

# ddPLEX *ESR1* Mutation Detection Kit User Guide

Catalog #	Description
17009501	<b>ddPLEX <i>ESR1</i> Mutation Detection Kit</b> , includes: ddPLEX <i>ESR1</i> Mutation Detection Assay, ddPLEX <i>ESR1</i> Positive Control, ddPCR™ Multiplex Supermix, and Dithiothreitol (DTT).

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

## Product Description

The *ESR1* gene encodes estrogen receptor (ER) subtype alpha. Variants in the ligand binding domain of the estrogen receptor have emerged as an important driver of resistance to endocrine therapies such as aromatase inhibitors, which are used in the treatment of ER-positive, HER2-negative breast cancer.

The ddPLEX *ESR1* Mutation Detection Kit detects seven common variants in the *ESR1* gene (*E380Q*, *S463P*, *L536R*, *Y537C*, *Y537N*, *Y537S*, and *D538G*) and their wild-type counterparts (see Table 1), which allows for the calculation of variant allele frequencies. The kit can be used to test DNA extracted from plasma and tumor tissues, including formalin-fixed paraffin-embedded (FFPE) tissue samples.

The ddPLEX *ESR1* Mutation Detection Kit includes sufficient reagents for a total of 120 reactions: 60 reactions of the mutant assay and 60 reactions of the wild-type 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 for use with the Bio-Rad QX600™ Droplet Digital™ PCR (ddPCR) System or QX600 AutoDG™ Droplet Digital PCR System. The ddPLEX *ESR1* Mutation Detection Kit is compatible with QX Manager Software, Standard Edition, v2.1 and later, and QX Manager Software, Premium Edition.

**Table 1. Targets of the ddPLEX *ESR1* Mutation Detection Kit.**

<i>ESR1</i> Exon #	Wild-Type Assay Allele	Mutant Assay Allele
5	380-Wild Type	<i>E380Q (c.1138G&gt;C)</i>
7	463-Wild Type	<i>S463P (c.1387T&gt;C)</i>
8	53X-Wild Type	<i>L536R (c.1607T&gt;G)</i>
		<i>Y537C (c.1610A&gt;G)</i>
		<i>Y537N (c.1609T&gt;A)</i>
		<i>Y537S (c.1610A&gt;C)</i>
		<i>D538G (c.1613A&gt;G)</i>

## ddPLEX *ESR1* Mutation Detection Kit and Contents

The ddPLEX *ESR1* Mutation Detection Kit consists of the ddPLEX *ESR1* Mutation Detection Assay, ddPLEX *ESR1* Positive Control, ddPCR Multiplex Supermix, and DTT, each of which can also be purchased separately. Please see Table 2. The more detailed contents of the *ESR1* Mutation Detection Assay and other kit reagents are listed in Tables 3, 4, and 5.

**Table 2. ddPLEX *ESR1* Mutation Detection Kit (#17009501) contents.**

Catalog Number	Description	Quantity
12019648	ddPLEX <i>ESR1</i> Mutation Detection Assay	1
12019606	ddPLEX <i>ESR1</i> Positive Control, 100 µl	1
12005909	ddPCR Multiplex Supermix, 1.2 ml (2 x 0.6 ml)	1
12012171	Dithiothreitol (DTT), 2 ml (2 x 1 ml vials)	1

The ddPLEX *ESR1* Positive Control (#12019606) is a mixture of synthetic DNA consisting of all *ESR1* variants and all wild-type targets covered by the ddPLEX *ESR1* Mutation Detection Kit (refer to Table 1). The ddPLEX *ESR1* Positive Control, in addition to verifying the performance of the reaction, serves as a guide for data thresholding, as it produces positive droplet clusters for each target. It is designed to resemble a sample with low-input amount and low allele frequency. The positive control has sufficient volume for 12 reactions of both the mutant and wild-type assays using the recommended input amounts.

The ddPLEX Internal Control is included as a component of the ddPLEX Mutation Detection Assay (#12019648). It consists of a synthetic, non-human DNA sequence in solution. The ddPLEX Internal Control serves as an exogenous control that is introduced into the *ESR1* Mutant Assay reaction mixture for monitoring ddPCR reactions.

All reagents should be stored in a constant temperature freezer at -15°C to -25°C. The ddPLEX *ESR1* Mutation Detection Assay and ddPLEX *ESR1* Positive Control can be frozen and thawed up to three times. Repeated freezing and thawing more than three times is not recommended. For DTT, multiple freeze-thaw cycles should be avoided, and it is recommended to prepare single-use aliquots. 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.

## ddPLEX *ESR1* Mutation Detection Assay and Contents

As the key component of the ddPLEX *ESR1* Mutation Detection Kit, the ddPLEX Mutation Detection Assay (#12019648) comprises two different assays. The wild-type assay measures the wild-type loci in three exons of the *ESR1* gene (see Table 4). The mutant assay quantifies seven common variants (*E380Q*, *S463P*, *L536R*, *Y537C*, *Y537N*, *Y537S*, and *D538G*) and includes an internal control to verify assay performance. These assays can be used to test DNA extracted from plasma and tumor tissues, including formalin-fixed paraffin-embedded (FFPE) tissue samples.

**Table 3. ddPLEX *ESR1* Mutation Detection Assay contents (#12019648).**

Kit Component	# Vials	Volume, $\mu$ l	Storage Conditions
ddPLEX <i>ESR1</i> Wild-Type Assay	1	85	-20°C, limit exposure to light
ddPLEX <i>ESR1</i> Mutant Assay	1	85	-20°C, limit exposure to light
ddPLEX Enhancer	1	90	-20°C
ddPLEX Internal Control	1	500	-20°C <sup>1</sup>

<sup>1</sup> It is recommended to store DNA samples in a separate area from the other reaction components.

**Table 4. ddPLEX *ESR1* Positive Control contents (#12019606).**

Kit Component	# Vials	Volume, $\mu$ l	Storage Conditions
ddPLEX <i>ESR1</i> Positive Control	1	100	-20°C <sup>1</sup>

<sup>1</sup> It is recommended to store DNA samples in a separate area from the other reaction components.

**Table 5. Additional reagents.**

Description	Vendor	Catalog Number
ddPCR Multiplex Supermix, 1.2 ml	Bio-Rad	12005909 <sup>1</sup>
Dithiothreitol (DTT), 2 ml	Bio-Rad	12012171

<sup>1</sup> Other packaging configurations include part numbers 12005910 and 12005911.

### Additional Reagents, Equipment, and Consumables

The QX600 System with either the manual or automated droplet generator is required for use with the ddPLEX *ESR1* Mutation Detection Kit. The equipment, consumables, and reagents necessary for the QX600 System are listed in Tables 6, 7, and 8.

