
dd-Check STEC Solution

User Guide

Test for the detection and colocalization of virulence genes
in Shiga toxin-producing *Escherichia coli* (STEC)

Catalog #17004826



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Section 1

Overview

The dd-Check STEC test is designed to detect the coexistence of virulence genes in Shiga toxin–producing *E. coli* bacteria.

This user guide includes the following:

- Introduction to the STEC test and Droplet Digital PCR (ddPCR) technology
- Kit components that are provided, along with shelf life timelines and additional required equipment and supplies
- Best practices for setting up and running the test
- Test protocols
- Data analysis information
- Confirmation indicators for serogroups and presumptive positive iQ-Check STEC Kits
- Troubleshooting information for minor issues
- Reference documentation
- ddPCR mix calculation guide

Section 2

Introduction

E. coli bacteria are normal flora in human and animal intestines and are usually harmless. However, some strains (such as STEC) are highly pathogenic to humans. They can cause diseases and lead to hemorrhagic colitis and hemolytic uremic syndrome. STECs are defined by the presence of *stx1* or *stx2* (Shiga toxin genes) in their genome. The *eae* (intimin) gene is an additional virulence marker.

Important: While this assay detects Shiga toxin gene sequences, it does not differentiate between *stx1* and *stx2*, nor does it detect *stx2f*.

The most common STEC strain is *E. coli* O157:H7, but isolation of non-O157 STEC strains has been increasing. Outbreaks are commonly associated with the consumption of raw meat and dairy products, and more recently, produce. Pathogenic STEC is defined as a single *E. coli* bacterium carrying both the *stx* genes and an intimin-coding gene, such as *eae*.

One of the challenges with STEC screening using traditional PCR or ELISA-based methods is the inability to distinguish the presence of virulence factors coming from a single *E. coli* bacterium (true positive) or from multiple *E. coli* bacteria (false positive). This can lead to a high rate of presumptive positives that cannot be culturally confirmed.

The dd-Check STEC Kit uses Droplet Digital PCR, Bio-Rad's unique digital PCR methodology, to distinguish double-positive linked virulence factors in samples from single-positive or unlinked virulence factors, and to differentiate samples that are true-positive STEC from false-positive STEC. This detection and linkage verification of targets in a single bacterium enhances the accuracy of food safety testing by reducing the number of false-positive reactions.

Droplet Digital PCR employs advanced microfluidics technology to generate thousands of highly uniform nanoliter-sized droplets in each sample, achieving sample partitioning on a massive scale. Once generated, the droplets can be amplified, read, and analyzed. In traditional PCR, a single measurement is performed on a single sample, while in Droplet Digital PCR, thousands of measurements can be performed on each sample.

Following enrichment and sample preparation, in which a highly optimized amplification mix and ready-to-use PCR reagents are combined with enriched and purified samples, Droplet Digital PCR is conducted using the following processes:

- **Droplet generation:** In each sample, droplet generation produces thousands of nanodroplets encapsulating intact *E. coli* bacteria
- **Thermal cycling:** Through repeated heating and cooling processes, PCR amplification creates multiple copies of DNA strands in each droplet
- **Droplet reading:** The droplet reader scans each droplet, counting it as positive or negative based on measured fluorescence that identifies each of the 2 targets (*stx* and *eae*)
- **Droplet analysis:** Bio-Rad's Qx IDE Manager Software, when connected to the QX200 Droplet Reader during droplet reading, does the following:
 - Analyzes the binary data for coexistence of both virulence markers in a single droplet
 - Determines whether the target genes are linked to the same bacterium
 - Produces visual analysis data

You can also use Droplet Digital PCR to confirm linked virulence factors after screening with the iQ-Check STEC VirX Kit. For information, see Confirmation of Presumptive Positive iQ-Check STEC Kit on page 22.

For information about preparing samples and using the ddPCR instruments, see section 5, Test Protocols.

Section 3

Test Components and Requirements

Kit Components

The reagents listed in Table 1 are included in each dd-Check STEC Kit.

Table 1. dd-Check STEC Kit components.

Reagent	Description	Quantity Provided
ddA	Amplification mix	2 tubes, 330 µl
ddP	Primer-probe mix	2 tubes, 660 µl
ddC	Positive control	1 tube, 200 µl
DG oil	Droplet generation oil	1 bottle, 7 ml

Shelf Life and Storage

Observe the following requirements when storing the kit components:

- You must store the amplification mix, primer-probe mix, and positive control at -20°C . The Droplet Generation Oil must be stored at room temperature ($20\text{--}25^{\circ}\text{C}$)

Note: You can use the reagents stored at this temperature until the expiration date indicated on the tubes.

- For each tube, a maximum of 3 freeze-thaw cycles are allowed
- You can store thawed reagents for up to 2 weeks at $2\text{--}8^{\circ}\text{C}$

Materials Required but Not Supplied in the Kit

This section lists the equipment and supplies you must have on hand to complete the test. Catalog numbers are listed for items available from Bio-Rad.

Equipment

- Lab paddle blender for homogenizing test samples
- Incubator for sample microbiological enrichment
- 20 μl pipet for sample loading (Rainin L-20, L8-20)
- 50 μl pipet for droplet transfer (Rainin L-50, L8-50)
- 200 μl pipet for oil (Rainin L200, L8-200)
- Benchtop centrifuge
 - Minimum speed for tube protocol 6,000 x g
 - Minimum speed for plate protocol 2,250 x g
- Deep well plate rotor (such as Eppendorf A-2-DWP Rotor for an Eppendorf Centrifuge), which allows the centrifugation of a plate with dimensions 8.3 x 8.7 x 12.5 cm (W x D x H) (used specifically for the sample preparation plate protocol)
- DG8 Cartridge Holder (catalog #1863051)
- QX200 Droplet Generator (#17005227)
- QX200 Droplet Reader (#17005228)
- Laptop for QX200 Droplet Digital PCR System (#17005230)
- PX1 PCR Plate Sealer (#17005226)
- CFX96 Touch Deep Well Real-Time PCR Detection System (#3600037) or similar Bio-Rad system

Note: Bio-Rad recommends using an uninterrupted power supply with the thermal cycler and droplet reader. Contact Bio-Rad Technical Support for information about recommended instruments.

Supplies

- Enrichment medium — buffered peptone water (BPW):
 - 500 g (#3564684)
 - 225 ml x 6 bottles (#3554179)
 - 3 L x 4 bags (#3555795)
 - 5 L x 2 bags (#3555790)
- Selective STEC Supplement, 1 g (#3564005)
- Filter sample bags
- Specific for the tube protocol sample preparation:
 - Syringe, luer lock tip, 2–5 ml
 - Syringe filter, 10 µm (Pall Laboratory AP-4001 or equivalent)
- Specific for the plate protocol sample preparation:
 - Filtration plate, 10–12 µm melt blown polypropylene, 10 ml (Whatman 7700-9917 or equivalent)
 - Sample collection plate, V-shaped well bottom, 10 ml (Corning P-DW-10ML-24-C-S or equivalent)
 - Adhesive film for plate
- Sterile sample tubes, 5 ml
- 1.5 ml Conical Tubes, screwcap, sterile (#2240110XTU)
- DG8 Cartridges for QX200 Droplet Generator (#17005222)
- DG8 Gaskets for QX200 Droplet Generator (#17005223)
- If needed: ddPCR Buffer Control for Probes (#1863052)
- ddPCR 96-Well Plates (#17005224)
- Pierceable Foil Heat Seals (#17005225)
- ddPCR Droplet Reader Oil (#17005221)
- Powder-free gloves
- Filtered pipet tips (Rainin GP-L10F, GP-L200F)
- Bleach, 5%
- Cleaning agent, such as DNA AWAY or RNase AWAY

Section 4

Best Practices

Bio-Rad strongly recommends that you follow the general requirements described in the standard EN ISO 22174 (Microbiology of food and animal feeding stuffs — Polymerase chain reaction (PCR) for the detection of food-borne pathogens — General requirements and definitions).

This section explains precautions and recommendations for obtaining the best test results.

General

- This test must be performed by trained personnel
- Handle samples and enrichment cultures as potentially infectious material and discard them according to local rules and regulations
- Autoclave all potentially infectious material before disposal
- The quality of PCR results depends on strict compliance with good laboratory practices (for example, the EN ISO 7218 standard):
 - Always use a positive control and a negative control for each series of amplification reactions
 - Never circulate laboratory equipment (pipets, tubes, etc.) from one workstation to another
 - Shake the kit reagent containers before using them to ensure homogeneity
 - Do not use reagents after their expiration date, or if stored at the wrong temperature and condition
 - Routinely perform the following tasks:
 - Verify the accuracy and precision of pipet
 - Check instruments to ensure they are functioning correctly
 - Clean work spaces with 5% bleach and other decontaminating agents, such as DNA AWAY
 - Use powder-free gloves to avoid interference with data acquisition
 - Change gloves often, especially if you suspect they are contaminated

dd-Check STEC Kit

- All substances or mixtures in the test kit are classified products, according to the Globally Harmonized System of Classification and Labeling of Chemicals (GHS)
- If used correctly, no special precautions are necessary
- If the product is inhaled, supply fresh air and consult a doctor in case of complaints
- After eye contact with the product, rinse opened eye for several minutes under running water
- If the products are swallowed, induce vomiting and call for medical help

CFX96 Touch Deep Well Real-Time PCR Detection System and QX200 Droplet Digital PCR System

Refer to the documentation for each instrument for more comprehensive information.

