Absolute Q™ Liquid Biopsy dPCR Assays

USER GUIDE

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Revision B.0

ThermoFisher SCIENTIFIC

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For descriptions of symbols on product labels or product documents, go to thermofisher.com/symbols-definition.

Revision history: Pub. No. MAN0025690

Revision	Date	Description
B.0	27 July 2022	• Information was added regarding assay ordering and specifications ("Assay ordering and specifications" on page 4). • Instructions were revised for preparing the dPCR reaction mix ("Prepare the dPCR reaction mix" on page 6). • Instructions were revised for loading the MAP plate ("Load the reagent mix into the MAP plate" on page 8). • Instructions were revised for setting up the experiment ("Set up and run the experiment" on page 11). • Instructions were revised for setting up the plate for analysis ("Post-analysis procedure" on page 17).
A.0	13 September 2021	New publication documenting the use of the Absolute Q™ Liquid Biopsy dPCR Assays with the QuantStudio™ Absolute Q™ Digital PCR System.

The information in this guide is subject to change without notice.

Contents

1 Product Information

Note: For safety and biohazard guidelines, refer to the "Safety" section in the *QuantStudio™ Absolute Q™ Digital PCR System Installation, Use, and Maintenance Guide* (Pub. No. MAN0025621).

WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from **thermofisher.com/support**.

For detailed instructions on preparing and running digital PCR (dPCR) experiments, see the *QuantStudio™ Absolute Q™ Digital PCR System Installation, Use, and Maintenance Guide* (Pub. No. MAN0025621).

Product description

The Absolute Q™ Liquid Biopsy dPCR Assays enable the detection of somatic mutations with high sensitivity and specificity. For liquid biopsy analysis, dPCR assays are a precise cost-effective and rapid method for cancer research, including studying the potential of response monitoring and resistance to potential treatment. These assays are formulated to work seamlessly with the QuantStudio™ Absolute Q™ Digital PCR System.

Assay ordering and specifications

For a complete list of available Absolute Q™ Liquid Biopsy dPCR Assays, contact your local sales representative or go to **http://www.thermofisher.com/dpcr-liquidbiopsy**.

Absolute Q™ Liquid Biopsy dPCR Assay specifications:

- 1 tube at 20X concentration
- Store at –20°C

Required materials not supplied

Unless otherwise indicated, all materials are available through **thermofisher.com**. "MLS" indicates that the material is available from **fisherscientific.com** or another major laboratory supplier.

Catalog numbers that appear as links open the web pages for those products.

Item	Source
Digital PCR System	
QuantStudio™ Absolute Q™ Digital PCR System	A52864
Equipment	
Centrifuge, bench top	MLS
Vortex mixer	MLS
Pipettes, P20 or P200	MLS
Filter pipette tips, P20 or P200	MLS
NanoDrop™ 2000/2000c Spectrophotometer *or* Qubit™ 4 Fluorometer	ND-2000 Q33238
Other consumables	
QuantStudio™ Absolute Q™ MAP16 Plate Kit includes: • 12 QuantStudio™ Absolute Q™ MAP16 Digital PCR Plates • 60 QuantStudio™ Absolute Q™ MAP plate gasket strips • 3 mL of QuantStudio™ Absolute Q™ Isolation Buffer	A52865
Absolute Q™ DNA Digital PCR Master Mix (5X)	A52490
Control DNA (from CEPH Individual 1347-02) (CEPH gDNA control) (used as a wild-type control)	403062
Low bind microcentrifuge tubes	MLS
Microcentrifuge tube rack	MLS
Nuclease-free water	MLS

2 Prepare and run an experiment

This section provides a procedure for preparing a dPCR reaction mix with an Absolute Q™ Liquid Biopsy dPCR Assay.

For detailed instructions on preparing and running digital PCR (dPCR) experiments, see the *QuantStudio™ Absolute Q™ Digital PCR System Installation, Use, and Maintenance Guide* (Pub No. MAN0025621).

Mutant alleles versus wild-type alleles

For liquid biopsy dPCR assays, copies of the mutant allele are positive in the FAM™ channel, while copies of wild-type alleles are positive in the VIC™ channel. For details on using a wild-type control to calculate the mutant allele frequency percentage of unknown samples, see “Normalize the background and calculate the mutant allele frequency percentage (%MAF)” on page 19.

