DTT (300 mM)	0.29	8.0
HindIII (20 U/ μL)	0.28	7.7
ddPLEX Internal Control [†]	2	55.2
Sample [†]	5 (variable up to 10 μL)	
Total volume	22	

* Prepared with 15% overage to account for liquid loss during pipetting. Preparing a total reaction volume of 22 μL is recommended.

[†] Add in an area appropriate for handling DNA materials.

Table 10. Preparation of the Mutant Assay master mix, for FFPE samples only.

Component	Volume per Reaction, μL 1 Well	Volume per 24 Reactions + Overage,* μL
Nuclease-free water	6.19 (variable for total reaction volume = 22 μL)	170.8 (adjust based on sample volume)
ddPCR Multiplex Supermix	5.5	151.8
ddPLEX EGFR/KRAS/BRAF Mutant Assay	2.2	60.7
ddPLEX Enhancer	0.32	8.8
DTT (300 mM)	0.29	8.0
HindIII (20 U/ μL)	0.28	7.7
UDG (5 U/ μL)	0.22	6.1
ddPLEX Internal Control [†]	2	55.2
Sample [†]	5 (variable up to 10 μL)	
Total volume	22	

* Prepared with 15% overage to account for liquid loss during pipetting. Preparing a total reaction volume of 22 μL is recommended.

[†] Add in an area appropriate for handling DNA materials.

4. Vortex the master mix thoroughly at maximum speed for at least 10 sec. Centrifuge briefly to collect the contents at the bottom of the tube.

Note: Thorough vortexing of the master mix is essential for optimal assay performance.

5. The reagents and volumes for one reaction of the Total Quant Assay are shown in Tables 11–12 for non-FFPE and FFPE samples, respectively. Calculate the volumes required for a master mix based on the total number of controls and samples to be tested. Tables 11–12 also show an example of calculated volumes for 24 reactions with 15% overage, which is recommended to account for liquid loss during pipetting. Refer to Table 11 or 12 as appropriate for the sample type to be tested, and assemble the total quantification master mix including all reagents in a DNA template-free area, except for the samples.

Table 11. Preparation of the Total Quant Assay master mix, for non-FFPE samples.

Component	Volume per Reaction, μL 1 Well	Volume per 24 Reactions + Overage,* μL
Nuclease-free water	12.83 (variable for total reaction volume = 22 μL)	354.1
ddPCR Multiplex Supermix	5.5	151.8
ddPLEX EGFR/KRAS/BRAF Total Quant Assay	1.1	30.4
DTT (300 mM)	0.29	8.0
HindIII (20 U/ μL)	0.28	7.7
Sample [†]	2 (variable 2–10 μL)	
Total volume	22	

* Prepared with 15% overage to account for liquid loss during pipetting. Preparing a total reaction volume of 22 μL is recommended.

[†] Add in an area appropriate for handling DNA materials.

Table 12. Preparation of the Total Quant Assay master mix, for FFPE samples only.

Component	Volume per Reaction, μL 1 Well	Volume per 24 Reactions + Overage,* μL
Nuclease-free water	12.61 (variable for total reaction volume = 22 μL)	348.0
ddPCR Multiplex Supermix	5.5	151.8
ddPLEX EGFR/KRAS/BRAF Total Quant Assay	1.1	30.4
DTT (300 mM)	0.29	8.0
HindIII (20 U/ μL)	0.28	7.7
UDG (5 U/ μL) [†]	0.22	6.1
Sample [†]	2 (variable 2–10 μL)	
Total volume	22	

* Prepared with 15% overage to account for liquid loss during pipetting. Preparing a total reaction volume of 22 μL is recommended.

[†] Add in an area appropriate for handling DNA materials.

6. Vortex the master mix thoroughly at maximum speed for at least 10 sec. Centrifuge briefly to collect the contents at the bottom of the tube.
7. Move to an area appropriate for handling DNA materials. The ddPLEX Internal Control and samples should be completely thawed, vortexed briefly, then centrifuged prior to use.
8. Add the ddPLEX Internal Control volume (calculated in step 2) only to the Mutant Assay master mix. Vortex the master mix thoroughly at maximum speed for at least 5 sec. Centrifuge briefly to collect the contents at the bottom of the tube.

Note: When the ddPLEX Internal Control is added to the Mutant Assay master mix, the NTC reaction will be positive for this target. If desired, a subportion of the master mix specifically for NTC wells may be reserved for contamination testing, prior to adding the ddPLEX Internal Control. Additional nuclease-free water should be added to compensate for the missing volume.
9. Dispense each assay master mix into a 96-well plate or other microwell reservoir. It is recommended to dispense the Mutant Assay master mix in the wells before the Total Quant Assay master mix. Figure 2 shows an example plate layout for 24 reactions each of the Mutant and Total Quant Assays. A repeater pipet is recommended but not required for this step.

Note: The master mix volume to be dispensed is different depending on the assay and sample volumes. For the Mutant Assay with 5 μL sample volumes, dispense **17 μL** of the master mix per well. For the Total Quant Assay with 2 μL sample volumes, dispense **20 μL** of the master mix per well. A total reaction volume of 22 μL is recommended.
10. For Mutant Assay wells, dispense **5 μL** of the positive control into the designated wells. If the master mix was formulated for sample volumes larger than 5 μL , nuclease-free water should be added to the wells containing the positive control to make up the difference. Dispense the predetermined sample volume of each of the other controls and samples into the appropriate wells. A single-dispense pipettor is recommended for this step to prevent cross-contamination from well to well.
11. For Total Quant Assay wells, dispense **2 μL** of the positive control into the designated wells. If the master mix was formulated for sample volumes larger than 2 μL , nuclease-free water should be added to the wells containing the positive control to make up the difference. Dispense the predetermined sample volume of each of the other controls and samples into the appropriate wells. A single-dispense pipettor is recommended to prevent cross-contamination from well to well.
12. Fill up the entire column in the ddPCR Plate with samples or ddPCR Buffer Control. For any unused wells in a column where droplets will be generated, ddPCR Buffer Control must be added. Dilute ddPCR Buffer Control for Probes from 2x to 1x with water. Dispense 22 μL of the 1x solution into each unused well.
13. For manual droplet generation, seal the reaction reservoir with an appropriate seal. If using the Automated Droplet Generator cover the plate with a pierceable foil PCR Plate Heat Seal and seal the plate using the PX1 PCR Plate Sealer at 180°C for 5 sec. For both droplet generator options, vortex the reaction reservoir thoroughly. If using a plate, vortex at maximum speed at three different positions of the plate for at least 15 sec per position to ensure thorough mixing. Centrifuge the reaction reservoir for 1 min at 1,150 rcf. Visually verify that all the liquid is at the bottom of the wells.
14. Proceed to droplet generation, either using the QX200 Droplet Generator or the AutoDG instrument.