- Improper use of these instruments can cause personal injury or damage to the instrument
- If improperly handled, some components can pose a risk of personal injury due to excessive heat
- For safe use, only qualified and appropriately trained laboratory personnel should operate these instruments
- Instruments must be serviced by Bio-Rad field service engineers only. Service performed by other entities might void the warranty

Enrichment

- Always follow standard laboratory safety practices
- The user should read, understand, and follow all safety information in the instructions for the dd-Check STEC Kit
- Retain all safety instructions for future reference

Important: Biological samples, such as enrichments, can potentially transmit infectious diseases.

- Conduct all work in properly equipped facilities, using the appropriate safety equipment (for example, physical containment devices)
- To reduce the risks associated with exposure to chemicals and biohazards, perform pathogen testing in a properly equipped laboratory under the control of trained personnel
- Wear appropriate protective equipment, which includes, but is not limited to, protective eyewear, face shield, clothing/lab coat, and gloves, while handling reagents and contaminated samples
- After amplification, avoid contact with the enrichment media and reagent tubes
- Dispose of enriched samples according to current industry standards and applicable local, state/provincial, and national regulations for disposal of biological waste

Important: Pathogenic *E. coli* is a Biosafety Level 2 or Level 3 organism, depending on the regulations of the country or state/province where the test is being conducted.

- Before working with potentially infectious samples, train all individuals in accordance with applicable regulatory and company/institution requirements

When testing is complete, decontaminate all materials and media that might contain pathogens; follow current industry standards for the disposal of contaminated waste (autoclave for 20 min at 120°C).

- Consult the Safety Data Sheet for additional information and local regulations for disposal

Section 5

Test Protocols

Bio-Rad strongly recommends that you read the entire Test Protocols section before starting the test.

This section contains information and instructions for the process steps shown below, from sample enrichment through droplet reading. Information about analysis and interpretation is in section 6.

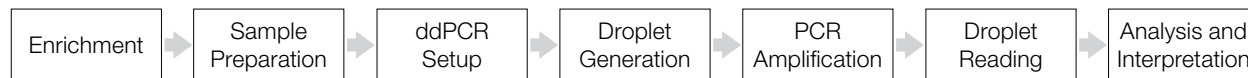


Table 2 specifies the protocols that can be used for screening samples directly with the dd-Check STEC Kit.

Table 2. Sample screening protocols.

Scope (matrices)	Enrichment
Raw meat (375 g)	1,125 ml prewarmed BPW plus 15 ml STEC Supplement at 37 ± 1°C for 16–24 hr
Raw meat (25 g)	225 ml prewarmed BPW plus 2.5 ml STEC Supplement at 37 ± 1°C for 16–24 hr
Leafy greens (200 g)	450 ml prewarmed BPW plus 6.5 ml STEC Supplement at 37 ± 1°C for 16–24 hr
Flour, sprouts, cilantro, parsley (25 g)	225 ml prewarmed BPW plus 2.5 ml STEC Supplement at 37 ± 1°C for 16–24 hr

Sample Enrichment Protocol

Important: Enrichment media must be at the appropriate incubation temperature before use. For a larger volume, an overnight period is recommended to ensure the medium is at the proper prewarmed temperature.

- Selective STEC Supplement (catalog #3564005) is used with BPW in certain matrices. To prepare the Selective STEC Supplement, use the instructions provided in the technical sheet
- For large raw beef sample sizes (375 g), homogenize by stomaching in 1,125 ml of prewarmed BPW plus 15 ml STEC Supplement in a stomacher bag with incorporated filter
- For large leafy green sample sizes (200 g), homogenize (without blending or stomaching) in 450 ml of prewarmed BPW plus 6.5 ml STEC Supplement in a stomacher bag with incorporated filter
- For flour and other raw meat or produce sample sizes of 25 g, homogenize in 225 ml of prewarmed BPW plus 2.5 ml STEC Supplement in a stomacher bag with incorporated filter. Flour should be homogenized without blending or stomaching
- Incubate, without shaking, in accordance with the times and temperatures indicated in Table 2

Note: Enrichment samples can be kept at 2–8°C up to 72 hr.

Sample Preparation Protocols

General Instructions

- Avoid shaking the enrichment bag and collecting large fragments of food debris. For food samples with a fatty supernatant, collect the sample just below this layer
- Change gloves often, especially if you suspect they are contaminated
- Periodically clean work spaces with 5% bleach and other decontaminating agents, such as DNA AWAY

Tube Setup Protocol

You must analyze prepared samples within 60 min, or keep them at 2–8°C for up to 24 hr.

To set up a tube for screening:

1. Collect 2 ml of enriched sample from the top of the bag and place it in a sterile tube.
2. Aspirate 1 ml of enrichment with a syringe and attach a 10 μm filter.
3. Push the sample through the filter and collect in a new tube.
4. Centrifuge at 6,000 \times g for 5 min. You can wait a maximum of 15 min at the end of centrifugation.
5. Completely discard the supernatant, taking care not to disturb the pellet.
6. For leafy greens and raw beef, add 200 μl of BPW to the pellet and pipet up and down to resuspend thoroughly.
7. For flour, add 1,000 μl of BPW to the pellet and pipet up and down to resuspend thoroughly.

Note: Obtain at least 1 ml in the tube after filtration. In case of clogging, use a second filter and a new sampling from the enrichment bag.

Plate Setup Protocol

You must analyze prepared samples within 60 min, or keep them at 2–8°C for up to 24 hr.

To set up a plate for screening:

1. Add the 24-well 10 μm filter plate above the 24-well collection plate and tighten them with tape. Make sure they are placed in the same direction (A1 above A1).
2. Collect 1 ml of each enriched sample into a well of the filtration plate. Seal the plate with plastic film.
3. Centrifuge the plates at 2,250 \times g for 10 min. You can wait a maximum of 15 min at the end of centrifugation.
4. Completely discard the supernatant, taking care not to disturb the pellet. Aspects of the pellet may differ, depending on localization in the plate. See Figure 1, which shows plates after centrifugation.

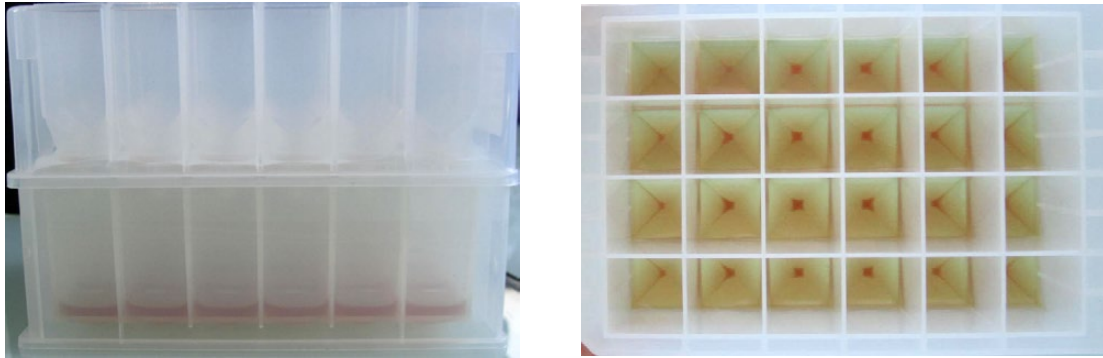


Fig. 1. Plates after centrifugation.

5. For leafy greens and raw beef, add 200 μ l of BPW to the pellet and pipet up and down to resuspend thoroughly.
6. For flour, add 1,000 μ l of BPW to the pellet and pipet up and down to resuspend thoroughly.

Note: In case of clogging, use another well with a new sampling from the enrichment bag.

ddPCR Protocols

General Instructions

- Remove kit from -20°C storage 30 min prior to use and ensure reagents are completely thawed before use
- Do not use reagents after their expiration date
- Do not use reagents after a maximum of 3 freeze-thaw cycles
- To avoid possible cross-contamination, open tubes and carefully remove sealing film from plates

ddPCR Mix Protocol

Bio-Rad recommends using the PCR mix (reagent ddA + ddP) within 3 hr at room temperature ($20-25^{\circ}\text{C}$).

Important: After adding the sample to the PCR mix, you must initiate droplet generation within 30 min.

To prepare your ddPCR mix:

1. Prepare a ddPCR mix containing the amplification mix (reagent ddA) and the primer-probe mix (reagent ddP).

Important: The volume of PCR mix depends on the number of samples and controls to be analyzed. You must include at least one positive and one negative control in each PCR run. For the correct volumes to use for each reagent, see Appendix: ddPCR Mix Calculation Guide in section 11.