**Table 6. Droplet generation and reader equipment, consumables, and reagents.**

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	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	-
Micropipettes (1–10 $\mu$ l, 2–20 $\mu$ l, 20–200 $\mu$ l, and 100–1,000 $\mu$ l)	Any	-
Barrier pipette tips (sterile, RNase- and DNase-free)	Rainin or Eppendorf	Various
Nuclease-free water	Any	-
MseI restriction enzyme (optional)	Any	-
ddPCR Buffer Control for Probes (2x)	Bio-Rad	1863052
Microcentrifuge tubes, 1.5 ml, low-DNA binding	Any	-
Vortexer	Any	-
Centrifuge for 96-well plates	Any	-
Benchtop microcentrifuge	Any	-

**Table 7. Reagents and consumables for automated droplet generation only.**

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

**Table 8. Reagents and consumables for manual droplet generation only.**

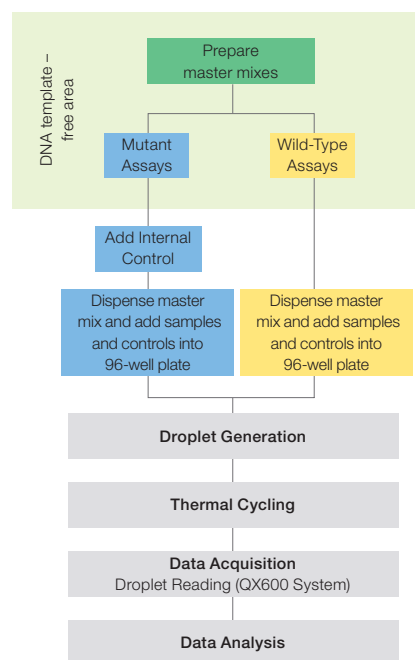
Description	Vendor	Catalog Number
Droplet Generation Oil for Probes	Bio-Rad	1863005
DG8 Cartridge Holder	Bio-Rad	1863051
DG8 Cartridges for QX200/QX100 Droplet Generator	Bio-Rad	1864008
DG8 Gaskets for QX200/QX100 Droplet Generator	Bio-Rad	1863009

### 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.

### ddPLEX *ESR1* 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 *ESR1* Wild-Type and Mutant Assays vary slightly but have similar workflows. Both assays can be run with the same negative and positive controls and on the same plate. See Figure 1 for an overview of the workflow.

**Fig 1. Overview of *ESR1* mutation detection workflow.**

## Protocol

### Sample Considerations

The sample input into the ddPLEX *ESR1* Mutant Assay may be 2–10  $\mu$ l and 2–132 ng. Quantification of samples by quantitative PCR (qPCR) or ddPCR is recommended. Note that higher sensitivity can be obtained with input amounts at the higher end of the range but amounts above 132 ng should be avoided as this increases the risk of false positives. An input of 0.8–53 ng with a volume of 2  $\mu$ l is recommended for the wild-type assay to conserve sample volume while allowing for pipetting accuracy. Refer to Table 9 for a summary of the input amounts for each assay.

**Table 9. Input amounts for the ddPLEX *ESR1* Assays.**

Assay	Sample Input Volume, $\mu$ l	Sample Input Amount, ng
Mutant	2–10 $\mu$ l	2–132 ng
Wild-type	2 $\mu$ l	0.8–53 ng

### Reagent Preparation

Multiple freeze-thaws should be avoided for DTT, so it is recommended to thaw and aliquot in volumes appropriate for single use. Refer to Tables 10 and 11 for the volume required per reaction. The Positive Control and Internal Control contain DNA and should not be taken into any DNA template-free areas.

### Reaction Setup

- Determine the number of controls and samples to be tested prior to setting up the reaction mixes. It is recommended to include the following controls: at least two positive controls (PC); two no template controls (NTC) consisting of nuclease-free water, sample elution buffer, or similar; and two negative controls, consisting of a known negative DNA sample (no *ESR1* mutation).

**Tip:** Steps 2–5 should be carried out in a DNA template-free area.

- Thaw all assay reagents and equilibrate to room temperature except for MseI (if used, see note), which should be left on ice or in a cold block during preparation.

**Note:** MseI is optional but may be required for optimal performance with non-fragmented DNA such as from fresh frozen tissue samples and with FFPE DNA.

Mix all tubes (except for MseI) thoroughly by vortexing at maximum speed for 10 sec to ensure homogeneity because a concentration gradient may form during  $-20^{\circ}\text{C}$  storage. For MseI, flick the tube to mix. Centrifuge all tubes briefly to collect the contents at the bottom of each tube.

**Tip:** Thorough vortexing (maximum speed for 10 sec) of each component at this step is essential.

- The reagents and volumes for one reaction of the mutant assay are shown in Table 10. Calculate the volumes required for a master mix based on the total number of controls and samples to be tested. It is recommended to add an overage amount of 12–15% to account for losses during pipetting. Table 10 also shows an example of calculated volumes for 24 reactions with 15% overage. Assemble the mutant master mix including all reagents in Table 10, except for the internal control DNA and samples. These components will be added in subsequent steps.

**Table 10. Preparation of the mutant assay master mix.**

Component	Volume per Reaction, $\mu$ l	
	1 Well	Volume per 24 Reactions + Overage <sup>1</sup> , $\mu$ l
Nuclease-free water	7.72 (adjust as needed to bring total volume to 22 $\mu$ l)	213
ddPCR Multiplex Supermix	5.5	151.8
ddPLEX Enhancer	0.35	9.7
DTT	0.23	6.5
MseI, 50 U/ $\mu$ l (optional)	0.1	2.6
ddPLEX <i>ESR1</i> Mutant Assay	1.1	30.4
ddPLEX Internal Control	2	55.2
Sample	5 (or up to 10 $\mu$ l)	
Total Volume	22	

<sup>1</sup> Prepared with 15% overage to account for liquid loss during pipetting steps.

- Vortex the master mix thoroughly at maximum speed for at least ten seconds. Centrifuge briefly to collect the contents at the bottom of the tube.

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

- The reagents and volumes for one reaction of wild-type assay are shown in Table 11. Calculate the volumes required for a master mix based on the total number of controls and samples to be tested. It is recommended to add an overage amount of 12–15% to account for losses during pipetting. Table 11 also shows an example of calculated volumes for 24 reactions. Assemble the wild-type master mix including all reagents in Table 11, except for the samples.

**Table 11. Preparation of the wild-type assay master mix.**

Component	Volume per Reaction, $\mu$ l	
	1 Well	Volume per 24 Reactions + Overage <sup>1</sup> , $\mu$ l
Nuclease-free water	13.07 (adjust as needed to bring total volume to 22 $\mu$ l)	360.6
ddPCR Multiplex Supermix	5.5	151.8
DTT	0.23	6.5
MseI, 50 U/ $\mu$ l (optional)	0.1	2.6
ddPLEX <i>ESR1</i> Wild-Type Assay	1.1	30.4
Sample	2	
Total volume	22	

<sup>1</sup> Prepared with 15% overage to account for liquid loss during pipetting steps.