	Mutant Assay			Total Quant Assay								
	1	2	3	4	5	6	7	8	9	10	11	12
A	NTC	Sample 3	Sample 11	NTC	Sample 3	Sample 11						
B	NTC	Sample 4	Sample 12	NTC	Sample 4	Sample 12						
C	Negative Control	Sample 5	Sample 13	Negative Control	Sample 5	Sample 13						
D	Negative Control	Sample 6	Sample 14	Negative Control	Sample 6	Sample 14						
E	Positive Control	Sample 7	Sample 15	Positive Control	Sample 7	Sample 15						
F	Positive Control	Sample 8	Sample 16	Positive Control	Sample 8	Sample 16						
G	Sample 1	Sample 9	Sample 17	Sample 1	Sample 9	Sample 17						
H	Sample 2	Sample 10	Sample 18	Sample 2	Sample 10	Sample 18						

Fig 2. Suggested plate map for testing controls in duplicate and 18 samples in singlet.

Droplet Generation

Note: Maintain droplet generator equipment and consumables according to the respective instruction manual recommendations to maximize event counts and assay sensitivity.

For the QX200 Droplet Generator:

1. Load 20 μL of each reaction mix into the sample wells of a DG8 Cartridge preloaded in a DG8 Cartridge Holder. The orientation of the DG8 Cartridge should mirror the way in which the droplets will be transferred to the final 96-well plate. Use only 20 μL aerosol-barrier (filtered) Rainin pipet tips. Do not use 200 μL pipet tips.
2. Load 70 μL of Droplet Generation Oil for Probes into the oil wells of the DG8 Cartridge, then hook the gasket over the cartridge holder to prepare the cartridge for the droplet generation run. For detailed instructions on manual droplet generation, refer to the QX200 Droplet Generator Instruction Manual (10031907).
3. Insert the prepared cartridge into the instrument and generate droplets.
4. After droplet generation, place the cartridge holder on a flat surface.
5. Using a P50 with 200 μL tips, position the pipet tips at the top of each of the eight wells at a minus 30–45° angle to the junction where the side wall meets the bottom of the well. Do not position the pipet tip in a vertical orientation (90°) or against any flat surface of the well. Do not allow the tips to be flat against the well bottoms. Do not use wide or narrow bore tips.
6. Slowly draw 40 μL of droplets into the pipet tip. This should take 5 sec, and -5 μL air is expected. Do not aspirate more than 40 μL , as this causes air to percolate through the droplets. Pipet slowly. Apply a stable resistive force to the plunger to draw and aspirate droplets smoothly into pipet tips.
7. To dispense droplets into the 96-well plate, position the pipet tip along the side of the well, near—but not at—the bottom of the well, and then slowly dispense the droplets (5 sec).
8. Cover the plate with a pierceable foil PCR Plate Heat Seal and seal the plate using the PX1 PCR Plate Sealer at 180°C for 5 sec. Proceed immediately to thermal cycling. **Do not centrifuge the plate after droplet generation.**

For the Automated Droplet Generator:

1. Place the sealed plate in the Automated Droplet Generator and follow the instructions in the Automated Droplet Generator Instruction Manual (10043138).
2. Once droplet generation is complete, cover the plate with a pierceable foil PCR Plate Heat Seal and seal the plate using the PX1 PCR Plate Sealer at 180°C for 5 sec. Proceed immediately to thermal cycling. Do not centrifuge the plate after droplet generation.

Thermal Cycling

1. Place the plate into the Bio-Rad C1000 Touch Thermal Cycler or PTC Tempo Deepwell Thermal Cycler.
2. Use the thermal cycling conditions provided in Table 13. Set the heated lid to 105°C and the sample volume to 40 μL and begin the run.
3. Ensure that before ending the run, the plate is held at **4°C for at least 30 min** or until the lid temperature is $\leq 31^\circ\text{C}$. Optionally, the plate may be stored at 4°C overnight before data acquisition on the QX600 Droplet Reader.

Table 13. ddPLEX EGFR/KRAS/BRAF Mutant and Total Quant Assay cycling conditions.

Cycling Step	Temperature, °C	Time	Number of Cycles	Ramp Rate
Enzyme activation	95	10 min	1	
Denaturation	94	30 sec	42	2°C/sec
Annealing/extension	69	1 min		
Enzyme deactivation	98	10 min	1	
Hold	4	∞^*	1	None

*Hold the plate at 4°C for at least 30 min or until the lid temperature is $\leq 31^\circ\text{C}$, prior to ending the run.

Data Acquisition

1. After thermal cycling and the 4°C hold are complete, remove the plate from the thermal cycler.
2. **Hold the plate at room temperature for 10 min.**

Note: Holding the plate at room temperature for 10 min after thermal cycling completion is essential for optimal assay performance.
3. After the 10 min hold, follow the instructions in the QX600 Droplet Reader and QX Manager Software Standard Edition User Guide (10000153877) to insert the plate into the instrument and add the plate in the software.
4. In the Plate Information tab (Figure 3), enter a plate name, select **ddPCR Multiplex Supermix** from the Supermix dropdown menu, and enter a data filename.
5. In the Well Selection tab, select the wells to include and exclude for reading. Click **Include Selected Wells**.
6. The run may be started by clicking **Start Run** or optionally, wells can be configured in the Well Information tab using the settings in the next section (Data Analysis).

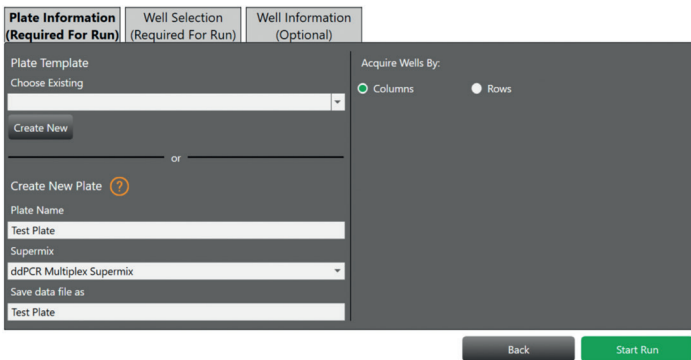


Fig. 3. QX Manager Plate Information tab setup.

Data Analysis: Plate Setup, Quality Checks, and Threshold Setting

Note: Plate setup may be performed by either manual entry or by importing a Plate Setup file. For additional information regarding import/export options see the QX600 Droplet Reader and QX Manager Software Standard Edition User Guide (10000153877).

Plate Setup with Manual Entry

1. In the Plate Editor tab, select **Direct Quantification (DQ)** from the Experiment Type dropdown menu.
2. In the Sample Description fields, enter desired descriptions to identify the samples in each well. Information may be entered for single wells or multiple wells simultaneously. Click **Apply** each time a description is entered to save it.
3. Select all wells in which the Mutant Assay was run and input the Assay Type as **Advanced Classification Method**. Press and hold the Control key to select noncontiguous wells of the plate together.
4. Input the following information under Target Info, as shown in Figure 4.