2. Pipet 18 μ l of the ddPCR mix into each well, according to your plate setup.
3. Add 6 μ l of sample, sterile BPW (negative control), and reagent ddC (positive control) into each well according to your plate setup.

Droplet Generation Protocol

For information about the QX200 Droplet Generator (including instrument setup, use, and maintenance) and laboratory practices, refer to the QX200 Droplet Generator Instruction Manual (#10031907).

Note: Droplet Generation Oil for Probes is stable for 12 months when stored at room temperature (20–25°C).

To prepare the sample for droplet generation:

1. Carefully mix the ddPCR mix with the sample by pipetting up and down a minimum of five times. Avoid creating bubbles.
2. Transfer 20 µl of each sample to the sample wells (middle row) of the DG8 Cartridge.

Important: All wells of the cartridge must be filled. For wells with no samples, use BPW with the PCR mix or the ddPCR Buffer Control for Probes (catalog #1863052). For information, see the instructions for use included with the buffer kit.

3. Fill each oil well (bottom row) with 70 µl of Droplet Generation Oil.
4. Hook a gasket over the cartridge holder. The gasket must be securely hooked on both ends of the holder; otherwise, pressure sufficient for droplet generation will not be achieved.

To generate droplets:

1. Press the button on the instrument lid to open the QX200 Droplet Generator.
2. Place the cartridge holder into the compartment.

When the holder is in the correct position, both the power (left light) and holder (middle light) indicator lights are green.

3. Press the button to close the lid and initiate droplet generation.

A manifold positions itself over the outlet wells, drawing oil and sample through the microfluidic channels where droplets are created. Droplets flow to the droplet well, where they accumulate. The droplet indicator light (at right) flashes green after 10 sec to indicate droplet generation is in progress. When all three indicator lights are solid green (indicating that droplet generation is complete), press the button to open the door, and then remove the plate holder (with DG8 Cartridge still in place) from the unit.

4. Remove the gasket from the holder and discard it. The top wells of the cartridge contain droplets, and the middle and lower wells are nearly empty with a small amount of residual oil.

Amplifying Droplets Using Thermal Cycling

For information about using the CFX96 Touch Deep Well Real-Time PCR Detection System and CFX Manager Software, Industrial Diagnostic Edition (IDE), see the corresponding user documentation.

Important: You must launch the PCR step within 30 min of initiating droplet generation.

To prepare the sample plate for thermal cycling:

1. Place the cartridge holder on a flat surface.
2. Using an 8-channel manual pipet, position the pipet tips in each of the eight top wells at approximately a 30–45° angle into the junction where the side wall meets the bottom of the well.

Important: Use Rainin tips to prevent droplet degradation. Do not position the pipet tip in a vertical orientation (90°) or against any flat surface of the well, and do not allow the tips to be flat against the bottoms of the wells.

3. Slowly draw 40 µl of droplets into the pipet tip; do not aspirate more than 40 µl, as this causes air to percolate through the droplets.
4. 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 slowly dispense the droplets.
5. To avoid evaporation, seal the PCR plate with foil immediately after transferring droplets using the PX1 PCR Plate Sealer. Follow the instructions in the PX1 PCR Plate Sealer Instruction Manual (#10023997).

Important: Do not use the metal frame to seal the plate.

To start thermal cycling for PCR amplification:

1. Place the sealed plate in the CFX96 Touch Deep Well System and close the reaction module.
2. Launch the CFX Manager Software, IDE on the computer connected to the instrument.
3. Select dd-Check STEC Amplification and start the run.

Reading the Droplets

For information about the QX200 Droplet Reader, including instrument setup, use, and maintenance, see the QX200 Droplet Reader and Qx IDE Manager Software User Guide (#10000110358).

Following thermal cycling amplification, you must launch the droplet reading process within 60 min.

To prepare the sample for droplet reading:

1. Place the 96-well PCR plate in the plate holder, ensuring well A1 is positioned in the top left corner.
2. Lift the release tabs of the plate holder and place the plate holder on the PCR plate.
3. Press the tabs to secure the plate, and then verify the three parts are firmly in place.
4. Press the button on the droplet reader lid to open the compartment.
5. Load the secure plate assembly in the compartment.
6. Press the button again to close the lid. Ensure the third indicator light is green to confirm the lid is closed.

To start the droplet reading run:

1. Launch Qx IDE Manager Software on the computer connected to the QX200 Droplet Reader.
2. Follow the instructions in the QX200 Droplet Reader and Qx IDE Manager Software User Guide to set up the plate layout and start the run.

You must include one positive control well and one negative control well. When the software recognizes a valid plate layout, the Run button is enabled and you can prompt the instrument to begin.

Note: Before starting the run, the system performs a status check on required instrument elements. The QX200 Droplet Reader cannot start the run unless all elements meet operational requirements. If issues are found during the status check, Qx IDE Manager Software displays an error message and you must resolve all issues before the instrument can start the run.

Section 6

Sample Data Analysis

To review the data analysis file immediately after droplet reading, click **Show Run Results** in the successful run completion dialog box.

To review the analysis file at a later time, click **x** in the upper-right corner to close the dialog box, and then refer to the QX200 Droplet Reader and Qx IDE Manager Software User Guide for information about opening analysis files.

Results Interpretation

Qx IDE Manager Software provides complete automated analysis for dd-Check STEC Kit assays. The results interpretation is based on the presence or absence of the two targets (*stx* and *eae*) and on the following linkage percentage formula:

$$\%AB = (2\lambda_{AB} / [\lambda_A + \lambda_B])100$$

where A is target A (*stx*); B is target B (*eae*); and λ is population mean.

Table 3 includes a summary of sample result interpretations that are shown in the Well Selector and Well Data table in the data analysis file.

Table 3. Data interpretation.

Result	Abbreviation	Interpretation	Action
Invalid	INV	Results cannot be determined.	See section 9, Troubleshooting.
Too concentrated, dilute 1/x	DIL	The colocalization of the gene on the same bacterium cannot be assessed, and the sample must be diluted.	From the postfiltered sample, perform a dilution in BPW recommended by Qx IDE Manager Software, and retest starting from section 5, Test Protocols, ddPCR Protocols subsection. If the second result appears as negative, retest using a 1 log lower dilution.
Negative	NEG	No targets or only one target is detected.	The sample is negative for <i>stx</i> and/or <i>eae</i> .
Negative: unlinked targets	NEG	Both targets are detected, but not from the same bacterium.	The sample is negative for linked virulence genes.
Positive: linked targets	POS	Both targets are detected and linked.	The sample is positive for linked virulence genes from the same bacterium.
Presumptive positive	PP	Both targets are detected but the linkage cannot be determined using our method.	Confirm by culture or dilute the sample to ensure a range of 15–10,000 copies for both targets.

Amplitude Chart Analysis

Qx IDE Manager Software shows amplitude data in one- and two-dimensional dot plots. 1D Amplitude data are also displayed in a histogram. An additional Plate 2D view shows a plate layout of all two-dimensional plots.

Selecting a Chart

1. Select an Amplitude tab.
2. Click a well in the Well Selector to display the corresponding data in the amplitude chart and well data table.

Tip: Press and hold the CTRL key to select multiple wells.

3. Figure 2 shows two selected wells in the 1D Amplitude tab.

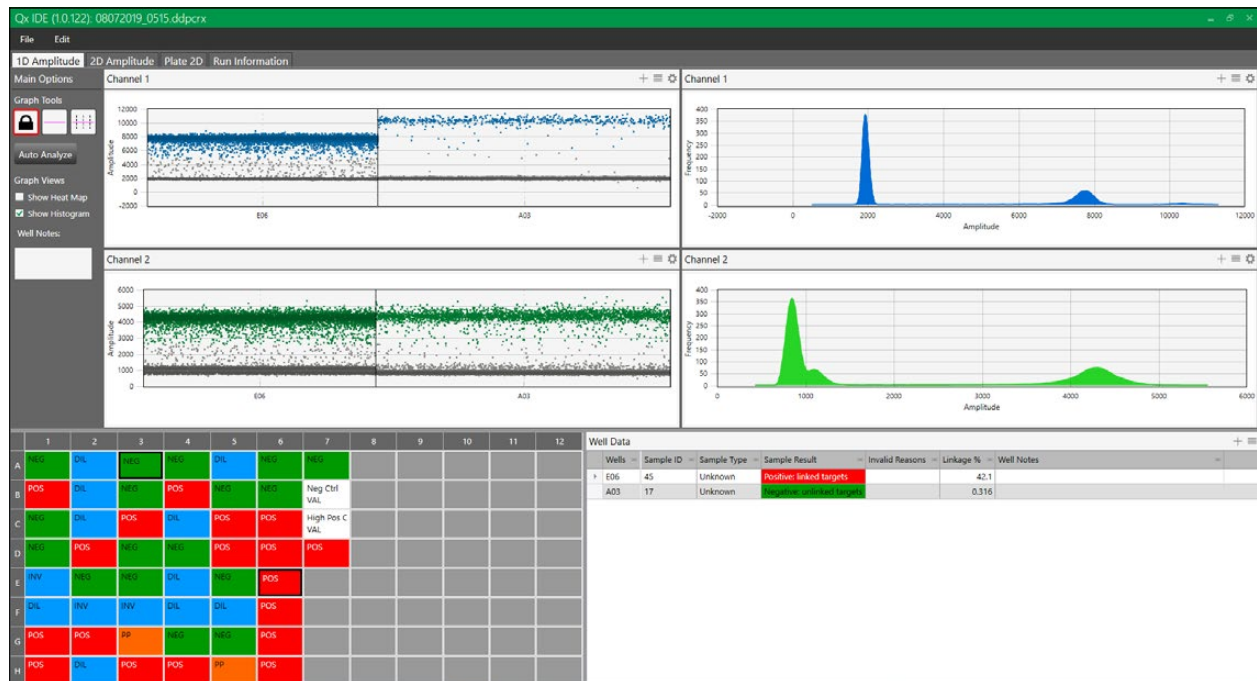


Fig. 2. Two selected wells in 1D Amplitude tab.