**Table 12. Example plate map for testing controls in duplicate and 18 samples in singlet.**

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

- Vortex the master mix thoroughly at maximum speed for at least ten seconds. Centrifuge briefly to collect the contents at the bottom of the tube.
- Move to an area appropriate for handling DNA materials. The ddPLEX Internal Control and samples should be completely thawed, vortexed briefly (three to five seconds), then centrifuged prior to use.
- Add the ddPLEX Internal Control volume (calculated in Step 3) only to the mutant assay master mix. Vortex the master mix thoroughly at maximum speed for at least five seconds. 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 sub-portion of the master mix, specifically for NTC wells, can be set aside before adding the ddPLEX Internal Control, keeping in mind that additional nuclease-free water will be needed to adjust for the missing volume.
- Dispense each assay master mix into a 96-well plate. Table 12 shows an example plate layout for 24 reactions of mutant and wild-type assays. A repeater pipette 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 and 5 µl sample volumes, dispense 17 µl of the master mix per well. For the wild-type assay, dispense 20 µl of the master mix per well for 2 µl sample volumes.
- For the mutant assay, 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.
- For the wild-type assay, 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 pipette is recommended to prevent cross-contamination from well to well.
- If there are any unused wells in a column for which 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.
- Cover the plate with a pierceable foil PCR Plate Seal and seal the plate using the PX1 PCR Plate Sealer at 180°C for 5 sec. Vortex the plate thoroughly at maximum speed at three different positions of the plate for at least 5–10 seconds per position to ensure thorough mixing. Centrifuge the plate for 1 min at 1,150 rcf. Visually verify that all the liquid is at the bottom of the wells.
- Proceed to droplet generation using either the QX200 Droplet Generator or the AutoDG Instrument.

### Droplet Generation

#### For the Manual Droplet Generator:

- 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.
- Load 70 µl of Droplet Generation Oil for Probes into the oil wells. For detailed instructions on manual droplet generation, refer to the QX200 Droplet Generator Instruction Manual (10031907).

- After droplet generation, carefully transfer 40 µl of the droplet emulsions into a clean ddPCR 96-Well Plate using a P50 or P200 multichannel pipette. Cover the plate with a foil, pierceable PCR Plate Seal and seal the plate using the PX1 PCR Plate Sealer at 180°C for 5 sec. Proceed immediately to thermal cycling.

#### For the AutoDG Instrument:

- Place the sealed plate in the Automated Droplet Generator and follow the instructions in the Automated Droplet Generator for Droplet Digital PCR Systems Instruction Manual (10043138).
- Once droplet generation is complete, cover the plate with a pierceable foil PCR Plate Seal and seal the plate using the PX1 PCR Plate Sealer at 180°C for 5 sec. Proceed immediately to thermal cycling.

### Thermal Cycling

- Place the plate into the C1000 Touch Thermal Cycler or PTC Tempo Deepwell Thermal Cycler.
- Use the thermal cycling conditions given in Table 12. Set the heated lid to 105°C and the sample volume to 40 µl and start the run.
- Ensure that before ending the run, the plate is held at 4°C for at least 30 minutes or until the lid temperature is 37°C or below. If desired, the plate may be moved out of the thermal cycler and into a refrigerator for incubation after this hold time and stored overnight in the refrigerator.

**Table 13. ESR1 mutant & wild-type assay cycling conditions.**

Cycling Step	Temperature, °C	Time	Number of Cycles	Ramp Rate
Enzyme activation	95	10 min	1	2°C/sec
Denaturation	95	15 sec	37	
Annealing/extension	66	1 min		
Enzyme deactivation	98	10 min	1	
Hold	4	∞*	1	None

\* The plate should be held at 4°C for at least 30 minutes or until the lid temperature is 37°C prior to ending the run.

### Data Acquisition

- After thermal cycling is complete, 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.
- In the Plate Information tab, enter a plate name and select ddPCR Multiplex Supermix from the dropdown menu.
- In the Well Selection tab, select the wells to include and exclude for reading.
- The run may be started by pressing Start Run or optionally, wells can be configured in the Well Information tab using the settings in the next section (Data Analysis).

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

### Plate Setup

- In the Plate Editor tab, select all wells of the plate and set the Experiment Type to Direct Quantification (DQ) if not already set. Click the Apply button if the value was changed.
- Select all wells in which the mutant assay was run and input the Assay Type as Advanced Classification Method.
- Input the following information under Target Info:

Target Name	Target Type	Signal Ch1	Signal Ch2	Signal Ch3	Signal Ch4	Signal Ch5	Signal Ch6
E380Q	Linkin	FAM 1	None	None	None	None	None
D538G	Linkin	FAM 2	HEX 1	None	None	None	None
V537S	Linkin	FAM 3	HEX 2	None	None	None	None
L336R	Linkin	None	HEX 3	None	None	None	None
V537N	Linkin	None	None	Cy5 1	None	None	None
V537C	Linkin	None	None	Cy5 2	Cy5.5 1	None	None
S463P	Linkin	None	None	None	None	ROX 1	None
IC	Linkin	None	None	None	None	None	ATTO590 1

**Fig. 2. Example of labeling and assigning channels to targets for mutant assay.**

- Click Apply to save the settings. Ignore the warning about using the Advanced Classification Method.
- Select all wells in which the wild-type assay was run and input the Assay Type as Single Target per Channel.
- Input the following information under Target Info:

Target Name	Target Type	Signal Ch1	Signal Ch2	Signal Ch3	Signal Ch4	Signal Ch5	Signal Ch6
S3X0WT	Linkin	FAM	None	None	None	None	None
S300WT	Linkin	None	HEX	None	None	None	None
A630WT	Linkin	None	None	Cy5	None	None	None
Empty	Linkin	None	None	None	Cy5.5	None	None