Target Name	Target Type	Signal Ch1	Signal Ch2	Signal Ch3	Signal Ch4	Signal Ch5	Signal Ch6
G719x	Unkn	FAM 1	None	None	None	None	None
S768I	Unkn	FAM 2	HEX 1	None	None	None	None
IC	Unkn	FAM 3	HEX 2	None	None	None	None
Ex20Ims	Unkn	None	HEX 3	None	None	None	None
L861Q	Unkn	None	None	Cy5 1	None	None	None
V600E	Unkn	None	None	Cy5 2	Cy5.5 1	None	None
L858R	Unkn	None	None	Cy5 3	Cy5.5 2	None	None
Ex19Del	Unkn	None	None	None	Cy5.5 3	None	None
G12C	Unkn	None	None	None	None	ROX 1	None
T790M	Unkn	None	None	None	None	ROX 2	ATTO590 1
T790M^{C797S}G	Unkn	None	None	None	None	ROX 3	ATTO590 2
T790M^{C797S}A	Unkn	None	None	None	None	ROX 4	ATTO590 3
C797S	Unkn	None	None	None	None	None	ATTO590 4

Fig. 4. Example of labeling and assigning channels to targets for the Mutant Assay.

5. Click **Apply** to save the settings. Click **OK** to close the pop-up shown in Figure 5.

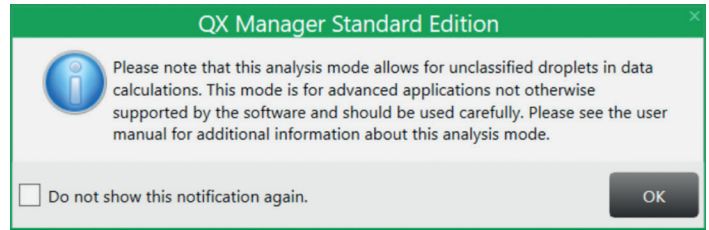


Fig. 5. Advanced classification warning.

6. Select all wells in which the Total Quant Assay was run and input the Assay Type as **Probe Mix Triplex**. Press and hold the Control key to select noncontiguous wells of the plate together.
7. Click the minus sign (highlighted in orange and outlined in red in Figure 6) to remove Targets 4–6 and Targets 7–9. Input the following information under Target Info, as shown in Figure 6.

Target Name	Target Type	Signal Ch1	Signal Ch2	Signal Ch3	Signal Ch4	Signal Ch5	Signal Ch6
KRAS	Unkn	FAM	None	None	None	None	None
EGFR	Unkn	None	HEX	None	None	None	None
BRAF	Unkn	FAM	HEX	None	None	None	None
4	Unkn	None	None	Cy5	None	None	None
5	Unkn	None	None	None	Cy5.5	None	None
6	Unkn	None	None	Cy5	Cy5.5	None	None
7	Unkn	None	None	None	None	ROX	None
8	Unkn	None	None	None	None	None	ATTO590
9	Unkn	None	None	None	None	ROX	ATTO590

Fig. 6. Example of labeling and assigning channels to targets for the Total Quant Assay.

8. Click **Apply** to save the settings.

Plate Setup Utilizing a Plate Setup CSV

1. Download the plate setup CSV file from the product page on the Bio-Rad website. Contact your sales or customer service representative for more information.
2. In the Plate Editor tab, import the ddPLEX EGFRKRASBRAF MT TQ Assays Template CSV file using the Import Plate Setup CSV button, as shown in Figure 7. This CSV file will autofill the Experiment Type, Sample Description 1, Assay Type, and Target Info for the first two columns. As shown in Figure 8, column 1 will default to the Mutant Assay and column 2 will default to the Total Quant Assay. If the plate layout differs, right clicking on any well after importing this CSV file will allow the user to copy and paste the associated well details and customize the plate layout.

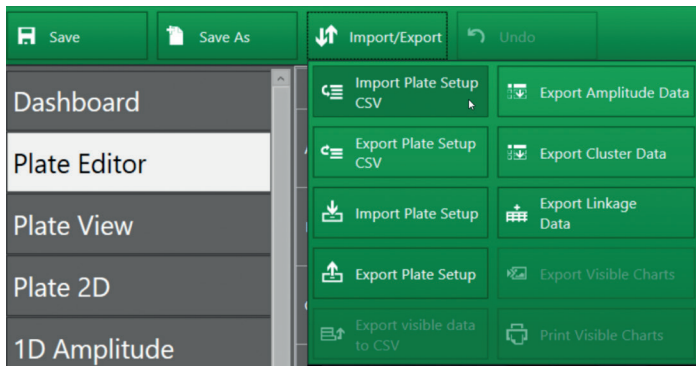


Fig. 7. Import Plate Setup button.

- Additional information can be captured in Sample Description fields 2–4 for single wells or multiple wells simultaneously by selecting the well(s) and entering the information in the fields. The **Apply** button must be clicked each time a description is entered to save it.

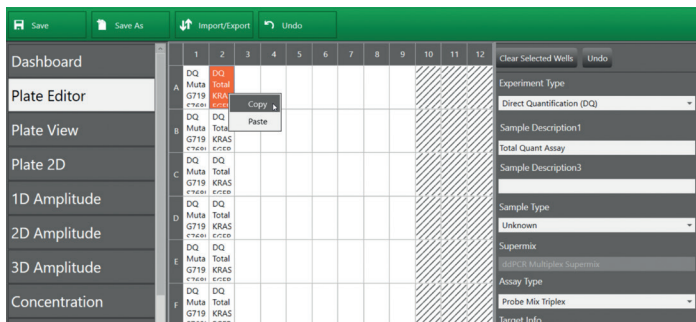


Fig. 8. Example of Plate Editor tab after importing Plate Setup CSV.

Initial Quality Check

- Navigate to the Event Counts tab, select all wells, and evaluate the event counts. If the accepted event count is <10,000 in any well, it is recommended to exclude the well from analysis.
- Select all wells in which the Mutant Assay was run. Navigate to the 2D Amplitude tab and examine the wells for uniformity. See Figure 9 for an example of 2-D plots for 24 overlaid wells. Any wells with shifted clusters, streaks, or any unusual features should be excluded from analysis. Figure 10 shows an example of shifted clusters, where seven out of eight overlaid wells have the same cluster positions and the eighth well has different cluster positions compared to the other wells. Figure 11 shows 2-D plots from a well with a streak, which is indicative of shredded droplets.

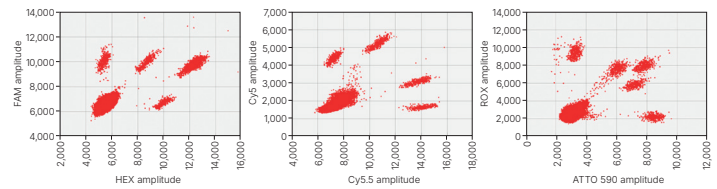


Fig. 9. 2-D plots for 24 overlaid wells with the Mutant Assay.

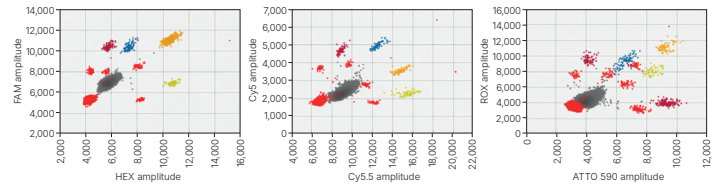


Fig. 10. 2-D plots for the Mutant Assay with shifted clusters. One well with shifted clusters (red) is excluded from analysis.

