Vericheck ddPCR *Mycoplasma* Detection Kit

Catalog #	Description
12013126	**Vericheck ddPCR *Mycoplasma* Detection Kit**

For research purposes only.

Product Description

The Vericheck ddPCR *Mycoplasma* Detection Kit is is a probe-based solution used to detect common *Mycoplasma* species found in cell culture or cell media during biopharmaceutical manufacturing processes. This kit uses Droplet Digital PCR (ddPCR) technology, which provides quantitative results without the use of standard curves.

Kit Contents

See Table 1 for kit contents. The kit contains reagents for 100 x 20 µl reactions. Kits and components can be stored at –20°C for 2 years or at 4°C for up to 2 weeks.

Table 1. Kit contents.

Product	Product Cap Color
ddPCR Supermix for Residual DNA Quantification (2x)	Teal
ddPCR *Mycoplasma* Assay (20x)	Green
ddPCR *Mycoplasma* Internal Control	Red
ddPCR *Mycoplasma* Positive Control	Blue
Nuclease-free water	Clear

Required Equipment, Reagents, and Consumables

See Table 2 for required materials that are not included in the kit.

Table 2. Additional required materials.

Instrument	Consumables and Reagents*
▪ QX200 Droplet Generator (catalog #1864002) **or** Automated Droplet Generator (#1864101) ▪ QX200 Droplet Reader (#1864003) ▪ C1000 Touch Thermal Cycler with 96–Deep Well Reaction Module (#1851197) ▪ PX1 PCR Plate Sealer (#1814000)	▪ Droplet Generation Oil for Probes (#1863005) **or** Automated Droplet Generation Oil for Probes (#1864110) ▪ ddPCR Droplet Reader Oil (#1863004) ▪ ddPCR 96-Well Plates (#12001925) ▪ DG8 Cartridges (#1864008) **or** DG32 Automated Droplet Generator Cartridges (#1864108, 1864109) ▪ ddPCR Buffer Control for Probes (#1863052) ▪ PCR Plate Heat Seal (#1814040)
▪ QX ONE Droplet Digital PCR System (#12006536)	▪ QX ONE Droplet Generation Oil for Probes (#12006058) ▪ QX ONE Droplet Reader Oil (#12006057) ▪ ddPCR 96-Well Plates (#12001925) ▪ GCR96 Cartridges (#12006858, 12006859)

* Adjustable pipettors (Rainin or Eppendorf) and pipet tips (#1864120, 1864121) can be used with the consumables and reagents listed.

Sample Preparation

Perform DNA extraction with the QIAGEN QIAamp DNA Mini Kit or a user-preferred extraction procedure. For optimal performance, extracted samples should be eluted into a total volume of 100 µl.

Optional: While designed as an internal control for ddPCR Assay performance, the ddPCR *Mycoplasma* Internal Control can also be spiked into the sample prior to extraction to serve as a control for extraction efficiency. The percentage recovery of the internal control in the extracted sample is a proxy indicator of the extraction efficiency.

Tip: The internal control tube should be thawed to room temperature, vortexed at maximum speed for 15 sec, and added to the concentrated sample or sample pellet prior to extraction.

Important: The volume of internal control added is variable and is based on the final elution volume of the extracted sample. Use the following equation to calculate the volume of internal control to add to the sample prior to extraction:

$$U = E/9$$

where

U = volume of internal control to spike into the sample prior to extraction, µl

E = elution volume, µl

For example, for a 100 µl elution volume:

$$U = 100\ \mu l/9\ \mu l = 11\ \mu l$$

That is, for a 100 µl elution volume, 11 µl of internal control should be added to the sample prior to extraction.

Reaction Setup

1. Determine the plate layout prior to setting up the reaction mix. Each plate should have three positive control wells and three negative control wells. If the optional internal control was spiked in during sample extraction, each plate should have at least one internal control well (three internal control wells are recommended). If an internal control was not used during sample extractions, it is not necessary to add internal control wells to the plate. Three replicate wells per sample are recommended. An example plate configuration is shown in Figure 1.

Tip: Not all wells on the ddPCR 96-Well Plate need to be used or filled up. We recommend laying out the sample wells in columns as droplet generation occurs in columns. Any unused well from which droplets will be generated must be filled with ddPCR Buffer Control for Probes prior to droplet generation. If the entire column is unused, no buffer control is required in those wells.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Positive control	Positive control										
B	Positive control	Buffer control										
C	Internal control (optional)	Internal control (optional)										
D	Internal control (optional)	Buffer control										
E	Sample #1	Sample #1										
F	Sample #1	Buffer control										
G	Nuclease-free water (negative control)	Nuclease-free water (negative control)										
H	Nuclease-free water (negative control)	Buffer control										

Fig. 1. Example plate configuration for one sample and controls. This is an example layout. Columns 3–12 can be used to run additional samples.

- **Sample (with internal control):** Extracted DNA sample, which includes the internal control spiked in prior to extraction, is added to the ddPCR reaction. The *Mycoplasma* signal will be read in channel 1 (FAM) and the internal control signal will be read in channel 2 (HEX).
- **Positive control (ddPCR positive control):** Instead of a sample, positive control is added to the ddPCR reaction. The positive control is provided at 10,000 copies/well (1,111 copies/µl ± 20%) for the FAM channel and 20,000 copies/well (2,222 copies/µl ± 20%) for the HEX channel.
- **Internal control (necessary only if internal control is used during extraction of samples):** Instead of a sample, internal control is added to the ddPCR reaction. The internal control is provided at 20,000 copies/well (20,000 copies/µl ± 20%).
- **Nuclease-free water (ddPCR negative control):** Instead of a sample, nuclease-free water is added to the ddPCR reaction.