1D Amplitude Chart

Use the 1D Amplitude analysis window to view how positive droplet concentrations are plotted for each selected well.

The 1D Amplitude charts illustrate:

- All accepted droplets, with a visual representation of the concentration of positive and negative droplets in each well. Positive droplets appear in color, while negative droplets appear in gray scale (Figure 3)
- Amplitude at specified frequencies in a histogram chart. The histogram displays frequency of droplets at specific fluorescence amplitudes for each well or group of wells selected

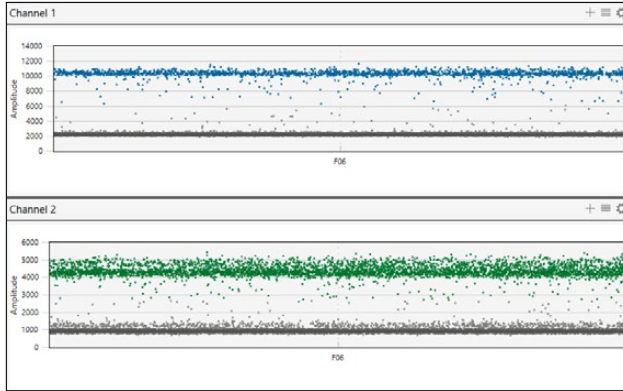


Fig. 3. Plot of positive droplet concentrations. Positive droplets in channel 1 for the *stx* target (■); positive droplets in channel 2 for the *eae* target (■); negative droplets (■).

1D Amplitude Tool Options

For experiments where enough droplets are recognized, Qx IDE Manager Software automatically configures an amplitude threshold based on the determination of positive and negative droplets by the droplet reader.

Note: Well data must meet certain quality metrics before the software will automatically calculate a threshold above which droplets are considered positive. You can use the manual thresholding tools to ensure correct quantification of positive and negative droplets (Table 4). To recover the original clustering, select **Auto Analyze**.

Important: Manual thresholding by inexperienced ddPCR users is strongly discouraged. Contact Bio-Rad Technical Support if you have questions regarding the automatic data analysis.

Table 4. 1D Amplitude tools.

Icon	Mode	Description
	Lock View	Data appear with the thresholds assigned by Qx IDE Manager Software. You cannot modify the thresholds while the icon is enabled.
	Single Well, Manual Threshold	For a single well, the user can manually place a threshold in the 1D Amplitude chart.
	Multiple Wells, Manual Threshold	For multiple wells, the user can manually place a threshold in the 1D Amplitude chart.
	Automatic Analysis	Click to have the software auto analyze clusters if the manual threshold was used.
	Heat Map	Shows the regulation of targets for an experimental sample compared to a control sample based on relative normalized expression and its location on a plate.
	Show Histogram	Enables the icon to display your data in histogram (volcano) plots.

2D Amplitude Chart

Use the 2D Amplitude analysis window to view the results of both targets (*stx* and *eae*) in a single chart. The chart shows individual cluster representations for droplets positive for the *stx* target, the *eae* target, droplets containing both *stx* and *eae* targets, and droplets containing neither target (Figure 4).

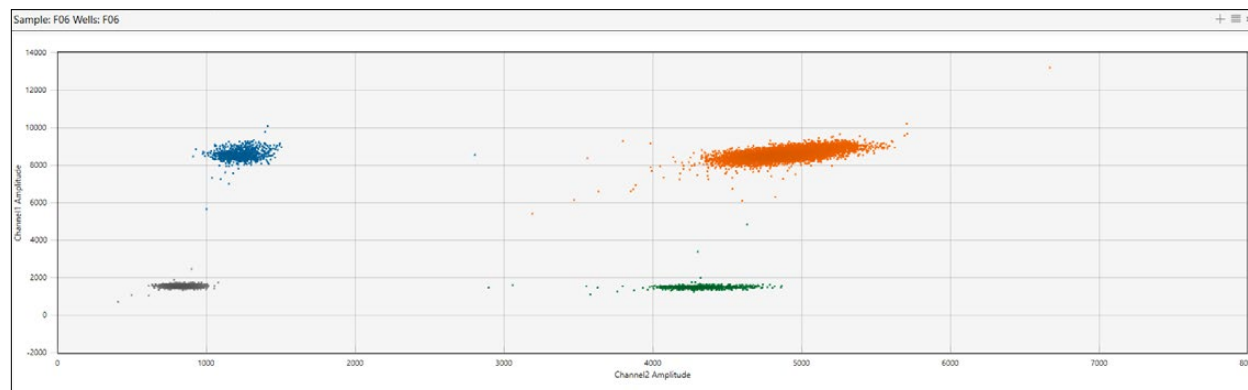


Fig. 4. 2D Amplitude histogram. Positive droplets for the *stx* target (■); positive droplets for the *eae* target (■); positive droplets for both targets (■); negative droplets (■).

2D Amplitude Tool Options

For experiments where enough droplets are recognized, Qx IDE Manager Software automatically configures an amplitude threshold based on the determination of positive and negative droplets by the droplet reader.

Note: Well data must meet certain quality metrics before the software will automatically calculate a threshold above which droplets are considered positive. You can use the manual thresholding tools to ensure correct quantification of positive and negative droplets (Table 5). To recover the original clustering, select **Auto Analyze**.

Important: Manual thresholding by inexperienced ddPCR users is strongly discouraged. Contact Bio-Rad Technical Support if you have questions regarding the automatic data analysis.

Table 5. 2D Amplitude tools.



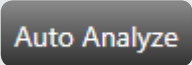
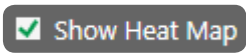
Icon	Mode	Description
	Lock View	Data appear with the thresholds assigned by Qx IDE Manager Software. You cannot modify the thresholds while the icon is enabled.
	Threshold Line	Use the Threshold line icons to establish thresholds across both Channel 1 and Channel 2, dividing droplets into distinct clusters separated by different colors.
	Automatic Analysis	Click to have the software auto analyze clusters if the manual threshold was used.
	Heat Map	Shows the regulation of targets for an experimental sample compared to a control sample based on relative normalized expression and its location on a plate.

Plate 2D

Select **Plate 2D** to see 2D Amplitude for each well in the plate layout (Figure 5).