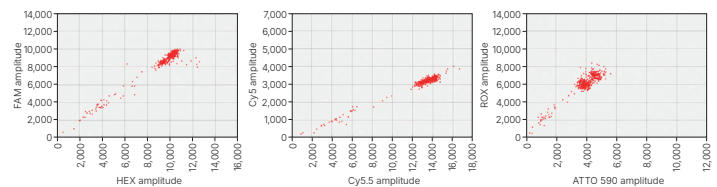


Fig. 11. 2-D plots for one well of the Mutant Assay with droplet shredding, which is excluded from analysis.

- Examine all wells for the internal control cluster in the FAM/ HEX (Ch1/Ch2) 2-D plot. If the internal control cluster is not present, this could indicate a compromised PCR process such as the presence of inhibitors in the reaction. Such wells should be excluded from further analysis.
- Check the Total Quant Assay wells for uniformity. Any wells with shifted clusters, streaks, or any unusual features should be excluded from analysis.

Threshold Setting for Mutant Assay

- It is ideal to apply the same thresholds to all wells run with the same assay to help ensure consistency of positive and negative droplet calls across the plate. However, depending upon sample types, variant concentration, or other experimental factors, the end user may need to manually adjust thresholds for certain samples. Begin with selecting all Mutant Assay wells in the 2D Amplitude tab. Press and hold the Control key to select noncontiguous wells of the plate together.
- From the tool bar, select an appropriate threshold cluster mode button. The threshold cluster square button is highlighted in Figure 12.



Fig. 12. Thresholding tool bar with dotted square icon outlined in orange.

- Starting with the FAM/HEX (Ch1/Ch2) plot, draw a square or rectangle surrounding all visible droplets. In the pop-up menu that appears after selecting a region, click the radio buttons to set the cluster assignment for all targets to negative. Click **Assign Cluster** to complete the assignment, which will classify the droplets as negative (gray color). Repeat for the Cy5/Cy5.5 (Ch3/Ch4) and ROX/ATTO 590 (Ch5/Ch6) plots (Figure 13). This initial assignment of all droplets as negative ensures that all droplets are thresholded and counted, and that none are unintentionally missed.

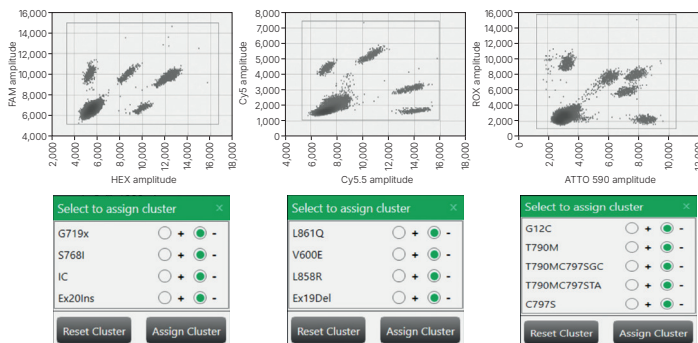


Fig. 13. Assigning all droplets as negative for 24 overlaid wells of the Mutant Assay. Ex19Del, exon 19 deletion; Ex20Ins, exon 20 insertion; IC, internal control; T790MC797SGC, T790M + C797S G>C; T790MC797STA, T790M + C797S T>A.

- From the tool bar, select an appropriate threshold cluster mode button. The threshold cluster freeform button (pencil icon) is highlighted in Figure 14.



Fig. 14. Thresholding tool bar with freeform button outlined in orange.

- In the FAM/HEX (Ch1/Ch2) plot, draw circles around each positive cluster using the appropriate tool. In the pop-up menu that appears after selecting a region, click the radio buttons to set the cluster assignment, referring to Figure 15 for the cluster designations. Each positive cluster should be set as positive for the assigned target and negative for the others. Click **Assign Cluster** to complete each assignment. It is recommended to threshold relatively tightly around the densest cluster regions.

Note: Any cluster to the right of the negative cluster in the HEX channel should be thresholded as positive for the target exon 20 insertion (Ex20Ins). Cluster amplitude may differ slightly from the positive control depending upon the sample and variant identity.

- In the Cy5/Cy5.5 (Ch3/Ch4) plot, draw thresholds and assign positive clusters.

Note: Any cluster to the right of the negative cluster in the Cy5.5 channel should be thresholded as positive for the target exon 19 deletion (Ex19Del). Cluster amplitude may differ slightly from the positive control depending upon the sample and variant identity.

- For ROX/ATTO 590 (Ch5/Ch6) plot, draw thresholds and assign positive clusters.

Note: There may be a few droplets present in the ROX channel to the left of the positive G12C cluster, as shown in Figure 15. It is recommended to leave these droplets thresholded as negative. Also refer to step 9 and Figure 16. To help distinguish this subpopulation of droplets from the positive cluster and determine the appropriate threshold location, it is recommended to review and compare the location of any droplets with amplitudes higher than the negative control cluster in the 2-D ROX/ATTO 590 cluster plot of the no template control (Figure 17) or wild-type negative control (Figure 19) to that of the positive control (Figure 18).

- Droplets with high fluorescence amplitude, those in between positive clusters, as well as higher order multiple occupancy droplets that are distinct from the dense positive or negative clusters should be reset by circling the droplet(s) and clicking **Reset Cluster** in the pop-up menu. These unassigned droplets will have a bright red color. See Figure 15 for an example.

Note: The assignment of droplets as positive and negative is a critical step and impacts the sensitivity and specificity of the assay.

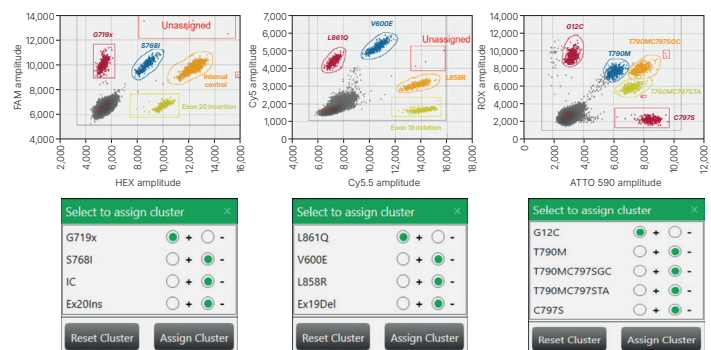


Fig. 15. Cluster assignments for 24 overlaid wells of the Mutant Assay with visible threshold lines and corresponding pop-up menus with radio buttons to assign clusters. Ex19Del, exon 19 deletion; Ex20Ins, exon 20 insertion; IC, internal control; T790MC797SGC, T790M + C797S G>C; T790MC797STA, T790M + C797S T>A.

- To remove threshold lines from view, select the lock button in the tool bar as shown in Figure 16.

Note: With fragmented DNA or high variant concentration there may be increased rain compared with positive control.