2. Thaw all kit components to room temperature. Mix all tubes thoroughly by vortexing at maximum speed for 15 sec to ensure homogeneity. Centrifuge briefly to collect contents at the bottom of each tube.

   Tip: Thorough vortexing (maximum speed for 15 sec) of each component at this step is essential for success.

3. If necessary, prepare samples to the desired concentration before setting up the reaction mix. Ensure samples are thoroughly mixed. Most extracted samples will not require dilution and can be added directly to the PCR reaction. The suggested input DNA is <100,000 copies/well.

4. Assemble a master mix containing all components, except the DNA sample or control, according to the master rmix setup guidelines in Table 3.

   Important: Mix the master mix thoroughly by vortexing the tube at maximum speed for 15 sec. Ensure there are no precipitates.

Table 3. Master mix and reaction mix volumes based on the number of wells.

	Number of Wells (wells + overage) Volume, µl			
Component	**Final 1x Volume for 1 Well***	**Volume for 1 Well* + Overage**	**Master Mix Setup for 12 Wells + Overage**	**Reaction Mix Tube Setup for 3 Wells + Overage**
ddPCR Supermix for Residual DNA Quantification (2x)	10	11	180	40
ddPCR *Mycoplasma* Assay (20x)	1	1.1	18	4
DNA sample or control	9	9.9		36
Total volume*	**20**	**22**	**198**	**80**

* The final 1x ddPCR reactions have a total volume of 20 µl. The reactions are prepared with overage to account for liquid loss during pipetting steps.

5. Prepare the reaction mix in a tube for each set of replicate wells and vortex thoroughly prior to transferring to the ddPCR 96-Well Plate. Each tube will contain enough reaction mix for three replicate samples or control wells.
   - Dispense 44 µl of master mix into each tube
   - Add 36 µl of the extracted DNA sample, positive control, or negative control (nuclease-free water) into the corresponding tube

     Important: For the internal control tube, add 4 µl internal control and 32 µl nuclease-free water.
   - Vortex each tube at maximum speed for 15 sec to mix thoroughly, and centrifuge briefly to collect the contents at the bottom of each tube

6. Dispense 22 µl of reaction mix into the wells of a ddPCR 96-Well Plate according to the plate layout created in step 1.

7. If necessary, add buffer control to unused wells from which droplets will be generated.
   - **For the QX200 and QX200 AutoDG Droplet Digital PCR Systems**, droplet generation occurs in columns. If necessary, add 22 µl of buffer control to any unused wells in a column from which droplets will be generated. If an entire column is unused, no buffer control is required in those wells

- **For the QX ONE ddPCR System**, droplet generation occurs in sets of 2 columns (for example, droplets for columns 1 and 2 are generated simultaneously). If necessary, add 22 µl of buffer control to any unused wells in each set of columns from which droplets will be generated

8. **Important:** Seal the ddPCR 96-Well Plate using the PX1 PCR Plate Sealer at 180°C for 5 sec, vortex at maximum speed for 15 sec, and centrifuge at 1,000 rcf for 1 min. Visually verify that all the liquid is at the bottom of the well.
9. Transfer the reaction mix from the sealed plate to the appropriate Droplet Generation Cartridge and generate droplets as follows:
   - **For the QX200 ddPCR System**, load 20 µl of each reaction mix into the sample wells of a DG8 Cartridge. Then load 70 µl of Droplet Generation Oil for Probes into the oil wells. For detailed instructions, refer to the QX200 Droplet Generator Instruction Manual (10031907)
   - **For the QX200 AutoDG ddPCR System**, place the sealed plate in the Automated Droplet Generator and follow instructions in the Automated Droplet Generator Instruction Manual (10043138)
   - **For the QX ONE ddPCR System**, load 20 µl of each reaction mix into the wells of a GCR96 Cartridge. Follow subsequent instructions for heat sealing, centrifuging, and loading the plate as specified in the QX ONE Droplet Digital PCR System and QX ONE Software User Guide (10000116512). Use the appropriate thermal cycling conditions as specified in Table 4

     Important: When setting up the plate template, first select the wells to include/exclude under the Exclude tab. Then move to the Edit tab and designate the following: Direct Quantification (DQ) as Experiment type, sample descriptions, Sample Type (ensure positive control wells are marked as Pos Ctrl), ddPCR Supermix for Residual DNA Quantification as the Supermix, Single Target per Channel as the Assay Type, Target Name(s), Unknown as Target Type, FAM for Signal Ch1, and HEX for Signal Ch2. Press Apply and then Save the template. Press Start Run.

Thermal Cycling

Follow the instructions for thermal cycling based on the system in use:

- **For the QX200 ddPCR System**, after droplet generation with the QX200 Droplet Generator, carefully transfer each column of the droplet emulsions into a clean ddPCR 96-Well Plate using a P50 multichannel pipettor. Seal the plate using the PX1 PCR Plate Sealer at 180°C for 5 sec. Proceed to thermal cycling. Use the appropriate thermal cycling conditions as specified in Table 4
- **For the QX200 AutoDG ddPCR System**, remove the droplet plate containing ddPCR droplets from the Automated Droplet Generator. Seal the plate using the PX1 PCR Plate Sealer at 180°C for 5 sec. Proceed to thermal cycling. Use the appropriate thermal cycling conditions as specified in Table 4
- **For the QX ONE ddPCR System**, thermal cycling is integrated into and sequentially performed by the system itself. Hence, no additional equipment or sample handling is required for this step

Table 4. Cycling conditions for the Bio-Rad C1000 Touch Thermal Cycler.*

Cycling Step		Temperature, °C	Time	Number of Cycles	Ramp Rate, °C/sec
Hold (QX ONE ddPCR System only)		25	3 min	1	2
Enzyme activation		95	10 min	1	
Denaturation		94	30 sec	40	
Annealing/extension		60	1 min		
Enzyme deactivation		98	10 min	1	
Hold	QX200 ddPCR System (optional)	4	∞	1	
	QX ONE ddPCR System (required)	25	1 min	1	

* For the C1000 Touch Thermal Cycler, use a heated lid set to 105°C and set the sample volume to 40 µl.