Note: For each run, we recommend 2–3 replicates of each unknown sample plus 1 wild-type control (CEPH gDNA control; see “Required materials not supplied” on page 5).

Prepare the dPCR reaction mix

Gather the following materials:

- P10 or P20 pipette and filter pipette tips
- Absolute Q™ DNA Digital PCR Master Mix (5X)
- Absolute Q™ Liquid Biopsy dPCR Assay (40X)
- CEPH gDNA control, if needed for your assay
- Nuclease-free water

The volume of the dPCR reaction can be adjusted depending on experimental requirements.

IMPORTANT!

- Throughout this procedure, protect reagents from light when not in use.
- For best results, perform the run within one hour of reaction preparation.

1. Isolate and prepare DNA from each liquid biopsy sample of interest. Prepare 500–2,000 copies of sample DNA in a volume of ≤7.75 µL per reaction.

   Note:
   - Do not freeze and reuse working DNA solution.
   - We recommend using the NanoDrop™ 2000/2000c Spectrophotometer or the Qubit™ 4 Fluorometer to determine quantity.
   - A DNA copy and dilution calculator can be found at **http://www.thermofisher.com/DNA-calculator**.

2. Prepare the CEPH gDNA control as the wild-type control. Dilute the CEPH gDNA control to the same concentration as your unknown sample.

3. Thaw and equilibrate all reagents to room temperature before use.

   Note:
   - Store reagents on ice when not in use.
   - Limit number of reagent freeze/thaw cycles.

4. Pulse vortex the Absolute Q™ DNA Digital PCR Master Mix (5X) and Absolute Q™ Liquid Biopsy dPCR Assay at high speed for 10 seconds.

5. Combine the following reagents in the order listed.

Reagent	Final concentration	Volume per reaction (with 10% overage)[1]
Absolute Q™ DNA Digital PCR Master Mix (5X)	1X	2 µL
Absolute Q™ Liquid Biopsy dPCR Assay (40X)	1X	0.25 µL
Liquid biopsy DNA sample (unknown sample) or CEPH gDNA control (wild-type control)	500–2,000 copies of unknown sample or same concentration of CEPH gDNA control	Variable, ≤7.75 µL
Nuclease-free water	—	Fill to 10 µL
Total	**—**	**10 µL**

[1] After calculating the number of reactions required, prepare dPCR mix for the appropriate number of reactions and scale those components by 10% for overage. Dilute assay accordingly to avoid pipetting less than 1 µL volumes.

6. Mix well by performing one of the following actions:
   - Pipette mix 10–20 times, or
   - Pulse vortex 3–5 times for 1 second each.

7. Using a benchtop centrifuge, centrifuge at 10,000 × *g* or the highest speed available for 1 minute.

8. Perform the run within one hour of reaction preparation.

Load the reagent mix into the MAP plate

At a clean lab bench, gather the following materials:

- P10 or P20 pipette and filter pipette tips
- Prepared dPCR reaction mix
- QuantStudio™ Absolute Q™ Isolation Buffer
- MAP plate with sufficient unused columns for the experiment
- MAP plate gasket strips (unused)

IMPORTANT! At least 1 column of the MAP plate must be run at a time. Columns cannot be reused, but a MAP plate with unused columns can be used for subsequent experiments. When the experiment is complete, if the MAP plate has unused columns, place it back into its pouch for storage.

1. Just prior to use, remove the MAP plate from its package.

   Note:
   - Leave the MAP plate in the package until ready to load sample.
   - Be careful to handle the MAP plate by its frame.
   - Place the MAP plate back into the package when not in use.