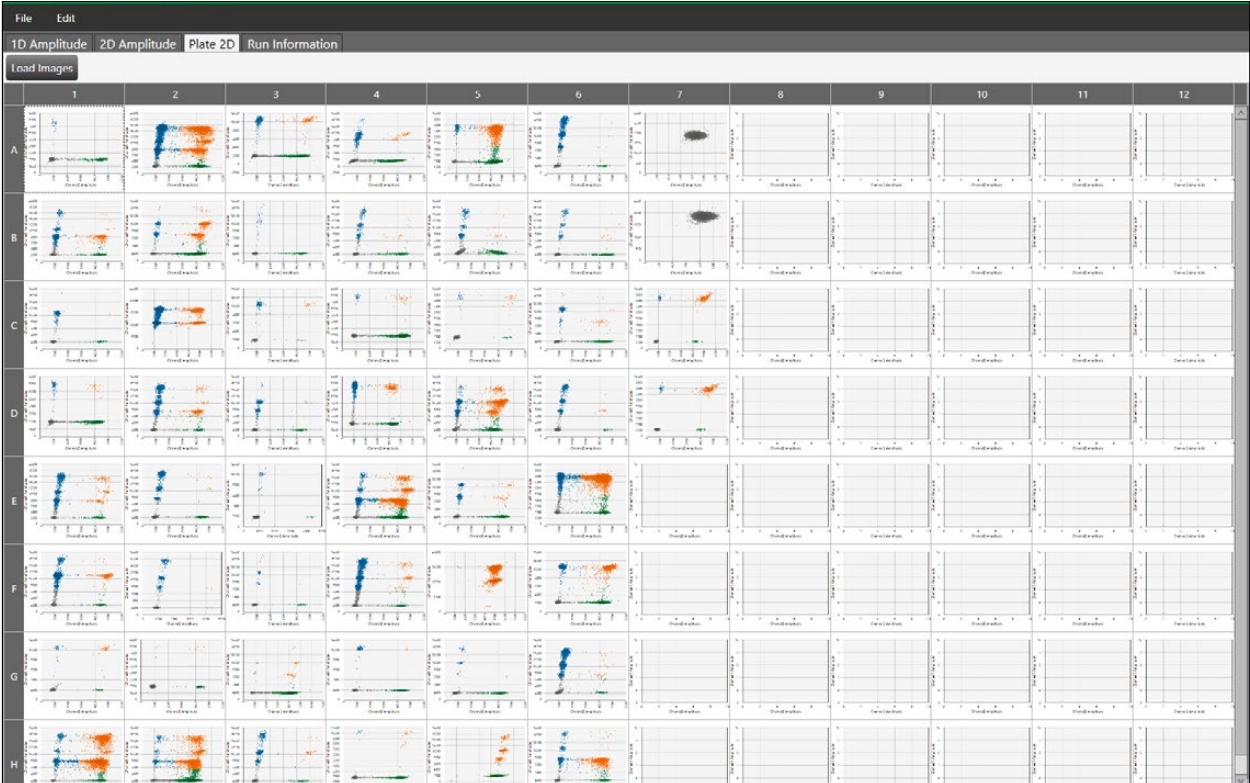


Fig. 5. Plate 2D histograms.

Well Data Table

When you open an analysis data file, Qx IDE Manager Software displays a well selector grid, from which you can select one or more sample wells, and a Well Data table containing data for the selected sample wells. Table 6 explains each available table column. Columns indicated by an asterisk (*) appear in the table by default, but you can use the Show/Hide option from the table menu to add or remove columns in the display.

Table 6. Well Data table columns.

Column	Description
Wells*	Identifies the well number for each sample
Sample ID*	Defines the sample identification number assigned by the user
Sample Type*	Distinguishes "Unknown" samples from positive and negative kit control samples
Sample Result*	Provides the final interpreted sample result
Invalid Reasons	Provides cause for invalid result
Linkage %*	Defines the value (in percentage) that gives the proportion of linked <i>stx</i> and <i>eae</i> targets
Well Notes*	Field for a description of the contents of the well
<i>stx</i> Value (Copies)	Defines the concentration in copies per well of <i>stx</i> targets
<i>eae</i> Value (Copies)	Defines the concentration in copies per well of <i>eae</i> targets
Accepted Droplets	Defines the number of droplets that are analyzable for Poisson calculation (not rejected by the reader)
Mean Amplitude of Positives (<i>stx</i>)	Defines, for <i>stx</i> target in FAM channel, the average fluorescence amplitude of the positive droplets
Mean Amplitude of Positives (<i>eae</i>)	Defines, for <i>eae</i> target in HEX channel, the average fluorescence amplitude of the positive droplets
Mean Amplitude of Negatives (<i>stx</i>)	Defines, for <i>stx</i> target in FAM channel, the average fluorescence amplitude of the negative droplets
Mean Amplitude of Negatives (<i>eae</i>)	Defines, for <i>eae</i> target in HEX channel, the average fluorescence amplitude of the negative droplets
Ratio	<i>stx</i> Value (Copies) divided by <i>eae</i> Value (Copies)
Ch1+Ch2+	Number of positive droplets in channel 1 (FAM) + channel 2 (HEX)
Ch1+Ch2-	Number of positive droplets in channel 1 (FAM) only
Ch1-Ch2+	Number of positive droplets in channel 2 (HEX) only
Ch1-Ch2-	Number of negative droplets in channel 1 (FAM) and channel 2 (HEX)

Control Well Data Compared to Sample Well Data

Testing for bacteria requires positive and negative control wells as part of the plate to be tested. For the STEC test used as an example in this document, the amplitude graphics show how Qx IDE Manager Software represents the high-positive and negative control well results, as well as the positive and negative unknown sample results.

The higher the linkage percentage, the more closely the dot scatter in a positive sample well approximates the scatter in the high-positive control well. In negative wells, a well with no targets most closely approximates the control well, but wells with low linkage percentages are also identified as negative.

Linkage percentages appear in the Well Data table. In Figure 6, all wells were selected to display the linkage percentages for each well.

Wells	Sample ID	Sample Type	Sample Result	Linkage %	Well Notes
A01	1	Unknown	Negative: unlinked targets	-0.337	
A07	49	Unknown	Negative		
B01	2	Unknown	Positive: linked targets	18.8	
B05	34	Unknown	Negative: unlinked targets	-1.31	
B07	50	NegCtrl	Valid ctrl		
C01	3	Unknown	Negative: unlinked targets	-0.3	
C05	35	Unknown	Positive: linked targets	42.1	
C07	51	HighPosCtrl	Valid ctrl	88	
D01	4	Unknown	Negative: unlinked targets	-0.113	
D05	36	Unknown	Positive: linked targets	49.4	
D07	52	Unknown	Positive: linked targets	90	
E05	37	Unknown	Negative: unlinked targets	-1.17	
G01	7	Unknown	Positive: linked targets	49.3	
G05	39	Unknown	Negative: unlinked targets	-0.279	
H01	8	Unknown	Positive: linked targets	56.5	

Fig. 6. Well Data table showing linkage percentages.

Results for Positive Wells

1D and 2D Amplitude results at different linkage percentages are shown in Figures 7–10. The positive control well is shown first (Figure 7), followed by unknown positive sample wells (Figures 8–10).

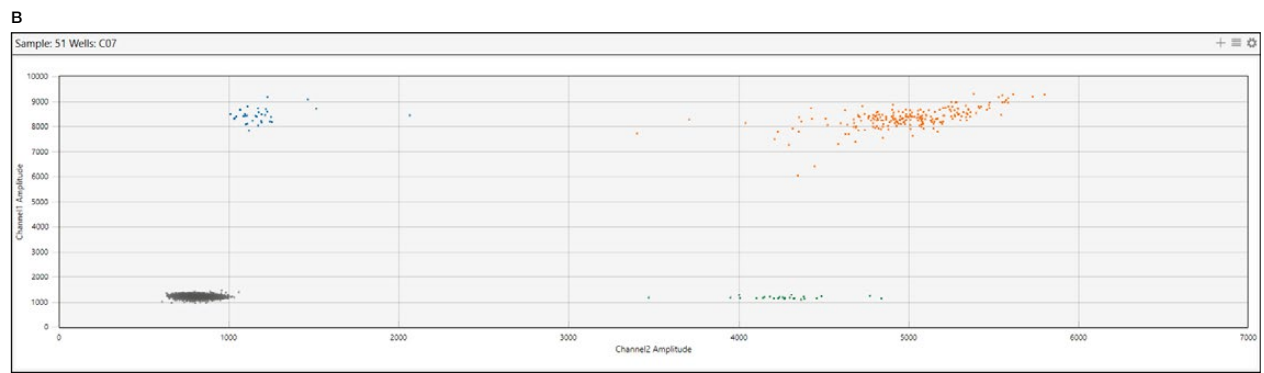
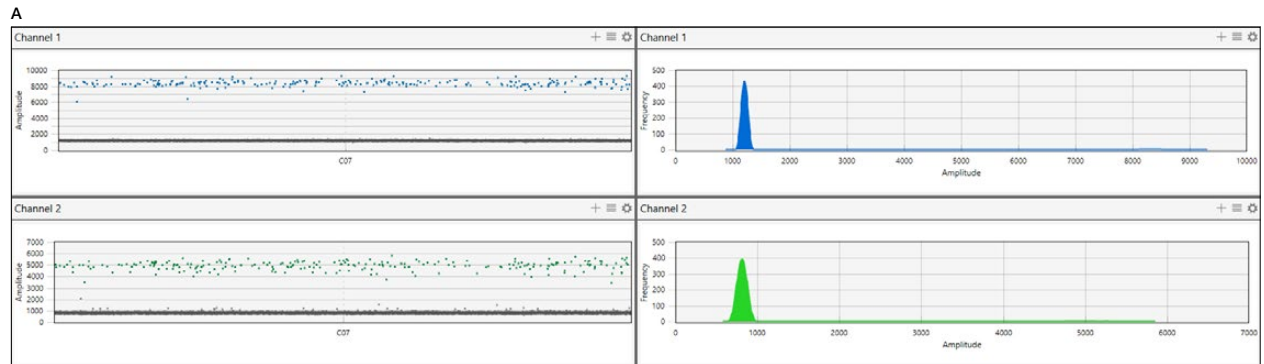


Fig. 7. Well C07: High-positive control at 88% linkage. **A**, 1D Amplitude; **B**, 2D Amplitude.