Data Acquisition

Follow the instructions for data acquisition based on the system in use:

- **For the QX200 and QX200 AutoDG ddPCR Systems**, place the sealed ddPCR 96-Well Plate in the QX200 Droplet Reader and set up the plate template. Under the Exclude tab, select the wells to include/exclude based on the plate configuration. Under the Edit tab, designate the following: Direct Quantification (DQ) as Experiment type, sample descriptions, Sample Type (ensure positive control wells are marked as Pos Ctrl), ddPCR Supermix for Residual DNA Quantification as the Supermix, Single Target per Channel as the Assay Type, Target Name(s), Unknown as Target Type, FAM for Signal Ch1, and HEX for Signal Ch2 (Figure 2). Press Apply and then Save the template. Press Start Run to begin droplet reading. Refer to the QX200 Droplet Reader and QX Manager Software Regulatory Edition (10000107224) User Guide if necessary
- **For the QX ONE ddPCR System**, data acquisition is integrated into and sequentially performed by the system itself. Hence, no additional equipment or sample handling is required for this step

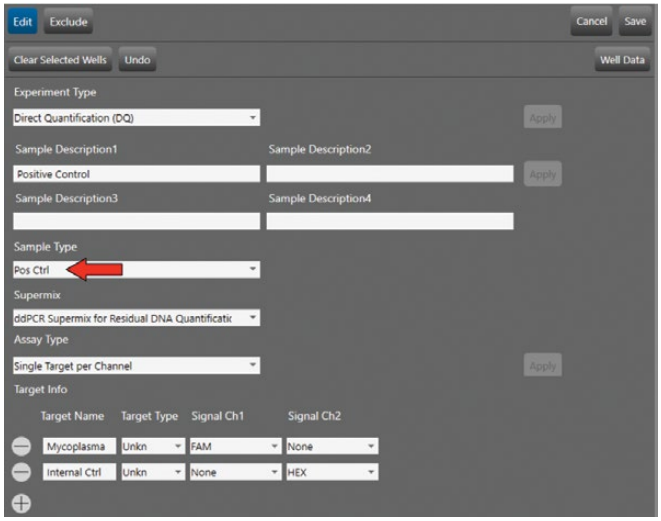

Fig. 2. Plate template setup example for a positive control well. It is important to select Pos Ctrl from the Sample Type dropdown menu (red arrow).

Data Analysis and Result Interpretation

The *Mycoplasma* signal is detected in the FAM channel (Figure 3A) and the internal control, if added, is detected in the HEX channel (Figure 3B). The concentration reported is copies/µl of the final 1x ddPCR reaction (Figure 4).

QX Manager Data Analysis: Positive Control–Based Autothresholding

QX Manager Software Regulatory Edition (#12012172) and QX Manager Software Standard Edition (#12010213) offer positive control–based autothresholding, which should be used for data analysis. Positive control–based autothresholding generates thresholds for each channel based on the currently selected wells marked as having the sample type Pos Ctrl. It then applies those thresholds to all currently selected wells. Refer to the QX200 Droplet Reader and QX Manager Software Regulatory Edition (10000107224) and QX200 Droplet Reader and QX Manager Software Standard Edition (10000107223) User Guides for detailed instructions about data analysis. See steps 1–3 for a quick guide to data analysis.

1. **Important:** Ensure that the positive control wells are marked as having the sample type Pos Ctrl. If necessary, navigate to the Plate Editor tab and select the positive control wells. Under Sample Type, use the dropdown menu to select Pos Ctrl and click Apply to mark the sample type for the selected wells. See Figure 2 for details.
2. Next, navigate to the 2D Amplitude tab and select all wells on the plate. Apply automatic thresholding by selecting Positive Control Wells (see Figure 5).

Important: This method of thresholding is accessible only if at least one of the selected wells has the sample type Pos Ctrl.