A



B

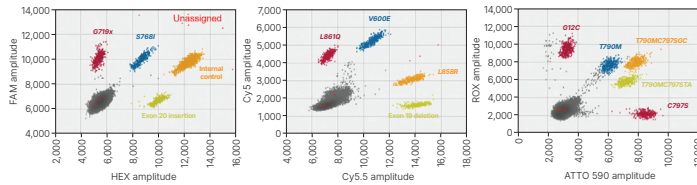


Fig. 16. Thresholding tool bar with lock button and 2-D plots. A, lock button outlined in orange; B, 24 overlaid wells of the Mutant Assay 2-D plots without visible threshold lines and with clusters labeled. *T790MC797SGC*, *T790M* + *C797S G>C*; *T790MC797STA*, *T790M* + *C797S T>A*.

Secondary Quality Check

1. Examine the no template control (NTC) wells to detect potential contamination. The internal control cluster will appear in the FAM/HEX channels. Ideally, all other droplets should be classified as negative. Tolerance to positive droplets should be determined by the end user. See Figure 17 for example 2-D plots.

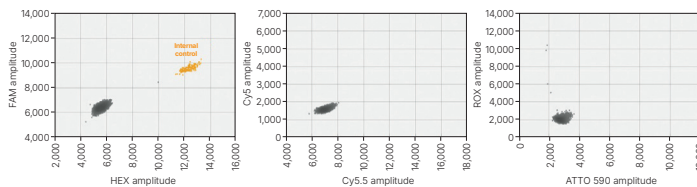


Fig. 17. 2-D plots for two wells of no template control.

2. Observe the positive control wells to ensure that each plot has the expected negative and positive clusters. Refer to Figure 18 for example 2-D plots.

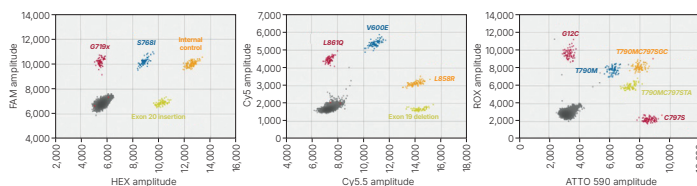


Fig. 18. 2-D plots for two wells of positive control. *T790MC797SGC*, *T790M* + *C797S G>C*; *T790MC797STA*, *T790M* + *C797S T>A*.

3. Wild-type-only (mutation negative) wells are critical for estimating the false-positive rate due to sample type and/or amount. Running several wild-type-only wells improves false-positive estimation. As false-positive rate is in part a function of the amount of sample loaded, wild-type-only wells should ideally be run at a concentration similar to that expected for the samples. This wild-type-only material should ideally be from a sample preparation analogous to samples tested (for example, FFPE samples).

4. Examine the wild-type negative control samples. A positive cluster for the internal control is expected in the FAM/HEX channels. For the other channels, the only cluster present should be the negative cluster, with a minimal number of droplets that stray far from the negative cluster such as those indicated by gray arrows in Figure 19. A small number of positive droplets in the *G719x* and *T790M* targets, such as those circled in Figure 19 D, may occur and increase in frequency with higher input DNA amounts. Refer to Figure 19 for example 2-D plots for two wild-type negative control wells run with 10, 30, 50, and 132 ng/reaction in Figures 19A–D, respectively. Tolerance to positive droplets should be determined by the end user.

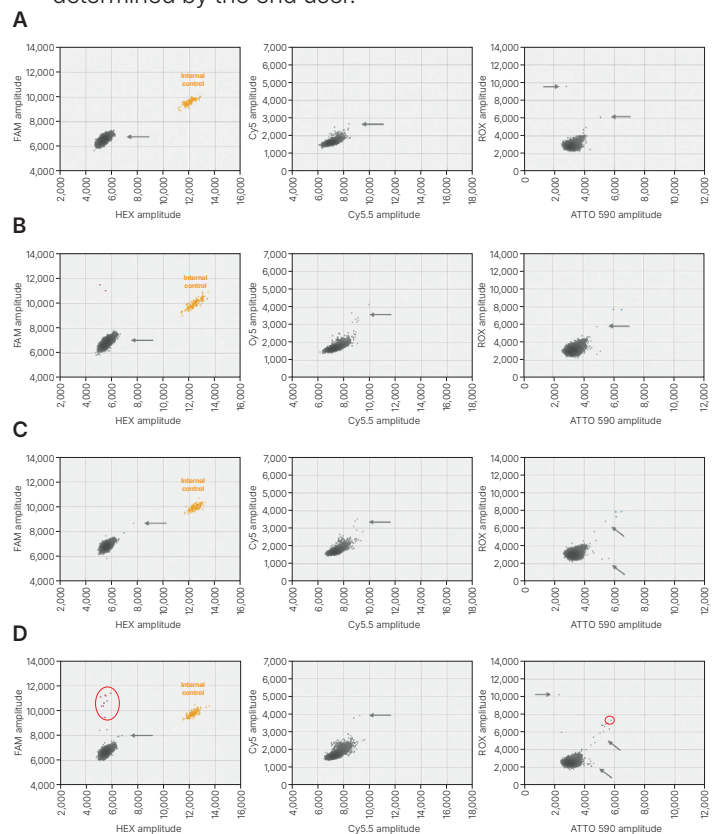


Fig. 19. 2-D plots for two wells each of wild-type negative control. Input wild-type DNA at A, 10; B, 30; C, 50; and D, 132 ng/reaction.

5. Inspect the replicate sample wells for uniformity and examine any outliers for problems. Do not include problem wells in the final analysis. If any clusters are cut off by the thresholds, the thresholds should be manually adjusted.

Interpretation for EGFR, T790M, and C797S Variants

1. This assay can identify whether the *C797S* variants are present in cis (on the same allele) or trans (on a different allele) with *T790M*. Trans-configured alleles partition independently into droplets, therefore copartitioning is governed by chance. Cis-configured alleles tend to cosegregate into the same droplets, because they are physically linked, and copartitioning greatly exceeds chance expectation.

2. Figure 20 displays clusters for the *T790M*, *T790M* + *C797S* *T>A* (cis and trans), *T790M* + *C797S* *G>C* (cis and trans), and *C797S* templates in the ROX/ATTO 590 channels. Figure 20A displays *T790M*. Figures 20B–C display *C797S* variants. Figure 20D displays cis-configured *T790M* and *C797S* *T>A*. Figure 20E displays cis-configured *T790M* and *C797S* *G>C*. Figures 20F–G display trans-configured *T790M* and *C797S*. Figure H displays *T790M*, cis-configured *T790M* and *C797S* *T>A*, cis-configured *T790M* and *C797S* *G>C*, and *C797S* *G>C*.