**Fig. 3. Example of labeling and assigning channels to targets for wild-type assay.**

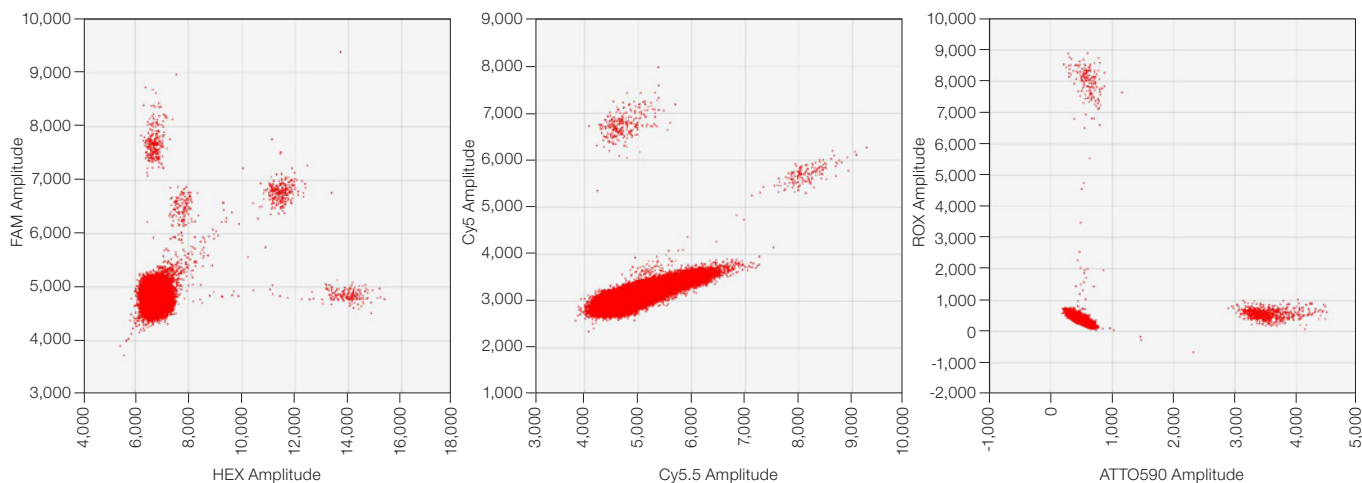
**Note:** The “Empty” target assigned to the Cy5.5 channel is required for generating 2-D plots for the Cy5 and Cy5.5 channels.

- Click Apply to save the settings.
- Sample information may be entered for single wells or multiple wells simultaneously by selecting the well(s) and entering information in the Sample Description fields. The Apply button must be clicked each time values are entered to save them.

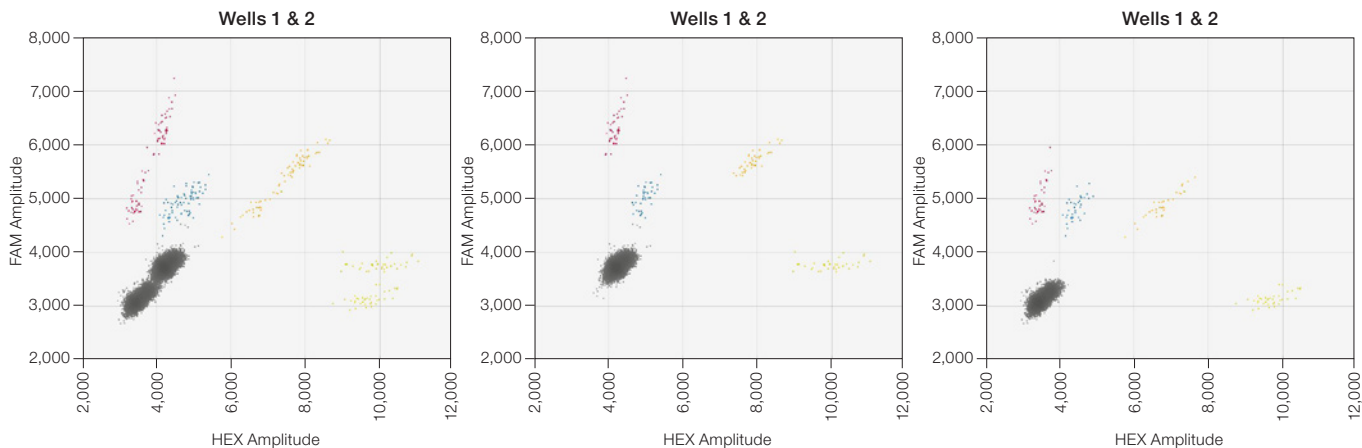
**Initial Quality Check**

1. 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.
2. Select all wells in which the mutant assay was run. Navigate to the 2-D Amplitude tab and examine the wells for uniformity. See Figure 4 for an example of 2-D plots for

27 wells overlaid. Any wells with shifted clusters, streaks, or any unusual features should be excluded from analysis. Figure 5 shows an example of shifted clusters, where wells 1 and 2, when overlaid, demonstrate duplicated clusters and individually, well 2 has different cluster positions compared to well 1 (and the other wells on the plate, not shown). Figure 6 is a plot of a valid well (well 1) and a well with a streak indicative of shredded droplets (well 2).



**Fig. 4. 2-D plots for 27 wells with the mutant assay.**



**Fig. 5. 2-D plots demonstrating one mutant assay well with shifted clusters.**

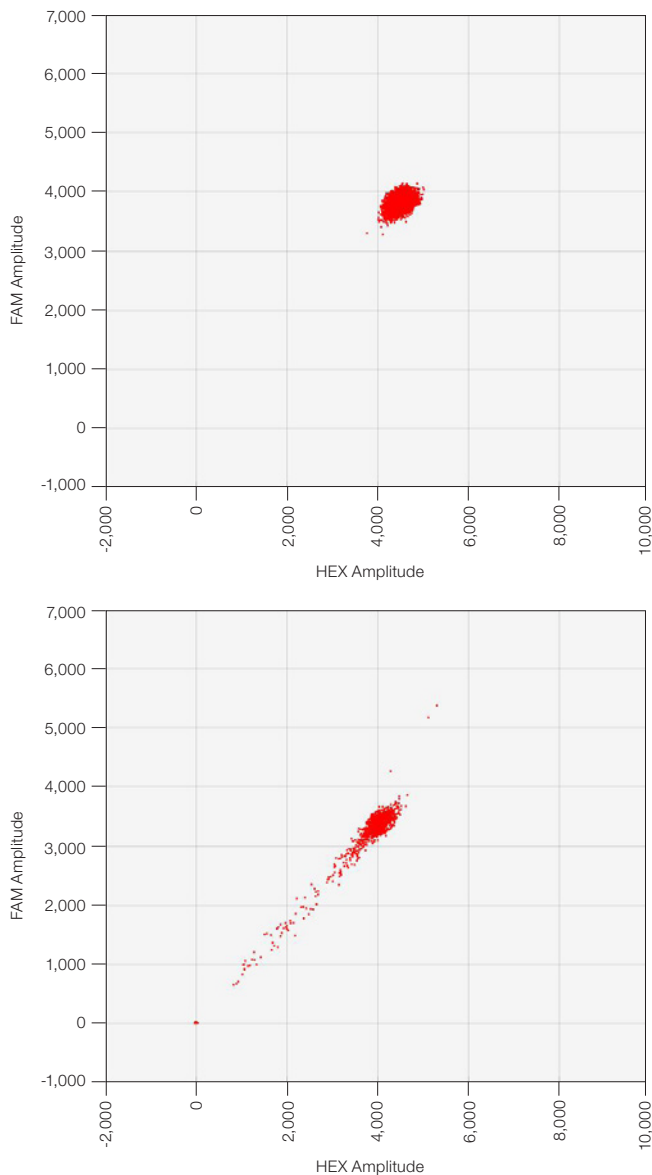


Fig. 6. 2-D plot of one valid well and one well with droplet shredding.