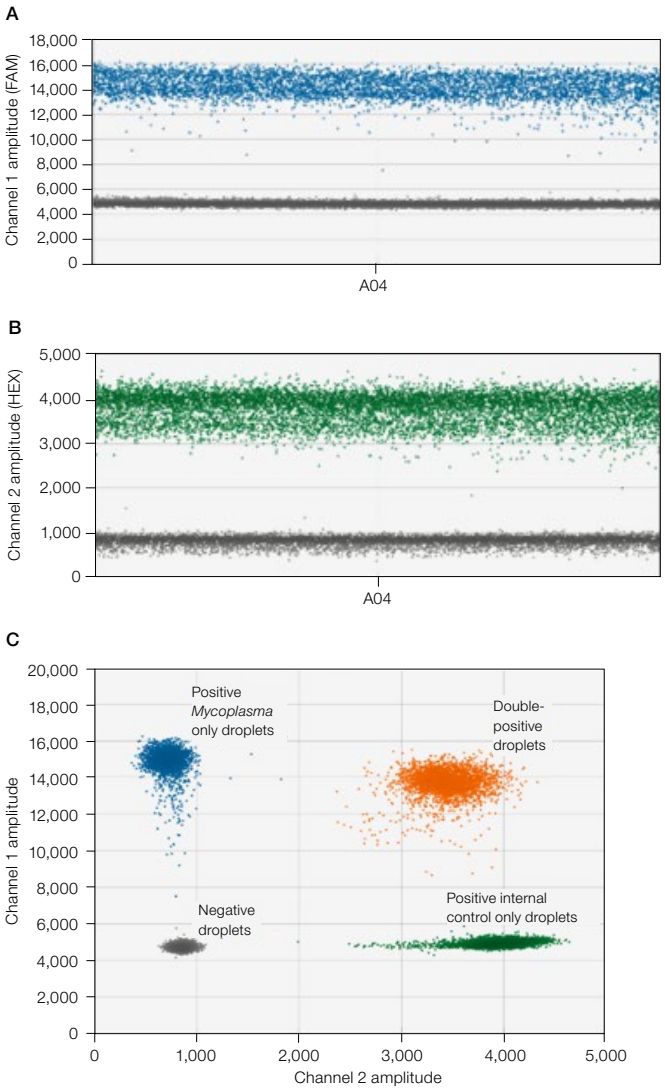

Fig. 3. 1-D and 2-D amplitude plots for positive control well. A, 1-D plot of a positive control well with *Mycoplasma* signal in channel 1 (FAM); **B**, 1-D plot of a positive control well with internal control signal in channel 2 (HEX); **C**, 2-D plot showing four droplet clusters. All positive control wells should have a signal in both channel 1 (FAM) and channel 2 (HEX). Data were analyzed in the QX Manager Software Regulatory Edition 1.2.

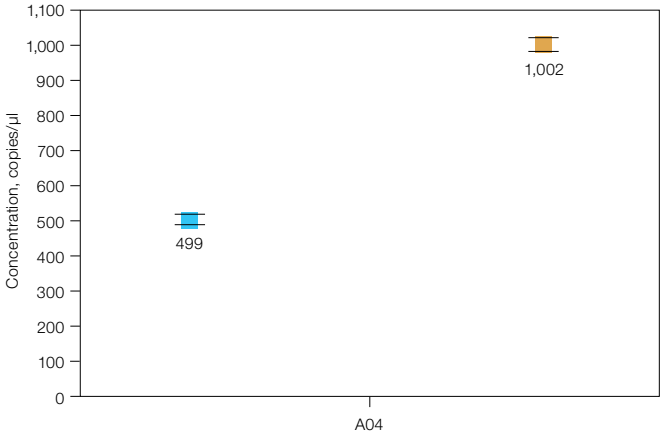

Fig. 4. Concentration plot for positive control well. *Mycoplasma* (channel 1) concentration in copies/µl (■); internal control (channel 2) concentration in copies/µl (■). For a positive control well, the channel 1 and 2 concentrations should both be between 200 and 2,000 copies/µl.

Fig. 5. Positive control–based autothresholding.

3. If automatic thresholding is unsatisfactory, follow the instructions in the QX ONE Data Analysis section to manually threshold the plate using the positive controls.

QX ONE Data Analysis: Positive Control–Based Manual Thresholding

1. Navigate to the 2D Amplitude tab and select all of the positive control wells. Manually threshold these wells using the pink manual thresholding tool on the 2D Amplitude tab (see Figure 6).

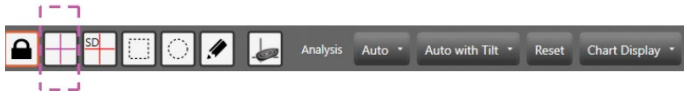

Fig. 6. Manual thresholding tool.

2. Copy the manual threshold applied to the positive control wells and paste it onto all other wells on the plate (that is, the sample, negative control, and internal control wells).

Tip: Refer to the QX ONE Software Regulatory Edition Instruction Manual (10000116656) and the Droplet Digital PCR Applications Guide (bulletin 6407) for detailed instructions and best practices for setting manual thresholds.

Result Interpretation

1. Navigate to the 2D Amplitude tab and examine the control wells. Ensure that all positive control wells have four distinct clusters (Figure 7A), all negative control wells have a single cluster (Figure 7B), and each internal control well has two clusters (Figure 7C).

   Tip: Examine the positive control wells for uniformity. If a positive control well does not have four distinct clusters, the quality of automatic thresholding may be impacted. Deselect the poor-quality positive control well(s) and threshold the plate again using Positive Control Well autothresholding. Only one positive control well is required for accurate thresholding.

2. Examine the replicate sample wells for uniformity and examine any outliers for problems. Do not include problem wells in the final analysis.

3. Navigate to the Event Counts tab.

   Recommended best practice: If the droplet count is <10,000 in any well, fail that well. If more than 5% of the wells on the plate have low droplet counts, fail the plate and refer to the Troubleshooting section.

4. Next, navigate to the Concentration tab and examine the control wells.

   Recommended best practice: Positive control wells should have a channel 1 (FAM) concentration between 200 and 2,000 copies/µl and a channel 2 (HEX) concentration between 200 and 2,000 copies/µl. Negative control wells should have channel 1 (FAM) and channel 2 (HEX) concentrations of 0 copies/µl. See Figure 4 for an example concentration plot.

5. On the Concentration tab, examine each sample's replicate wells for uniformity. If all wells have similar concentrations, merge the wells for the final analysis (see Figure 8). Refer to the QX200 Droplet Reader and QX Manager Software Regulatory Edition (10000107224) or QX200 Droplet Reader and QX Manager Software Standard Edition (10000107223) User Guide or the QX ONE Software Regulatory Edition Instruction Manual (10000116656) for a detailed explanation of merged wells.

Fig. 8. Merged well selection from the Concentration tab.