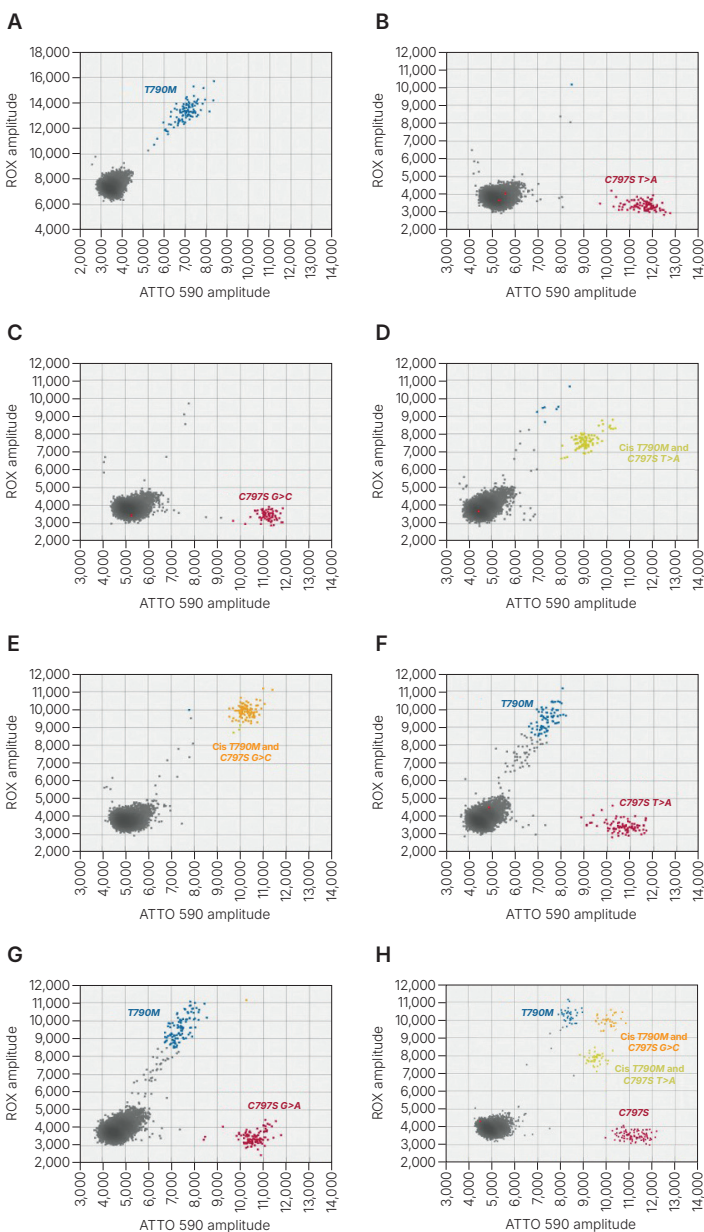


Fig. 20. Example 2-D plots for *T790M* and *C797S* variants. Clusters are displayed for **A**, *T790M*; **B–C**, *C797S* variants; **D**, cis-configured *T790M* and *C797S* *T>A*; **E**, cis-configured *T790M* and *C797S* *G>C*; **F–G**, trans-configured *T790M* and *C797S*; and **H**, *T790M*, cis-configured *T790M* and *C797S* *T>A*, cis-configured *T790M* and *C797S* *G>C*, and *C797S* *G>C*.

Examples of Mutant Assay 2-D Plots with Various Sample Types

Figure 21 shows example 2-D plots for cell-free DNA (cfDNA) and FFPE DNA samples positive for *L858R*. Note that these sample types are expected to have more rain than unfragmented DNA.

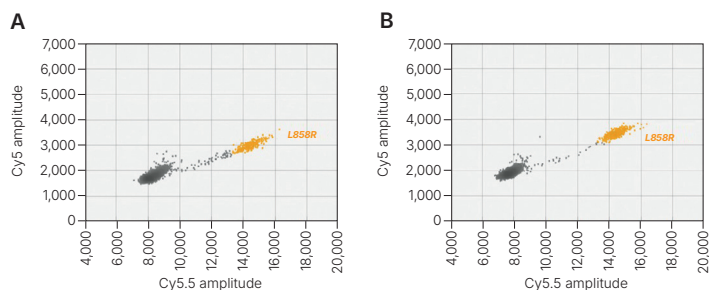


Fig. 21. Example 2-D plot for *L858R* variant. **A**, cfDNA; **B**, FFPE DNA.

Thresholding Guidelines for High Concentration Clusters with Multiple Occupancy Droplets

2-D plots of co-mutations in *G719x* and *S768I* targets detected in a cfDNA and FFPE sample are shown in Figure 22. When the concentration of these variants is high, multiple occupancy droplets (*G719x* + *S768I*, *G719x* + internal control, *S768I* + internal control) are also present. Multiple occupancy droplets with high fluorescence amplitude are reset during thresholding (red droplets). As expected, increased input amounts of fragmented DNA, as with the FFPE DNA sample compared to the cfDNA sample, has a higher occurrence of multiple occupancy droplets and rain between negative and positive clusters.

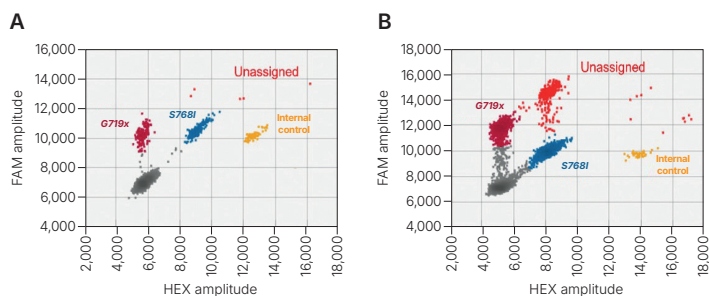


Fig. 22. Example 2-D plots for *G719x* and *S768I* variants. **A**, cfDNA; **B**, FFPE DNA.

Threshold Setting for Total Quant Assay

- It is ideal to apply the same thresholds to all wells run with the same assay to help ensure consistency of positive and negative droplet calls across the plate. However, depending upon sample types, variant concentration, or other experimental factors, the end user may need to manually adjust thresholds for certain samples. Begin with selecting all Total Quant Assay wells in the 2D Amplitude tab. Press and hold the Control key to select noncontiguous wells of the plate together.

- From the tool bar, choose the appropriate threshold cluster mode button. Use the threshold function to define single-positive clusters (*KRAS*, *BRAF*, *EGFR*), as illustrated in Figure 23. In this example, the threshold cluster freeform button (previously shown in Figure 14) is used. The *KRAS* threshold should include the FAM (Ch1) cluster just above the negative cluster and any upward spray in FAM (Ch1). The *EGFR* threshold should extend in the HEX (Ch2) direction from the negative cluster and include the HEX (Ch2) cluster. The *BRAF* threshold should also include any spray between the negative cluster and the *BRAF* single-positive cluster in FAM/HEX (Ch1/Ch2).

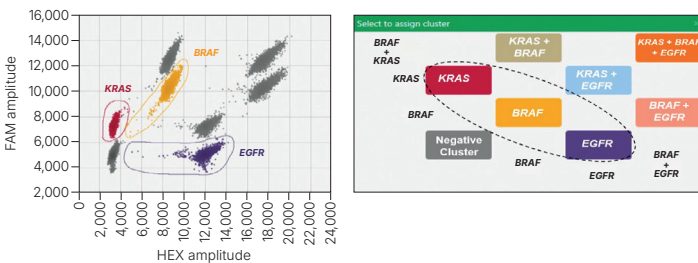


Fig. 23. Thresholding the Total Quant Assay single-positive clusters.