3. Examine all wells for the internal control cluster in the ATTO590 channel. If the internal cluster is not present, this could indicate the presence of inhibitors in the reaction. Such wells should be excluded from further analysis.

**Threshold Setting for Mutant Assay**

1. From the tool bar, select Threshold Cluster Mode (pencil icon) as shown in Figure 7.



Fig. 7. Thresholding tool bar with pencil icon outlined in orange.

2. It is recommended to set thresholds for all wells with the mutant assay together, which helps to ensure consistency of positive and negative droplet calls across the plate. Begin by selecting all mutant assay wells. Press and hold the Control key to select non-contiguous regions of the plate together.
3. Starting with the FAM/HEX (Ch1/Ch2) plot, draw circles around each positive cluster using the pencil tool. In the pop-up menu that appears after selecting a region, click on the radio buttons to set the cluster assignment and click "Assign Cluster" as shown in Figure 8. Each positive cluster should be set as positive for the appropriate target and negative for the others. Refer to Figure 9 for the cluster designations. The positive control wells are good indicators of the positions of each cluster. It is recommended to threshold relatively tightly around the densest cluster regions.

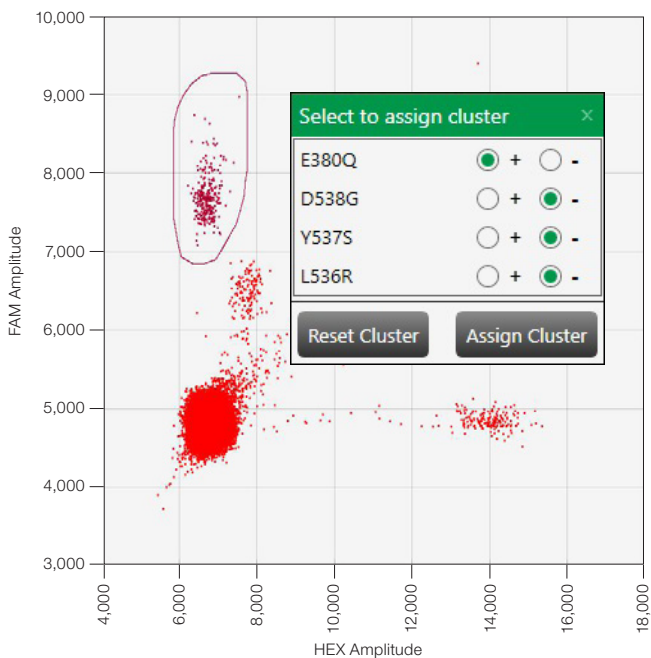


Fig. 8. Assigning clusters.

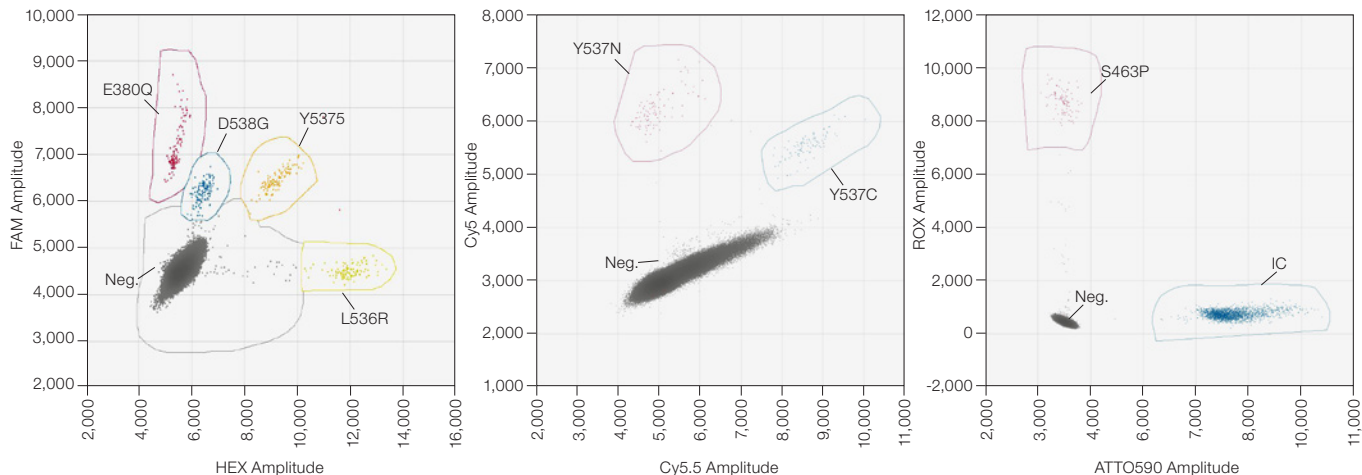


Fig. 9. Cluster assignments for the ESR1 mutant assay.

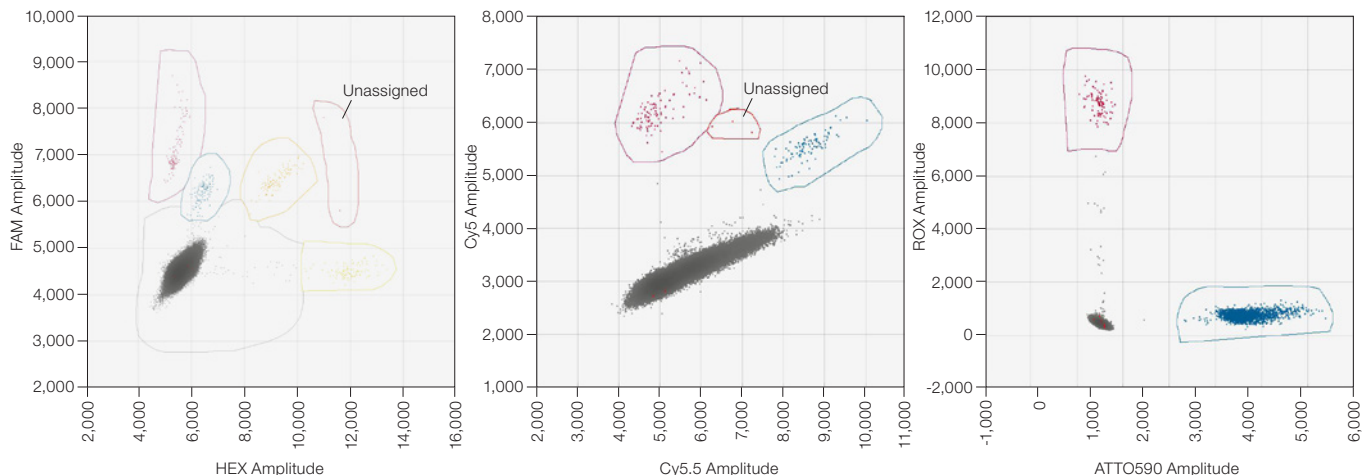


Fig. 10. Examples of unassigned droplets.