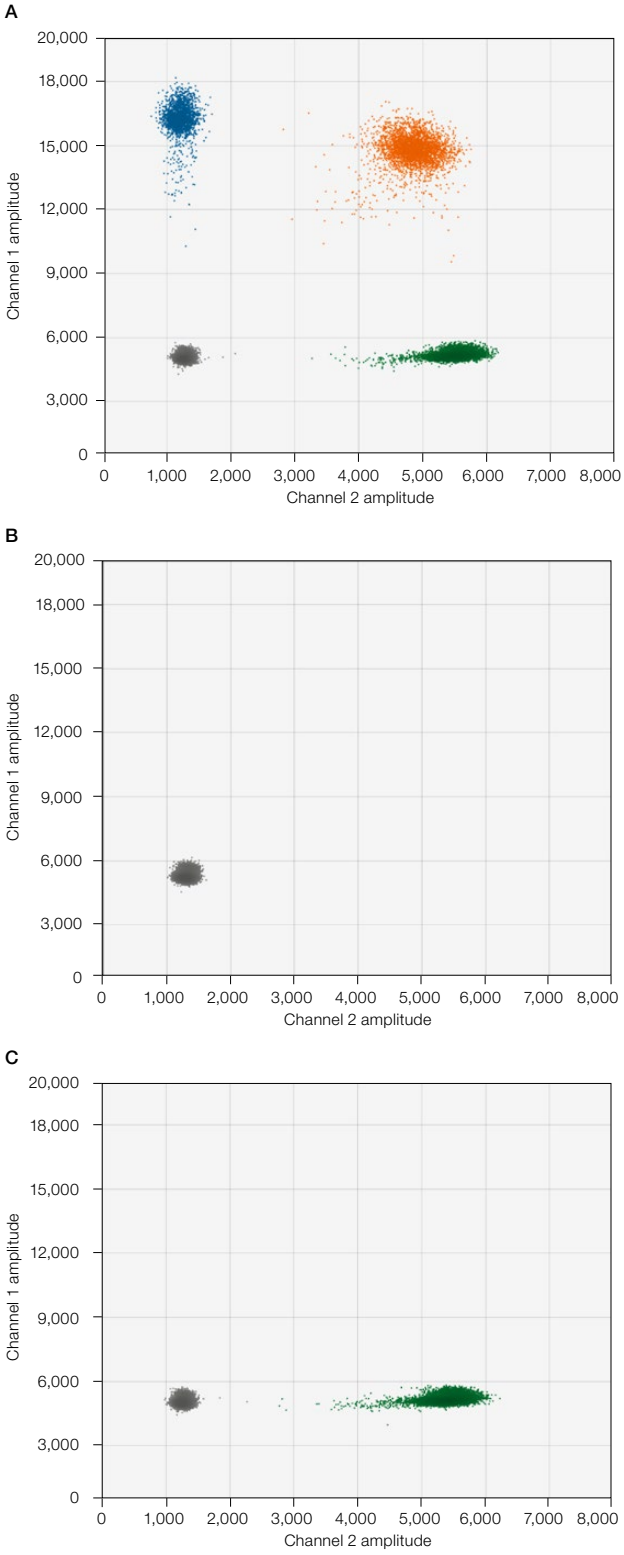

Fig. 7. Example 2-D amplitude plots for each of the control wells. A, all positive control wells have four clusters; **B**, all negative control wells have a single negative droplet cluster; **C**, all internal control wells have two clusters: a negative droplet cluster and a positive internal control only droplet cluster.

6. Sample concentrations and 2-D plots can be used to determine if the sample is *Mycoplasma* positive. Table 5 can be used as a guide to interpret merged well results.

Table 5. Vericheck ddPCR *Mycoplasma* Detection Kit merged well results interpretation guide.

Channel 1 (FAM) Result	Interpretation
Negative (single well) <0.1 copies/µl <2 genome copies (GC)/well* <2 positive droplets	*Mycoplasma* not detected
Negative (three wells merged) <0.033 copies/µl <0.66 GC/well* <2 positive droplets	
Positive (single well) ≥0.1 copies/µl ≥2 GC/well* ≥2 positive droplets	*Mycoplasma* detected
Positive (three wells merged) ≥0.033 copies/µl ≥0.66 GC/well* ≥2 positive droplets	

* GC/well = copies/µl x 20.

7. **Optional:** Examine each sample's merged well channel 2 (HEX) concentration. Also examine the internal control merged well channel 2 (HEX) concentration. The following formula can be used to determine percentage recovery of each sample. The percentage recovery of the internal control is an indication of the efficiency of extraction for each sample.

   R = (S/C) x 100%

   where

   R = percentage recovery

   S = sample well channel 2 (HEX) concentration, copies/µl

   C = internal control well channel 2 (HEX) concentration, copies/µl

Troubleshooting

This section lists some common failure modes with their phenotypes, descriptions, and suggested resolution. For a complete list of failure modes, refer to the Droplet Digital PCR Applications Guide (bulletin 6407) and the instruction manual of the instrument.

No Negative Droplets

Only high-amplitude clusters are present in a sample well. The sample is too concentrated (Figure 9) and is outside of the ddPCR dynamic range.

Resolution: Exclude well from analysis, dilute sample, and test again.