- Threshold the double-positive clusters (*KRAS + BRAF*, *KRAS + EGFR*, and *BRAF + EGFR*) as shown in Figure 24. *KRAS + BRAF* threshold should include the upper left cluster adjacent to the previously thresholded *BRAF* cluster and extend down to the previously thresholded *KRAS* cluster. The *KRAS + EGFR* cluster should contain the cluster above the previously thresholded *EGFR* cluster and extend toward the *BRAF* cluster tail. The *BRAF + EGFR* threshold should contain the cluster just below the unthresholded triple-positive cluster and the tails that extend toward the *BRAF* and *EGFR* clusters.

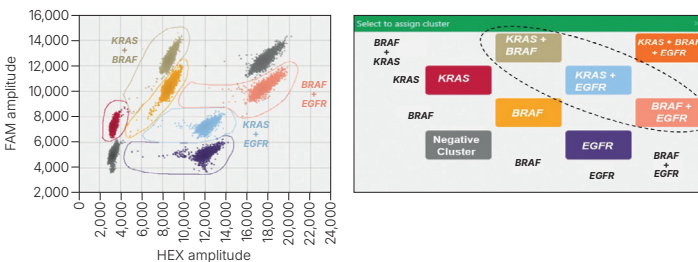


Fig. 24. Thresholding the Total Quant Assay double-positive clusters.

- Threshold the triple-positive cluster by drawing the threshold around the top right cluster, extending to the edges of the previously drawn adjacent thresholds, then assigning the orange *KRAS + BRAF + EGFR* cluster as shown in Figure 25. Note that the presence and density of the cluster corresponds to the input DNA amount and may be minimal or absent with lower input amounts.

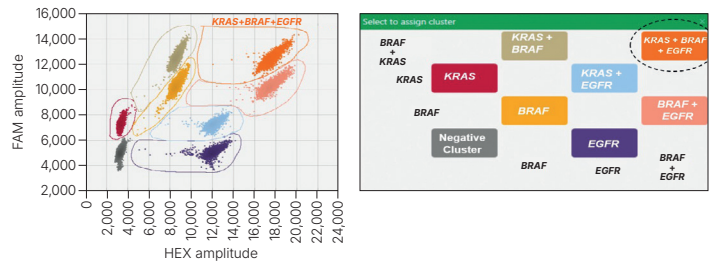


Fig. 25. Thresholding the Total Quant Assay triple-positive cluster.

- All droplets, excluding those of the negative cluster, should now be thresholded and all clusters should be assigned. Review all thresholded wells and replicate sample wells for uniformity and examine any outliers for potential issues. Do not include any problematic wells in the final analysis. If any clusters are cut off by the assigned thresholds, the thresholds may be manually adjusted as determined appropriate by the end user.

Tertiary Quality Check

- Examine the no template control (NTC) wells to detect potential contamination. Ideally, all other droplets should be classified as negative. Tolerance to positive droplets should be determined by the end user. See Figure 26 for an example 2-D plot of no template control.

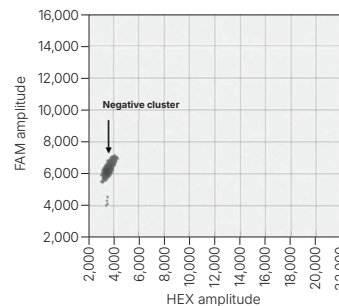


Fig. 26. 2-D plot for two wells of no template control.

- Observe the positive control wells to ensure that each plot has the expected negative and positive clusters. Refer to Figure 27 for an example 2-D plot of positive control. When variants are present it is expected for subclusters to occur, as designated in Figure 27 with gray arrows. These subclusters may increase in size and density as variant concentration increases.

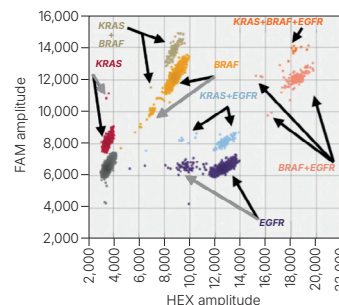


Fig. 27. 2-D plot for two wells of positive control.

- Wild-type human genomic DNA 2-D plots with an input of 1.3 ng and 53 ng per reaction are shown in Figure 28. Note that higher order clusters (double-positive and triple-positive) may not be seen with low input samples.

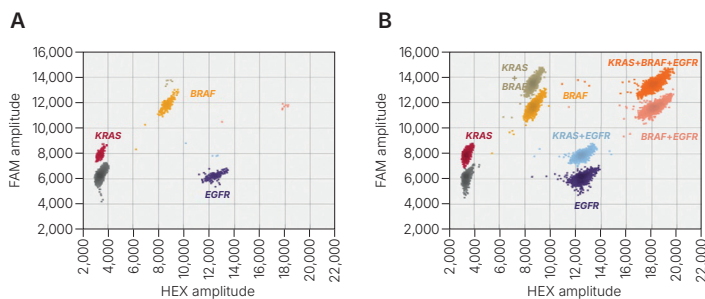


Fig. 28. 2-D plots for one well each of wild-type human genomic DNA. A, 1.3 ng/reaction; B, 53 ng/reaction.

- After the thresholding is complete, save the data file.

Examples of Total Quant Assay 2-D Plots with Various Sample Types

Note that these sample types are expected to have more rain than unfragmented DNA (Figure 29).

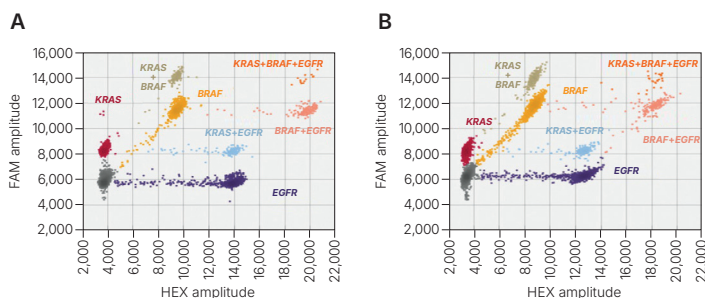


Fig. 29. 2-D plots for one well each of sample at 4 ng/reaction. A, wild-type cfDNA; B, wild-type FFPE DNA.

Data Export

- In the Data Table tab, select the dropdown menu in the upper right corner. Click **Export to CSV...** and save the exported file. Refer to Figure 30 to locate the dropdown menu.

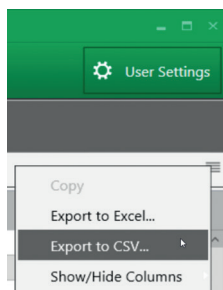


Fig. 30. Data Table dropdown menu.

- Once saved, the file may be opened with a spreadsheet application such as Microsoft Excel.

Data Analysis and Interpretation

- To determine when a positive value for a target is background or a true positive result, a laboratory would, for example, test many negative samples across multiple runs, operators, and lots to establish a threshold value for each target.
- Verify that the ddPLEX Internal Control has a positive reported concentration for all samples. The concentration observed in internal testing ranged from 2 to 11 copies/ μ L in individual reactions. In standard practice, end users would establish a suitable acceptance range for their laboratories.
- Determine which of the Mutant Assay targets are above background levels in known negative samples.
- If samples were tested in replicate, the mean concentration of positive targets can be calculated.
- The ddPLEX EGFR/KRAS/BRAF Positive Control, with a 5 μ L input into the Mutant Assay and 2 μ L input into the Total Quant Assay, produced values from 2 to 10 copies/ μ L for Mutant Assay targets, except for the internal control, and from 60 to 300 copies/ μ L for Total Quant Assay targets. Observed values may vary and end users should establish expected ranges for their own laboratories.