4. For the Cy5/Cy5.5 (Ch2/Ch3) and ROX/ATTO590 (Ch4/Ch5) plots, assign the positive clusters as done for the FAM/HEX plot.
5. Continuing with the FAM/HEX plot, use the pencil tool to surround the negative cluster and the lower amplitude droplets lying in between the positive clusters and the negative cluster. In the pop-up menu, set each target as negative then click on “Assign Cluster”. Once negative droplets are assigned in the FAM/HEX plot, all the droplets in the other plots except those left as unassigned will be classified as negative.
6. Droplets with high fluorescence amplitude that are distinct from the dense positive clusters may be left as unassigned, which removes them from analysis. If previously assigned to a cluster, they can be reset by circling the droplet(s) and clicking “Reset Cluster” in the pop-up menu. Unassigned droplets will have a bright red color. See Figure 10 for some examples.
7. In the ROX/ATTO590 plot, droplets positive for both the ROX and ATTO590 channels that are orthogonal to the S463P and IC positive clusters should be assigned as positive for both S463P and IC.

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



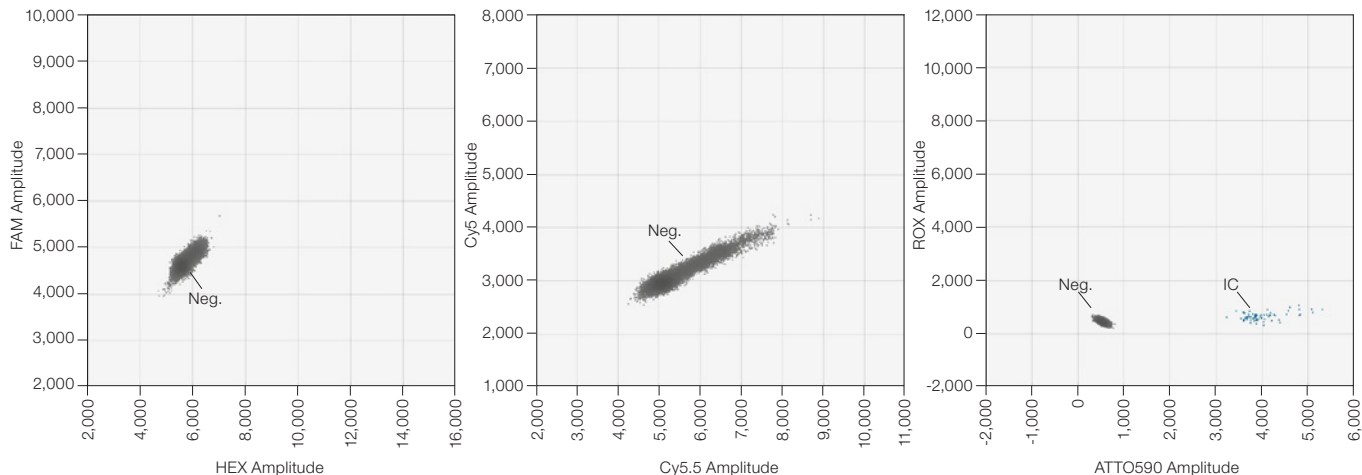


Fig. 11. 2-D plots for 1 well of no template control (NTC).

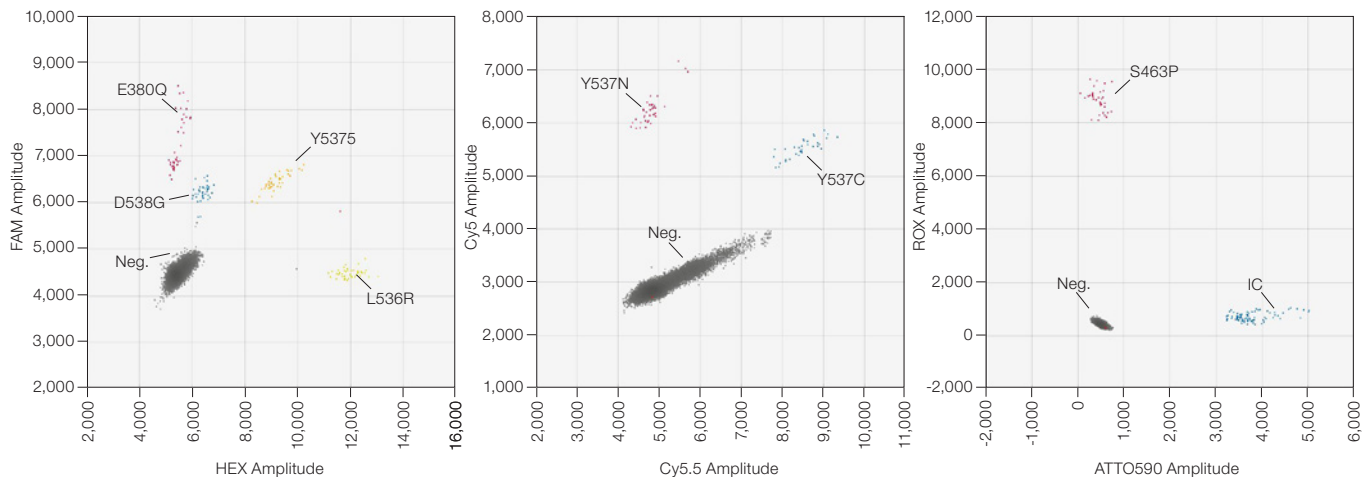


Fig. 12. 2-D plots for 1 well of positive control.

**Mutant Assay Quality Check**

1. Examine the NTC wells to detect potential contamination. The ddPLEX Internal Control cluster will appear in the ATTO590 channel. Ideally, all other droplets should be classified as negative. Tolerance to positive droplets should be determined by the end-user. The recommended allowable range is 0–2 positive droplets per target. See Figure 11 for an example 2-D plot.

2. Observe the positive control wells to ensure that each plot has the expected negative and positive clusters. Refer to Figure 12 for an example 2-D plot for one positive control well.