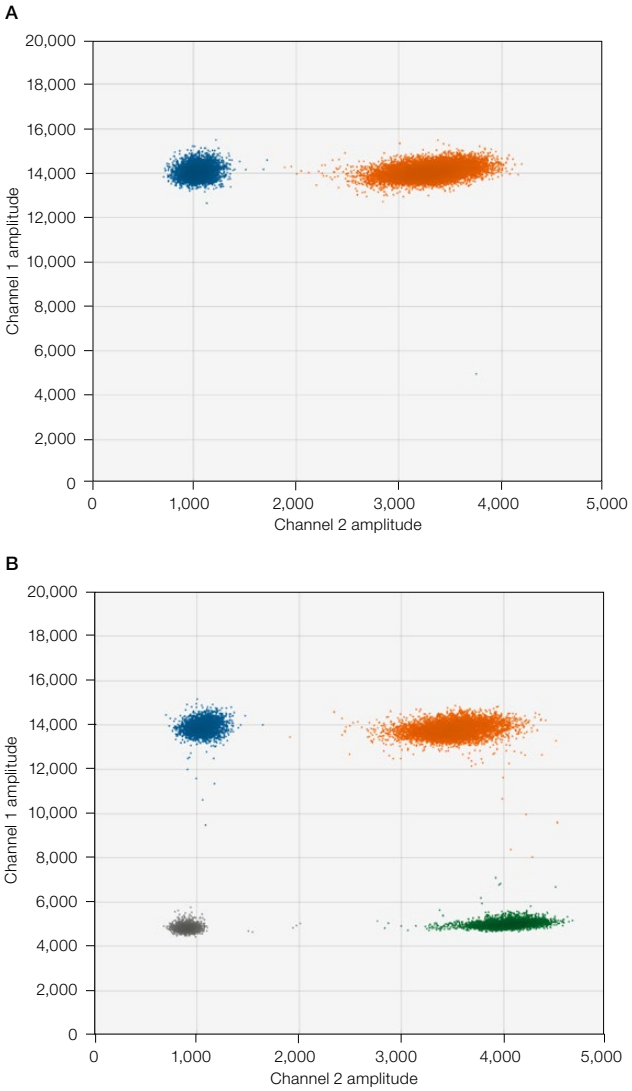

Fig. 9. No negative droplets. A, problem well; **B**, normal well.

No or Low Total Droplet Counts in Only Sample Wells

Multiple replicate sample wells have a droplet count <10,000 (Figure 10). If control wells have droplet counts >10,000, the issue is likely caused by inhibitors carried over from the starting matrix or extraction.

Resolution: Exclude sample wells with low droplet counts from analysis. If all replicate sample wells have low total droplet counts, dilute the sample to decrease inhibitor concentration and repeat Droplet Digital PCR. Alternatively, further purify the DNA sample to eliminate inhibitors.

No or Low Total Droplet Counts in Both Sample and Control Wells

More than 5% of the wells on the plate have a droplet count <10,000, including control wells (see Figure 10). If control wells also have low droplet counts, inhibition is not the likely cause of the low counts.

Resolution: Exclude wells with low total droplet counts from analysis. Repeat Droplet Digital PCR, preferably using a different lot of consumables. Refer to the Droplet Digital PCR Applications Guide and the instruction manual of the instrument for further troubleshooting suggestions.

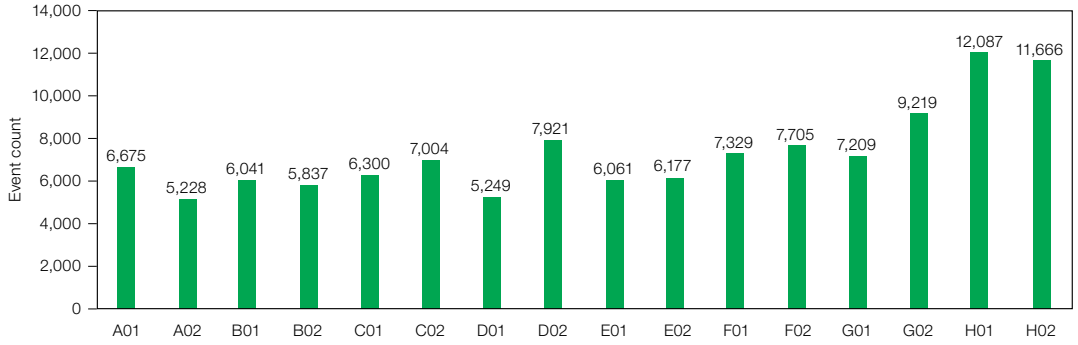

Fig. 10. Event count graph in which more than 5% of the wells have a total droplet count <10,000.

PCR Inhibition

Amplitude decreases and clusters spread out (Figure 11).

Resolution: If concentration cannot be automatically calculated, exclude the well from analysis. Dilute the sample to decrease inhibitor concentration and repeat Droplet Digital PCR. Alternatively, further purify the DNA sample to eliminate PCR inhibitors.

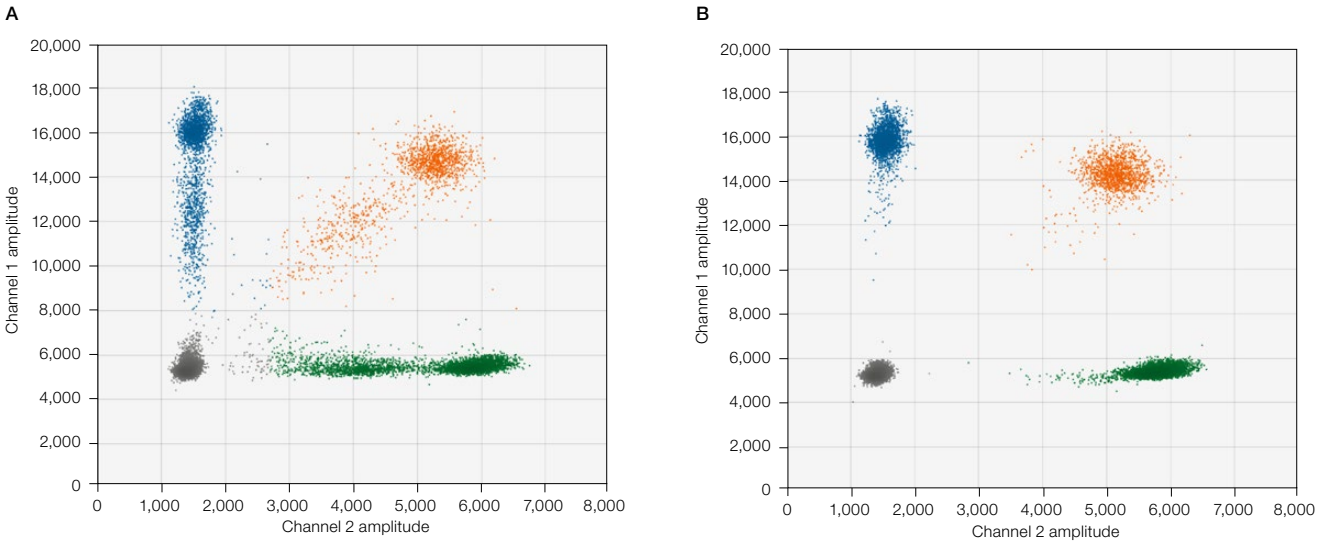

Fig. 11. PCR inhibition. A, problem well; **B**, normal well.