Calculation of Allele Frequencies (optional and for information only)

- For the Mutant Assay wells, use the reported concentration (copies/ μ L) to calculate the original input sample concentration for each target using the following formula:

Original sample concentration (copies/ μ L) =

$$\frac{\text{Reported concentration (copies}/\mu\text{L}) \times \text{reaction volume } (\mu\text{L})}{\text{Input sample volume } (\mu\text{L})}$$

Example for a sample positive for *L858R*:

L858R original sample concentration (copies/ μ L) =

$$\frac{2.5 \text{ copies}/\mu\text{L} \times 22 \mu\text{L}}{5 \mu\text{L}}$$

L858R original sample concentration (copies/ μ L) = 11 cp/ μ L

- For the Total Quant Assay wells, use the reported concentration (copies/ μ L) to calculate the original input sample concentration using the following formula. Note that the input sample volume may have been different between the Mutant and Total Quant Assays.

Original sample concentration (copies/ μ L) =

$$\frac{\text{Reported concentration (copies}/\mu\text{L}) \times \text{reaction volume } (\mu\text{L})}{\text{Input sample volume } (\mu\text{L})}$$

Example for a sample positive for *EGFR*:

EGFR original sample concentration (copies/ μ L)=

$$\frac{100 \text{ copies}/\mu\text{L} \times 22 \mu\text{L}}{2 \mu\text{L}}$$

EGFR original sample concentration (copies/ μ L)= 1,100 cp/ μ L

- For samples tested in replicate, the averages of these original sample concentrations may be calculated.
- Use the average original values to determine allele frequencies using the following formula:

Allele frequency =

$$\frac{\text{Average mutant allele concentration (copies}/\mu\text{L})}{\text{Average total allele concentration (copies}/\mu\text{L}) \text{ for the same gene}} \times 100\%$$

Example for a sample positive for *L858R*:

L858R allele frequency =

$$\frac{11 \text{ copies}/\mu\text{L}}{1,100 \text{ copies}/\mu\text{L}} \times 100\%$$

L858R allele frequency = 1.0%

Note: At very low variant concentrations, the allele frequency is expected to exhibit high variability.

Troubleshooting

Low Total Event Counts

Problem: Event counts are <10,000. Low event counts could arise due to insufficient vortexing, or issues with droplet generation or the QX600 Droplet Reader. Maintain droplet generator equipment and consumables according to the respective instruction manual recommendations to maximize event counts. However, if wells with event counts <10,000 are isolated to sample wells and not control wells, please confirm that input DNA is purified and free of carryover components that may interfere with droplet stability.

Resolution: Exclude sample wells with low event counts (<10,000) from the analysis. Ensure thorough vortexing was performed at all indicated steps. For errors and logs in the Automated Droplet Generator, refer to the Automated Droplet Generator Instruction Manual (10043138). If possible, dilute the sample to decrease the inhibitor concentration and repeat Droplet Digital PCR. Alternatively, re-extract or purify the sample to eliminate inhibitors.

Missing Targets in the Positive Control

Problem: Missing or indistinct target clusters in the positive control (see Figure 31) may indicate insufficient vortexing or inefficient PCR, possibly due to incorrect reagent amounts, improper storage, or expired components. Figure 31 shows effects of excess enhancer.

Resolution: Check PCR reagent volumes, storage conditions, and expiration dates, and resolve any issues found. Verify PCR cycling conditions. Ensure thorough vortexing was performed at all indicated steps.

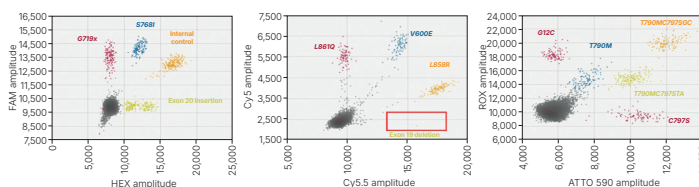


Fig. 31. 2-D plots of Mutant Assay reaction mix with elevated levels of enhancer added. Poorly amplified target is highlighted in red boxes. *T790MC797SGC*, *T790M* + *C797S* G>C; *T790MC797STA*, *T790M* + *C797S* T>A.

No Call Reported in the Conc (copies/ μ L) Column

Problem: No Call result is present for some targets in the column with concentration results (Figure 32).

Resolution: Utilize QX Manager Software, Standard Edition, v2.3 or later, for data analysis, as this is a known software issue with earlier versions. Alternatively, after verifying that other targets in the same well have values reported for the concentration, and the number of Positives is 0 (Figure 32), replace the No Call result with a 0 value.

Mirroring

Problem: Droplets exhibit two distinct sizes (Figure 33), which indicate a potential consumable failure or particulates from samples, environment, tips, or reagents.

Resolution: Exclude the well from analysis and repeat Droplet Digital PCR.

Well	Sample Description 1	Sample Description 2	Target	Conc (copies/ μ L)	Accepted Droplets	Positives	Negatives
A09	Mutant Assay	NTC	G719x	0.00	19,365	0	19,290
A09	Mutant Assay	NTC	S768I	0.00	19,365	0	19,290
A09	Mutant Assay	NTC	Internal control	4.88	19,365	75	19,290
A09	Mutant Assay	NTC	Exon 20 insertion	0.00	19,365	0	19,290
A09	Mutant Assay	NTC	L861Q	0.00	19,365	0	19,290
A09	Mutant Assay	NTC	V600E	0.00	19,365	0	19,290
A09	Mutant Assay	NTC	L858R	0.00	19,365	0	19,290
A09	Mutant Assay	NTC	Exon 19 deletion	0.00	19,365	0	19,290
A09	Mutant Assay	NTC	G12C	No Call	19,365	0	0
A09	Mutant Assay	NTC	T790M	0.00	19,365	0	19,290
A09	Mutant Assay	NTC	T790M + C797S G>C	0.00	19,365	0	19,290
A09	Mutant Assay	NTC	T790M + C797S T>A	0.00	19,365	0	19,290
A09	Mutant Assay	NTC	C797S	0.00	19,365	0	19,290

Fig. 32. Example of a No Call result. Conc, concentration; NTC, no template control.

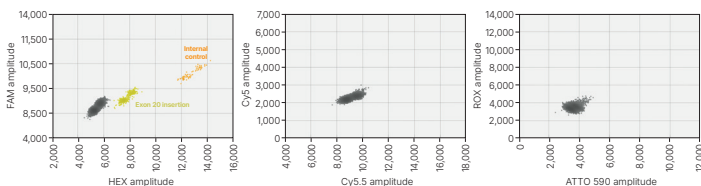


Fig. 33. Example of cluster mirroring with the Mutant Assay and a single sample positive for exon 20 insertion.

Visit [bio-rad.com/DropletDigitalPCRAssays](https://www.bio-rad.com/DropletDigitalPCRAssays) for more information.

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