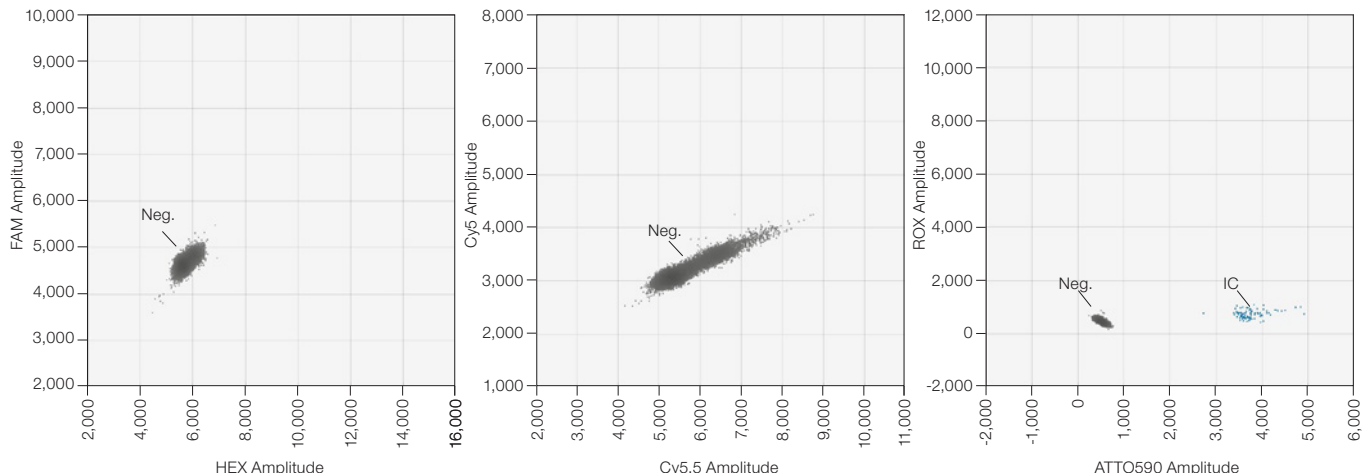


Fig. 13. 2-D plot for 1 well of wild-type negative control.

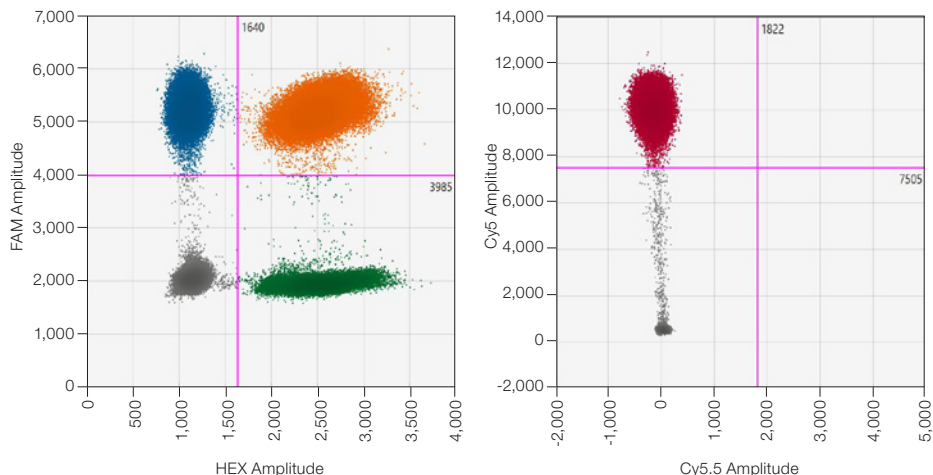


Fig. 14. Example of crosshair thresholds for the ESR1 wild-type assay.

3. Examine the wild-type negative control samples. A positive cluster for the IC is expected. 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. Refer to Figure 14 for an example 2-D plot for one wild-type negative control well. Tolerance to positive droplets should be determined by the end-user.
4. 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.
2. Select all wild-type assay wells in order to set a consistent threshold across the plate. Press and hold the Control key to select non-contiguous regions of the plate together.
3. Starting with the FAM/HEX plot, use the crosshairs tool to click in the center of the four-cluster quadrant. The bright pink thresholding lines that appear can be moved vertically and horizontally to make adjustments. It is recommended to set the threshold close to the positive clusters, leaving any minor clusters present between the positive cluster and the main negative clusters as negative. Refer to Figure 15 as an example of thresholds set with the line tool and for cluster assignments.

**Threshold Setting for Wild-Type Assay**

1. From the tool bar, select Threshold Line Mode (crosshairs icon without “SD”) as shown in Figure 15.



Fig. 15. Thresholding tool bar with crosshairs icon outlined in orange.

4. In the remaining 2-D plot for Cy5/Cy5.5, use the same line tool to threshold the remaining clusters. Use Figure 15 as a guide. Note that the positive control produces a minor cluster between the negative and positive clusters in the Cy5 channel. It is recommended to set the threshold above the position of this minor cluster.

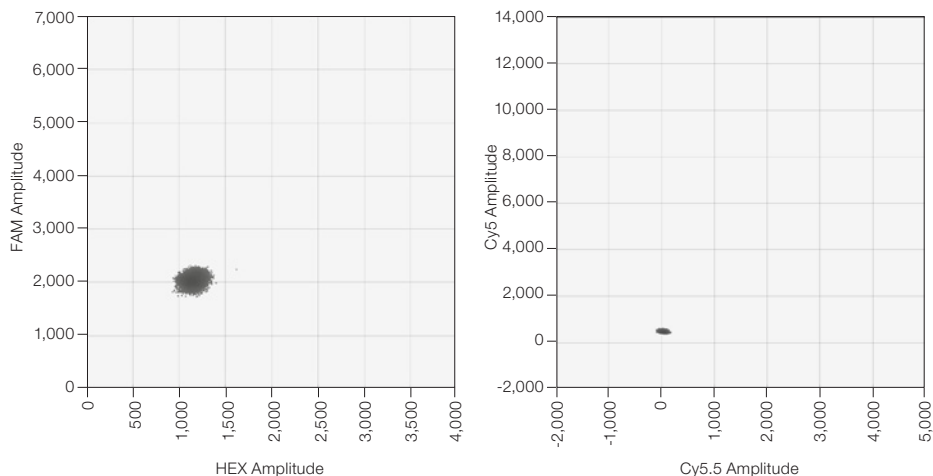


Figure 16. 2-D plots for 1 well of no template control.

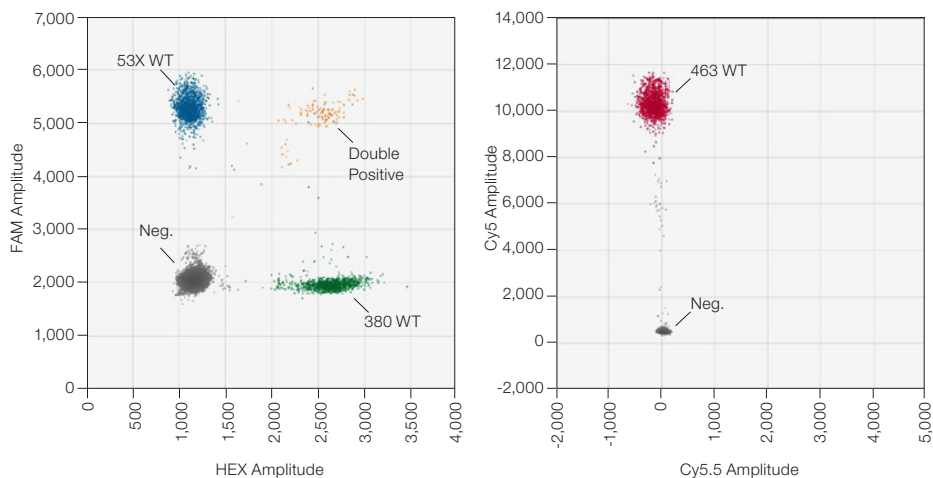


Fig. 17. 2-D plots for 1 well of positive control.