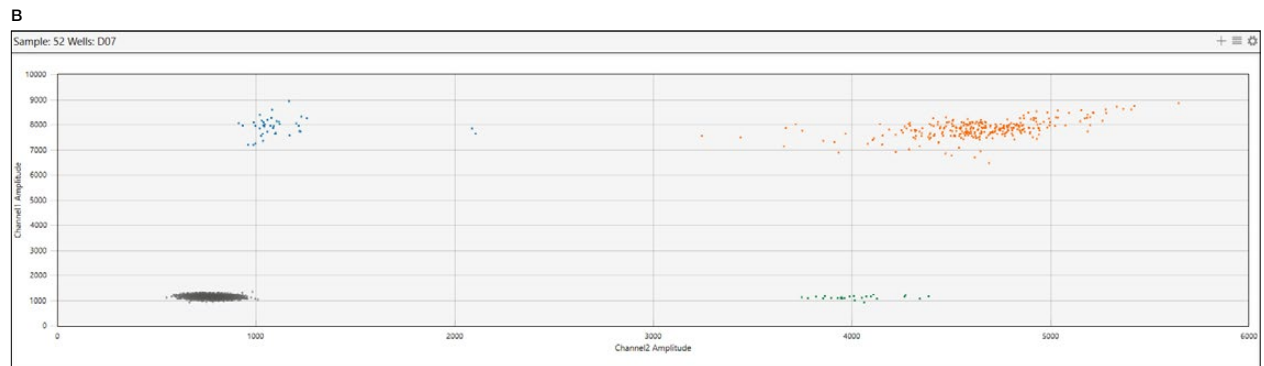
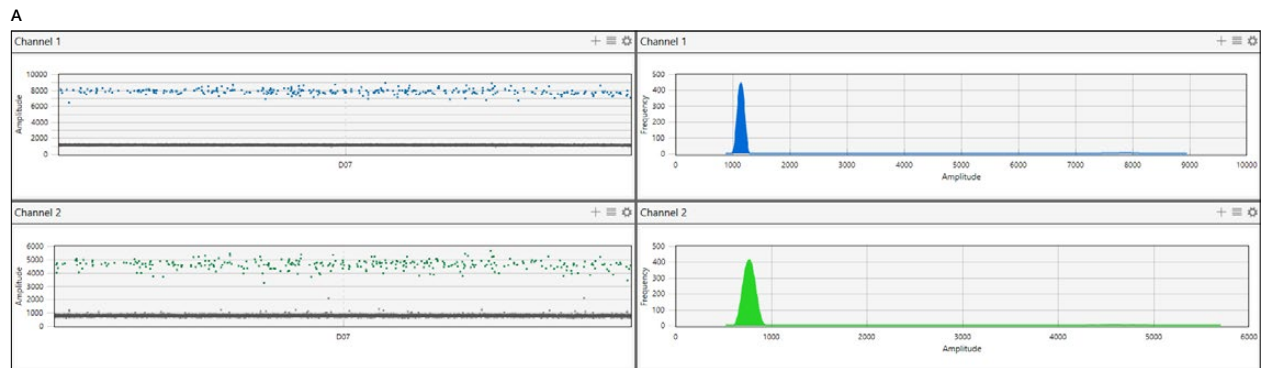


Fig. 8. Well D07: Unknown positive sample at 90% linkage. **A**, 1D Amplitude; **B**, 2D Amplitude.

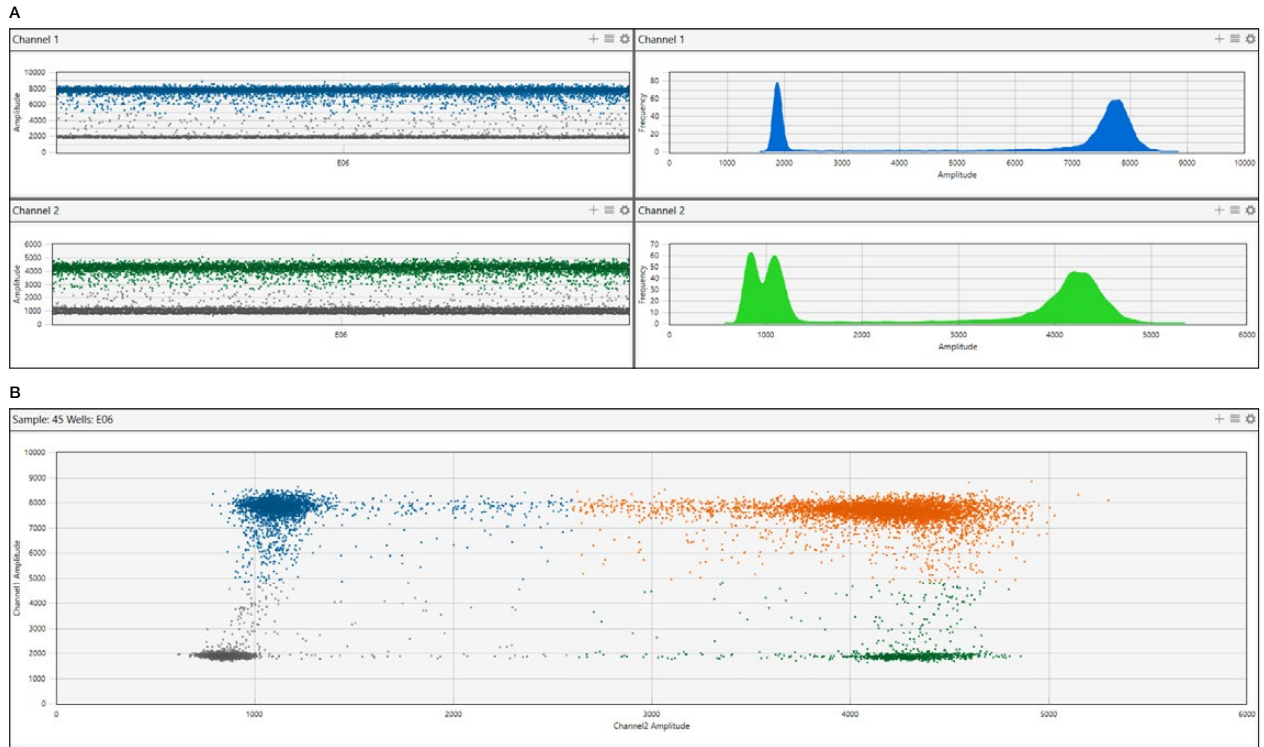


Fig. 9. Well E06: Unknown positive sample at 42.1% linkage. A, 1D Amplitude; B, 2D Amplitude.

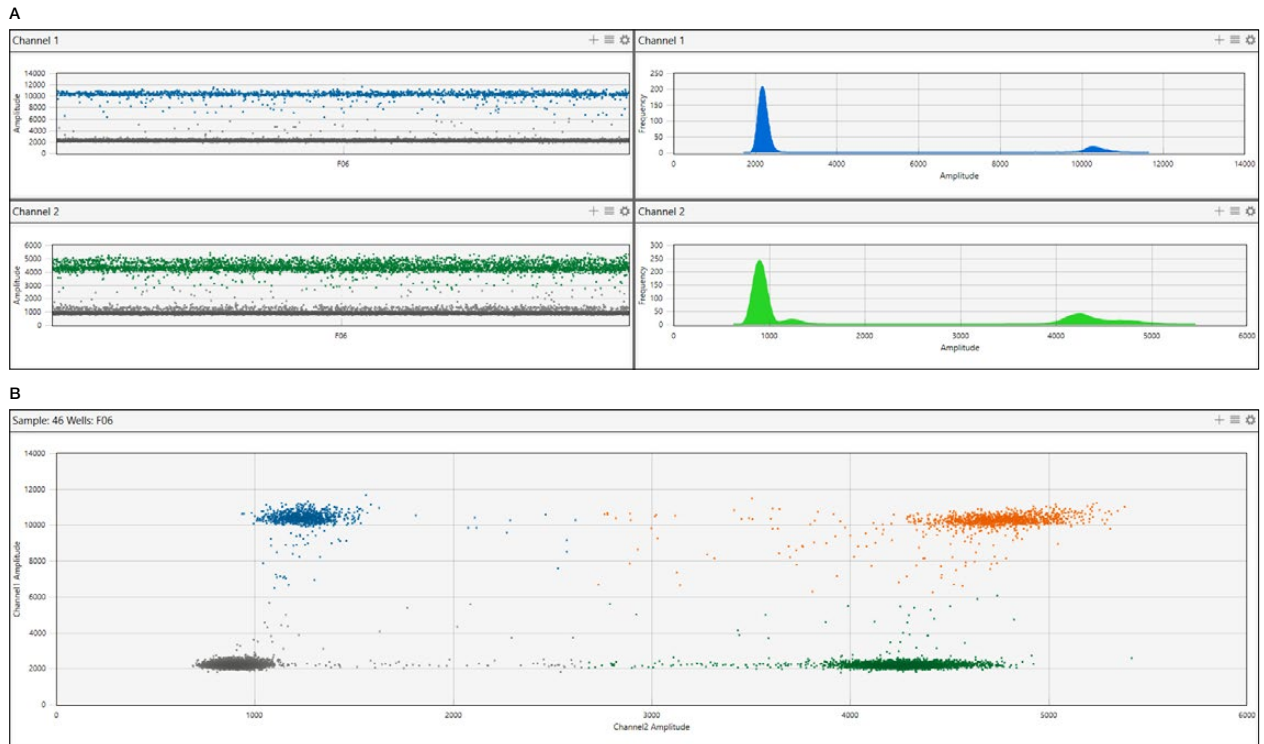
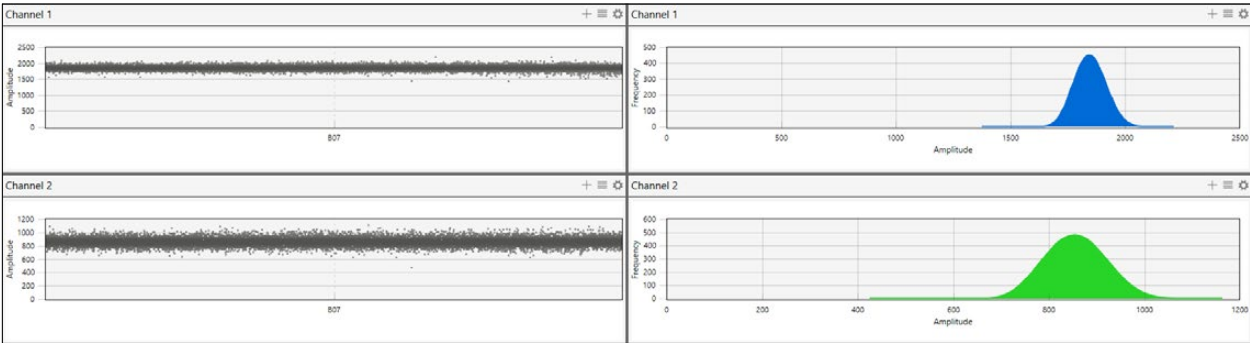