Mirroring

Droplets exhibit two distinct sizes (Figure 12), which indicates a potential consumable failure or particulates from samples, environment, tips, or reagents.

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

Droplet Shredding

Shredded droplets appear on the diagonal through the negative droplet cluster (Figure 13).

Resolution: Exclude well from analysis or repeat Droplet Digital PCR.

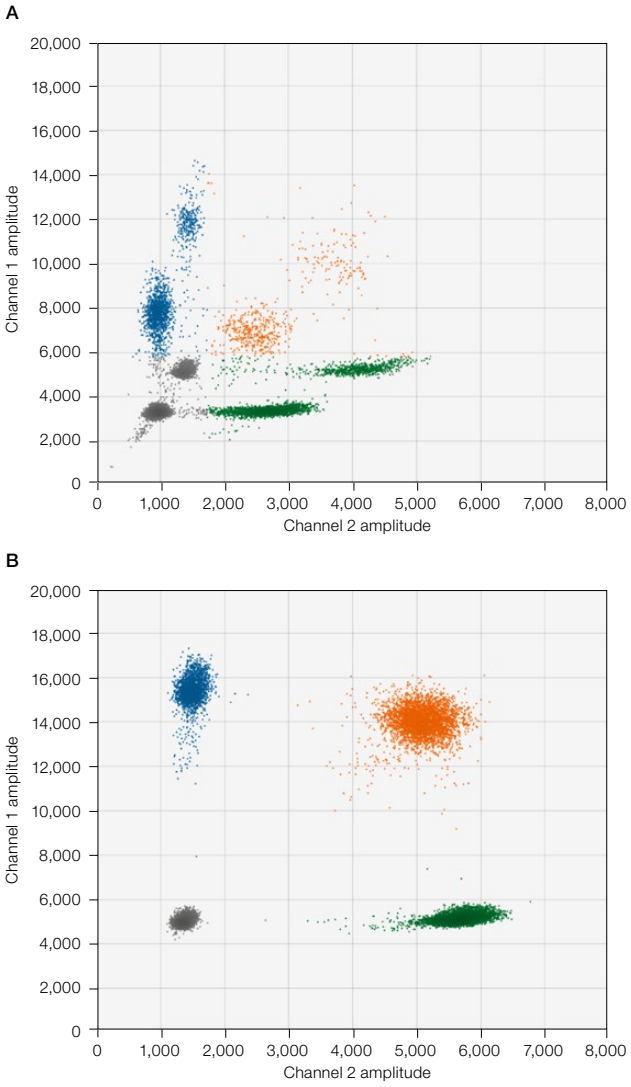

Fig. 12. Mirroring. A, problem well; **B**, normal well.

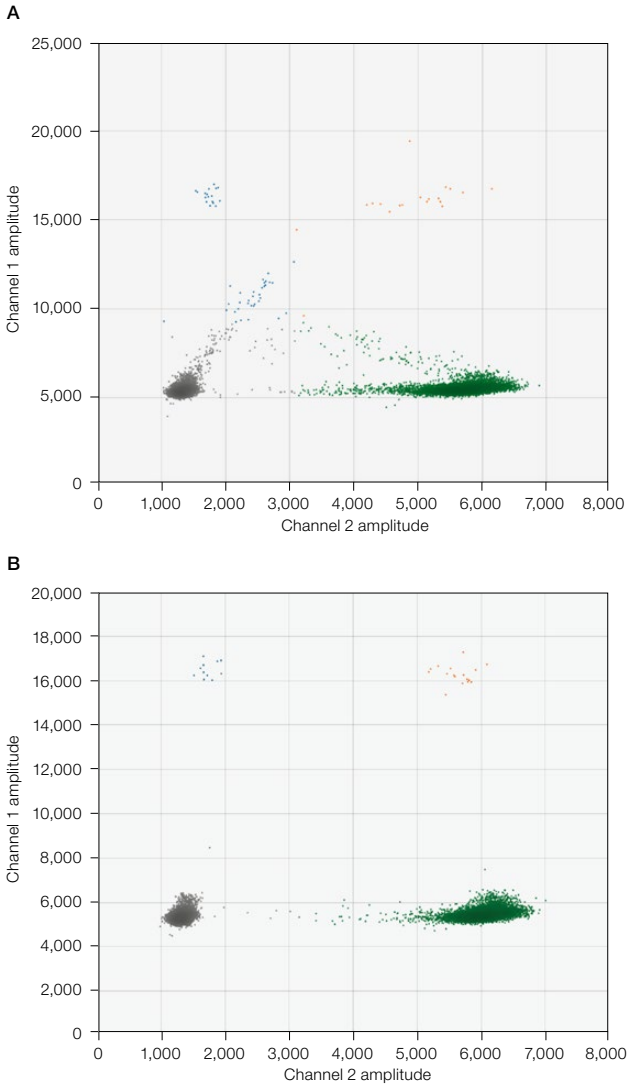

Fig. 13. Droplet shredding. A, problem well; **B**, normal well.

Incorrect Autothresholding

Droplets are incorrectly thresholded using autothresholding. Part of the negative droplet cluster is incorrectly gated as positive (Figure 14).