**Wild-Type Assay Quality Check**

1. Examine the 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. The recommended allowable range is 0–2 positive droplets per target. See Figure 16 for example 2-D plots.
2. Observe the positive control wells to ensure that each plot has the expected negative and positive clusters. Refer to Figure 17 for an example 2-D plot for one well of positive control.
3. Inspect the replicate sample wells for uniformity and note any problematic wells, which should be excluded from analysis. If any clusters are cut off by the thresholds, the thresholds should be manually adjusted.
4. After the thresholding is complete, save the data file.

**Data Export**

1. In the Data Table tab, select the dropdown menu in the upper right corner. Click on Export to CSV... and save the file. Refer to Figure 18 to locate the dropdown menu.

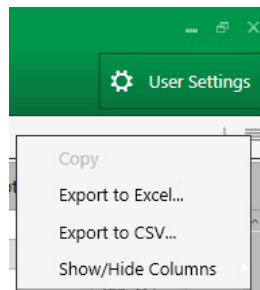


Fig. 18. Data table dropdown menu.

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

	A	D	E	F	G	U	V	W
1	Well	Sample description 1	Sample description 2	Target	Conc (copies/μL)	Accepted Droplets	Positives	Negatives
2	A01	NTC	ESR1 Mutant Assay	E380Q	0	17376	0	17301
3	A01	NTC	ESR1 Mutant Assay	D538G	0	17376	0	17301
4	A01	NTC	ESR1 Mutant Assay	Y537S	No Call	17376	0	0
5	A01	NTC	ESR1 Mutant Assay	L536R	0	17376	0	17301
6	A01	NTC	ESR1 Mutant Assay	Y537N	0	17376	0	17301
7	A01	NTC	ESR1 Mutant Assay	Y537C	0	17376	0	17301
8	A01	NTC	ESR1 Mutant Assay	S463P	0	17376	0	17376
9	A01	NTC	ESR1 Mutant Assay	IC	5.44	17376	75	17301

Fig. 19. Example of No Call Result.

### 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 threshold values for each target.
- For QX Manager Software, Standard Edition, v2.1, a “No Call” result may be present instead of a concentration value for some targets in the column with concentration results (see cell highlighted orange in the example below, Figure 19). If the number of “Positives” for the affected target(s) is 0 (see cell highlighted yellow in the example) and other targets in the same well have values reported for the concentration, the “No Call” result may be replaced with a 0 value.
- Verify that the ddPLEX Internal Control has a positive reported concentration for all samples. The concentration observed in internal testing ranged from 2.5–7 copies/μl in individual reactions, but end users should establish a suitable acceptance range for their laboratories.
- Determine which of the mutant targets are above the pre-determined background thresholds for each sample tested.
- If replicate samples were tested, the mean concentration of positive targets can be calculated.
- Internal testing of the ddPLEX Positive Control, with a 5 μl input into the mutant assay and 2 μl input into the wild-type assay, has produced values in the range of 1–5 copies/μl for mutant targets and 50–120 copies/μl for wild-type targets. Observed values may vary and end users should establish expected ranges for their own laboratories.

### Calculation of Allele Frequencies

- For the mutant assay wells, use the reported concentration (copies/μl) to calculate the original sample input concentration for each target using the following formula:

$$\text{Original sample concentration (cp/}\mu\text{l)} = \frac{(\text{Reported concentration (cp/}\mu\text{l)}) \times \text{Reaction volume (}\mu\text{l)}}{(\text{Sample input volume (}\mu\text{l)})}$$

Example for sample positive for E380Q:

$$\text{E380Q original sample concentration (cp/}\mu\text{l)} = \frac{2.5 \text{ cp/}\mu\text{l} \times 22 \mu\text{l}}{5 \mu\text{l}}$$

$$\text{E380Q original sample concentration (cp/}\mu\text{l)} = 11 \text{ cp/}\mu\text{l}$$

- For the wild-type assay wells, use the reported concentration (copies/μl) to calculate the original sample input concentration using the following formula. Note that the sample input volume may have been different between the mutant and wild-type assays.

$$\text{Original sample concentration (cp/}\mu\text{l)} = \frac{(\text{Reported concentration (cp/}\mu\text{l)}) \times \text{Reaction volume (}\mu\text{l)}}{(\text{Sample input volume (}\mu\text{l)})}$$

Example:

$$\text{380\_WT original sample concentration (cp/}\mu\text{l)} = \frac{99 \text{ cp/}\mu\text{l} \times 22 \mu\text{l}}{2 \mu\text{l}}$$

$$\text{380\_WT original sample concentration (cp/}\mu\text{l)} = 1,089 \text{ cp/}\mu\text{l}$$

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

$$\text{Allele frequency} = \frac{\text{Average mutant allele concentration (cp/}\mu\text{l)}}{\text{Sum of all average mutant and wild-type allele concentrations for the same exon}} \times 100\%$$

Example for sample positive for E380Q:

$$\text{E380Q allele frequency} = \frac{11 \text{ cp/}\mu\text{l}}{11 \text{ cp/}\mu\text{l} + 1,089 \text{ cp/}\mu\text{l}} \times 100\%$$

$$\text{E380Q allele frequency} = 1.0\%$$

**Notes:** For samples that are positive for multiple variants in the same exon, all positive variant concentrations should be summed with the wild-type allele concentration in the denominator. Refer to Table 1 for the corresponding wild-type allele for each variant.

For the positive control, the allele frequency should be around 1–1.5% for each variant.

### Troubleshooting Tips

- Wells with shifted clusters: A high frequency of wells showing shifted clusters (see Figure 5) may indicate insufficient vortexing of reaction components or the reaction mix. Carefully follow the vortexing guidelines in the procedure.
- Low droplet numbers: Exclude wells with low droplet counts (<10,000) from analysis. Ensure that after thermal cycling, the plate is held at 4°C for at least 30 minutes or until the lid temperature is 37°C. Increased hold times at 4°C may improve droplet numbers. If problem persists, call Technical Support.
- Low amplitudes: If positive clusters have low amplitudes and are difficult to separate from the negative cluster, this indicates poor amplification. Verify that the thermal cycling steps and ramp rates have been set according to this procedure. This could also indicate the presence of PCR inhibitors in the sample. Dilute the sample to decrease inhibitor concentration and repeat the test.
- Droplet shredding: Shredded droplets appear on the diagonal through the negative cluster (Figure 6). Exclude well from analysis or repeat the test. Ensure that PCR plates containing droplets are handled carefully.

Refer to the Droplet Digital PCR Application Guide (bulletin 6407) for additional troubleshooting tips.

Visit [bio-rad.com/ESR1](https://www.bio-rad.com/ESR1) for more information.

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