Fig. 10. Well F06: Unknown positive sample at 24.1% linkage. A, 1D Amplitude; B, 2D Amplitude.

Results for Negative Wells

Figure 11 shows the negative control well, followed by unknown negative sample wells with no linkage (Figure 12) and low linkage (Figure 13).

A



B

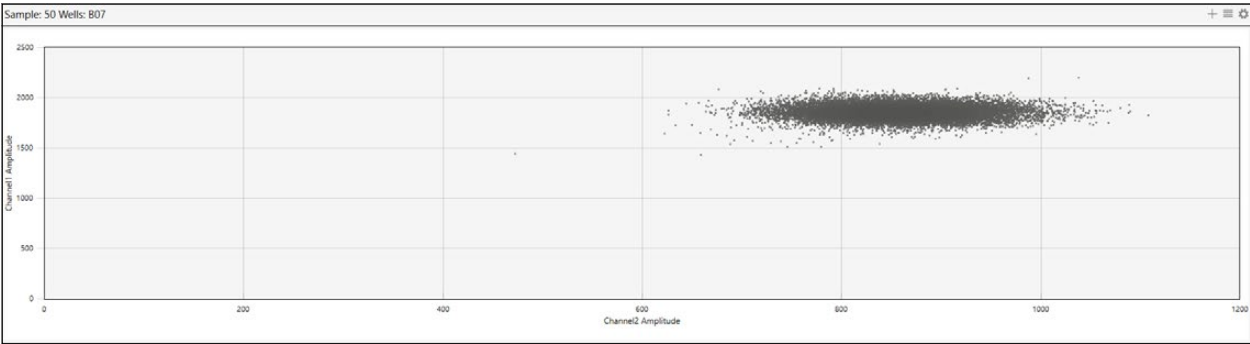
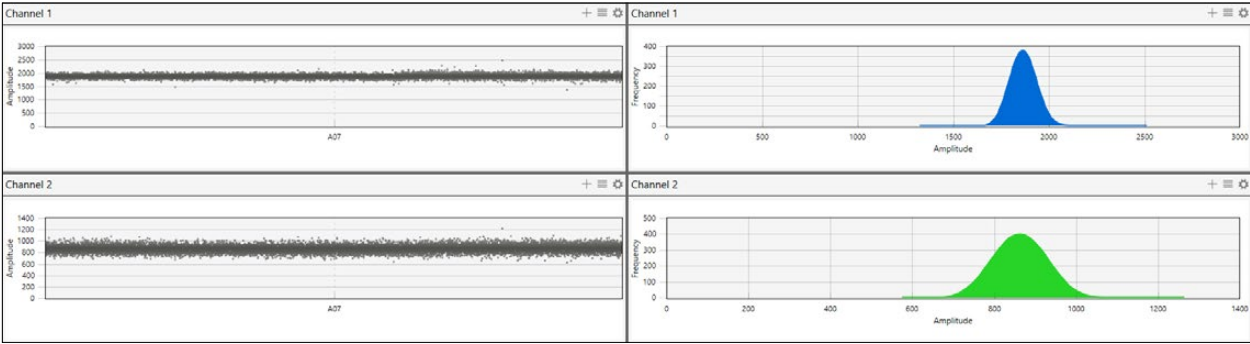


Fig. 11. Well B07: Negative control (no linkage). A, 1D Amplitude; B, 2D Amplitude.

A



B

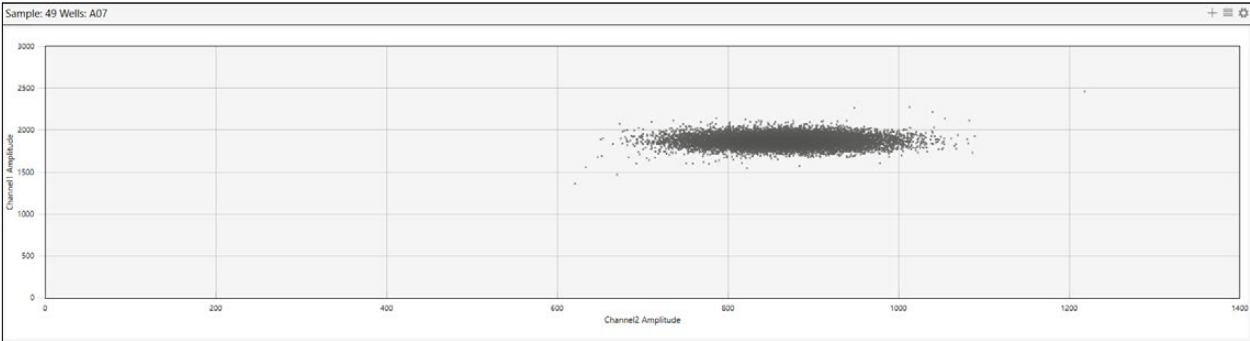


Fig. 12. Well A07: Unknown negative sample (no linkage). A, 1D Amplitude; B, 2D Amplitude.

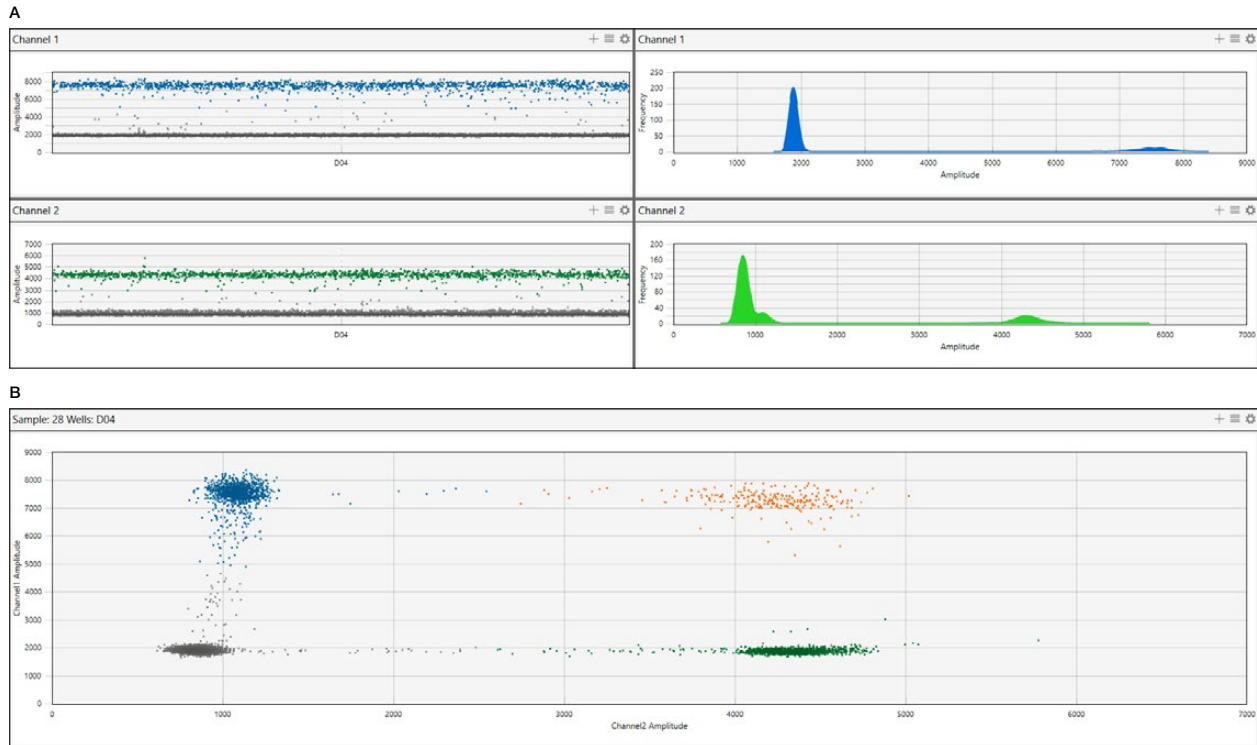


Fig. 13. Well D04: Unknown negative sample (low linkage). A, 1D Amplitude; B, 2D Amplitude.