Resolution: Ensure that wells were thresholded using the positive control–based autothresholding. If this does not resolve the issue, follow the instructions in the QX ONE Data Analysis: Positive Control–Based Manual Thresholding section. If necessary, refer to the Droplet Digital PCR Applications Guide for best practices setting manual thresholds.

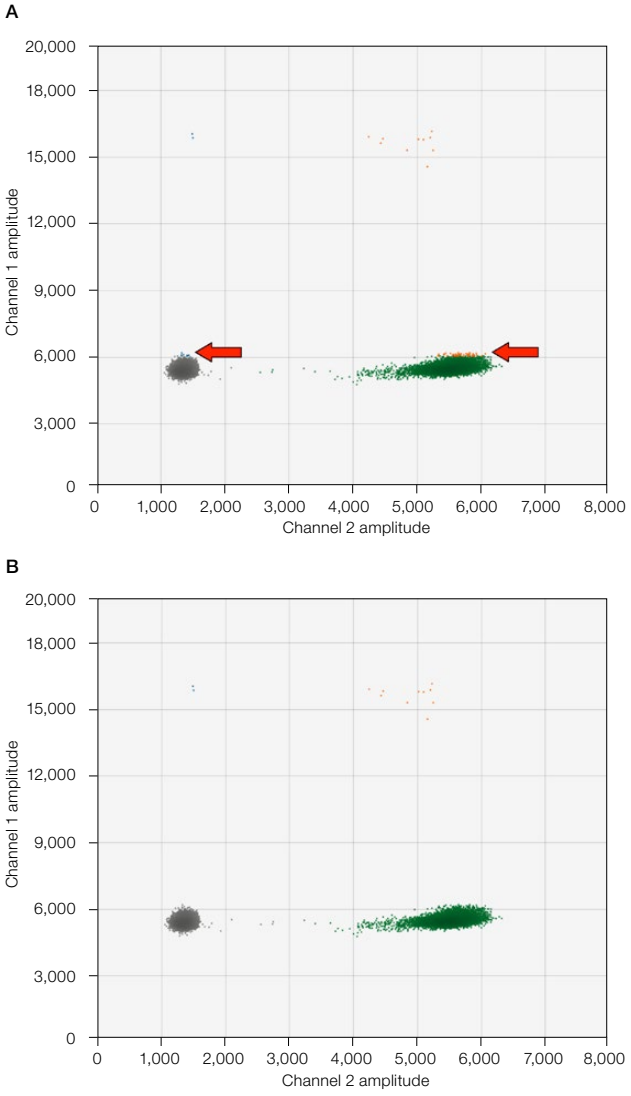

Fig. 14. Incorrect autothresholding. A, incorrect thresholding (red arrows); **B**, correct thresholding using positive control–based autothresholding.

High-Amplitude Positives

Some droplets have an amplitude ≥2x the amplitude of the positive droplet clusters in the positive control wells (Figure 15). These droplets can occur in the FAM and/or the HEX channel.

Resolution: These droplets are false positives and should be excluded from analysis (they should not be considered true positives).

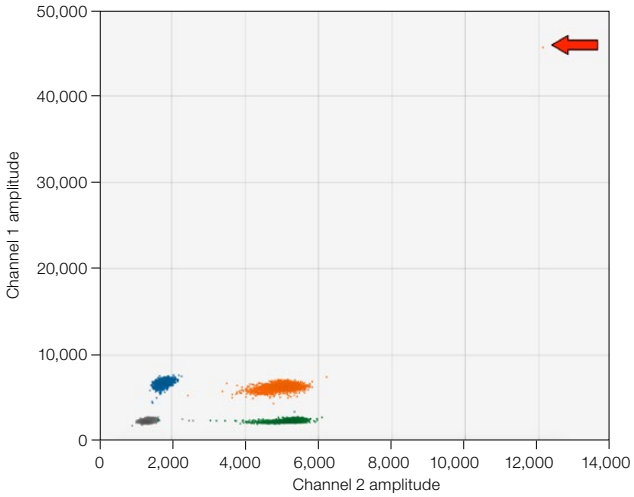

Fig. 15. High-amplitude positives. Droplets with amplitude ≥2x the amplitude of the positive droplet clusters (in either the FAM or HEX channel) should be excluded from analysis. The red arrow indicates a high-amplitude false-positive droplet.

Kit Performance

Sensitivity

Nine *Mycoplasma* species at ≥95% of the replicate wells were positive for *Mycoplasma* extracted at 1 colony forming unit (CFU)/ml. The limit of detection (LOD) in CFU/ml was 1. The LOD in GC/well was ≤6.

Specificity

The Vericheck ddPCR *Mycoplasma* Detection Kit can detect 112 *Mycoplasma* species. The kit does not detect *Clostridium sporogenes*, *Lactobacillus acidophilu*s, or *Streptococcus bovis*, which are bacterial species recommended by the European Pharmacopoeia as assay specificity examples.

Sample Extraction

The QIAGEN QIAamp DNA Mini Kit was used for development of the Vericheck ddPCR *Mycoplasma* Detection Kit. Other sample extraction methods may be compatible but will require verification by the customer.

Sample Matrix

The kit is compatible with suspended cell culture, cell culture supernatant, and cell culture media. Other matrices may be compatible, such as transformed cell culture, but the compatibility with the Vericheck ddPCR *Mycoplasma* Detection Kit requires verification by the customer.

Inhibitor Tolerance

The kit is tolerant to certain levels of common inhibitors, including dimethyl sulfoxide (5%), ethylenediaminetetraacetic acid (1.25 mM), sodium dodecyl sulfate (0.01%), human embryonic kidney cell lysate (1%), and cell culture media (10%).

Visit **bio-rad.com/VericheckddPCRMycoplasma** for more information.

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10000130316 Ver A 20-0668 1220