Section 7 Confirmation of Serogroups

Use the iQ-Check STEC SerO PCR Detection Kit (catalog #3578140) to test positive dd-Check STEC results for identification of the seven major *E. coli* serogroups (O26, O45, O103, O111, O121, O145, and O157).

To confirm serogroups:

1. Use 100 μ l of the enrichment to perform a DNA extraction, following the steps provided in the iQ-Check STEC VirX Kit User Guide (#808474) for the Easy II protocol in section VII.
2. Use the DNA extract and follow the steps provided in section VII, Protocol, of the iQ-Check STEC SerO Kit User Guide (#808475) to confirm the serogroups.

Results are provided for the seven major *E. coli* serogroups by CFX Manager Software, IDE.

Section 8

Confirmation of Presumptive Positive iQ-Check STEC Kit

Use the dd-Check STEC Kit to confirm presumptive positive samples that were screened using the iQ-Check STEC Solution or equivalent.

If BPW plus supplement was used for the original enrichment:

1. Follow instructions in section 5, Test Protocols (Sample Preparation and ddPCR Protocols subsections).
2. To dilute the sample, add the required BPW volume at the end of the sample preparation per Table 7.

Note: To directly target the analytical range of the dd-Check STEC Kit, the table identifies the dilution that can be applied according to the highest quantification cycle (Cq) values between the FAM and Cy5 channels.

Table 7. Dilution information.

Highest FAM or Cy5 Cq	Dilution Level in BPW
Cq ≥ 36	Perform regrowth per the following instructions
Cq ≥ 26	Undiluted
Cq ≥ 24	1/10
Cq ≥ 20	1/100
Cq ≥ 16	1/1,000
Cq < 16	1/10,000

Perform regrowth step if other media were used for the original enrichment or if the FAM or Cy5 Cq values are ≥ 36 after iQ-Check STEC VirX screening:

1. From the original enrichment, transfer 6 ml of the original enrichment to 18 ml of prewarmed BPW plus STEC Supplement, and incubate for 16–24 hr at 37 ± 1°C.
2. After enrichment, follow the instructions in section 5, Test Protocols (Sample Preparation and ddPCR Protocols subsections).

To interpret the data:

- Samples with results Positive: linked targets are confirmed positive for linked *stx* and *eae* virulence genes from the same bacterium
- Samples with results Negative: unlinked targets are not confirmed for colocalization of the two markers; the *stx* and *eae* genes came from two different bacteria

Note: Retest samples with results Negative using a dilution 1 log lower.

Section 9

Troubleshooting Guide

Table 8 includes information about troubleshooting and correcting minor issues you may experience.

Table 8. Troubleshooting guide.

Issue	Software Data to Verify	Affected Sample	Cause	Resolution
Invalid	Droplet count: <8,000	Positive and negative controls	<ol style="list-style-type: none"> 1. Improper pipetting technique 2. Cartridge not filled with 8 samples 3. Poor droplet generation by the droplet generator 4. Incorrect sealing of the PCR plate (foil, PX1 PCR Plate Sealer) 	<ol style="list-style-type: none"> 1. Training 2. Training 3. Instrument maintenance 4. See section 3 for information about supplies required but not provided and repeat
Invalid	Droplet count: <8,000	Unknown	<ol style="list-style-type: none"> 1. See causes above 2. Food matrix incompatibility 3. Stomaching of the flour before enrichment 4. Mixing of the bag after enrichment 5. More than 30 min between droplet generation and PCR 6. PCR inhibitors 	<ol style="list-style-type: none"> 1. See resolutions above 2. Use recommended protocol for matrices and repeat 3. Repeat without stomaching 4. Repeat without mixing postenriched sample 5. Repeat, ensuring less than 30 min between droplet generation and PCR 6. Repeat after diluting the enriched sample 1/5
Invalid	<i>stx</i> and <i>eae</i> value copies range: 50–3,500	Positive control not in range	<ol style="list-style-type: none"> 1. More than 3 freeze-thaw cycles of kit 2. 0 or 2 positive controls loaded 3. Use of an external positive control 4. Wrong clustering of the control 	<ol style="list-style-type: none"> 1. Repeat with new kit 2. Repeat the ddPCR experiment loading 1 positive control in the High Positive Control well 3. Repeat with kit positive control 4. Manually set positive control threshold
Invalid	<i>stx</i> and <i>eae</i> value copies range: <15	Negative control not in range	<ol style="list-style-type: none"> 1. Contamination of the BPW 2. Wrong clustering of the control 	<ol style="list-style-type: none"> 1. Repeat with fresh BPW or new kit 2. Manually set negative control threshold
Invalid	Linkage	Positive control less than 30%	<ol style="list-style-type: none"> 1. Degradation of the positive control 2. Use of an external positive control 	<ol style="list-style-type: none"> 1. Repeat with new kit 2. Repeat with kit positive control
Invalid	Linkage	Unknown less than –10%	Wrong clustering	Manually set threshold
Invalid	Interpretation of the positive control	Unknown	See all cases of invalid positive control above	See all cases of invalid positive control above
Clogged filter	None	Unknown	<ol style="list-style-type: none"> 1. Filter bag not used 2. Use of the wrong filter reference 3. Mixing of the bag after enrichment 4. Too much particulate from sample 	<ol style="list-style-type: none"> 1. Repeat with filter sample bag 2. See section 3 for information about supplies required but not provided and repeat 3. Repeat without mixing postenriched sample 4. Repeat from sample bag
Run aborted — Hardware	Warning message: All wells were not correctly processed	All	<ol style="list-style-type: none"> 1. Too many foil seals used to seal plate 2. QX200 ddPCR System hardware error 	<ol style="list-style-type: none"> 1. Repeat plate setup from enrichment 2. Contact Technical Support <p>Note: Droplet reading must be initiated in <3 hr</p>
Run aborted — Software	Warning message: All wells were not correctly processed	All	Computer issue	Contact Technical Support

Section 10

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Section 11

Appendix: ddPCR Mix Calculation Guide

To find the correct volumes to use when preparing the ddPCR mix, add the total number of samples and controls to be analyzed and find the corresponding volumes of reagent ddP and reagent ddA in Table 9.

Table 9. ddPCR mix calculation guide.

Total Number of Samples and Controls	Primer-Probe Mix Reagent ddP, μ l	Amplification Mix Reagent ddA, μ l	Total Number of Samples and Controls	Primer-Probe Mix Reagent ddP, μ l	Amplification Mix Reagent ddA, μ l	Total Number of Samples and Controls	Primer-Probe Mix Reagent ddP, μ l	Amplification Mix Reagent ddA, μ l
1	12	6	33	430	215	65	840	420
2	26	13	34	450	225	66	850	425
3	40	20	35	460	230	67	870	435
4	52	26	36	470	235	68	880	440
5	66	33	37	490	245	69	890	445
6	80	40	38	500	250	70	910	455
7	92	46	39	510	255	71	920	460
8	104	52	40	520	260	72	930	465
9	120	60	41	540	270	73	940	470
10	130	65	42	550	275	74	960	480
11	146	73	43	560	280	75	970	485
12	160	80	44	570	285	76	990	495
13	170	85	45	580	290	77	1,000	500
14	180	90	46	590	295	78	1,000	500
15	200	100	47	610	305	79	1,000	500
16	210	105	48	620	310	80	1,050	525
17	220	110	49	630	315	81	1,050	525
18	230	115	50	650	325	82	1,050	525
19	250	125	51	660	330	83	1,050	525
20	260	130	52	670	335	84	1,100	550
21	280	140	53	680	340	85	1,100	550
22	290	145	54	700	350	86	1,100	550
23	300	150	55	710	355	87	1,100	550
24	320	160	56	720	360	88	1,150	575
25	330	165	57	740	370	89	1,150	575
26	340	170	58	750	375	90	1,150	575
27	350	175	59	770	385	91	1,150	575
28	370	185	60	780	390	92	1,200	600
29	380	190	61	790	395	93	1,200	600
30	390	195	62	800	400	94	1,200	600
31	410	205	63	820	410	95	1,200	600
32	420	210	64	830	415	96	1,250	625

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