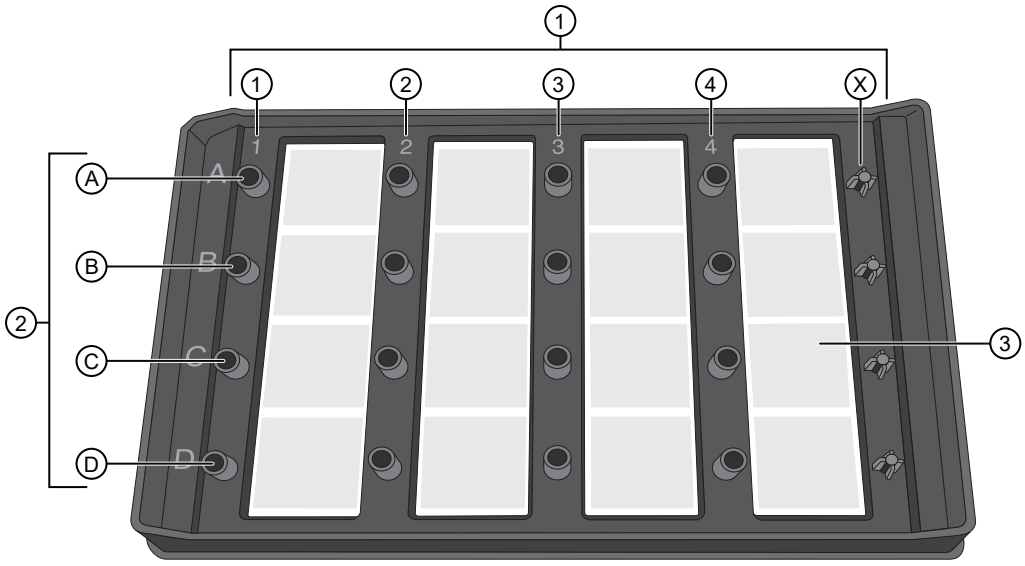


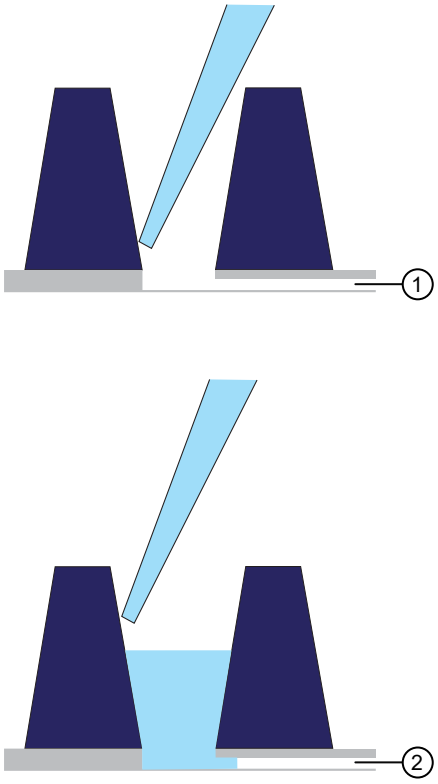
Figure 1 MAP plate without MAP plate gasket strips

① Columns 1–4 and column X
② A–D represent wells A1–D1 associated with column 1
③ Array associated with well 4C

2. Place the MAP plate on a level, dust-free, dry surface.

3. Using a new pipette tip for each well, at a 45° angle, load 9 µL of the dPCR reagent mix to the bottom of the well. Pipette the mixture only to the first stop to prevent bubble formation.

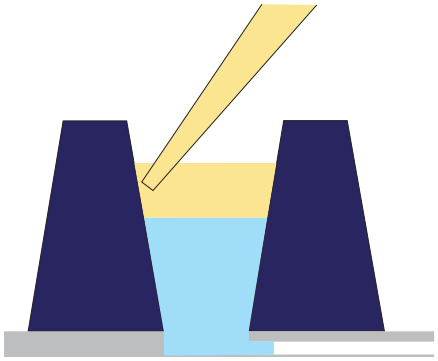
IMPORTANT! Do not contact bottom of well with the pipette tip or puncture the thin film at the bottom of the well.



① Microfluidic channel to the microreaction chamber array
② Reagent remains in the well until the instrument pushes it into the microreaction chamber array during the run

4. Using a new pipette tip for each well, at a 45° angle, load 15 µL of the Absolute Q™ Isolation Buffer on the side of the well above the top of the reagent mix. Carefully overlay the buffer on top of the reagent mix to prevent mixing or bubble formation. Pipette only to the first stop.

   The isolation buffer sits on top of the reagent, preventing contamination and evaporation.



5. Place a total of 5 MAP plate gasket strips on all 4 columns of wells and the X-shaped posts on the column X on the right side of the plate. Orient the MAP plate gasket strip so that the side labeled A–D aligns with rows A–D marked on the plate. Be sure to cover the columns completely and press the MAP plate gasket strips firmly into place.

IMPORTANT! MAP plate gasket strips must be placed on all columns, including unused columns. Failure to do so can produce poor results.

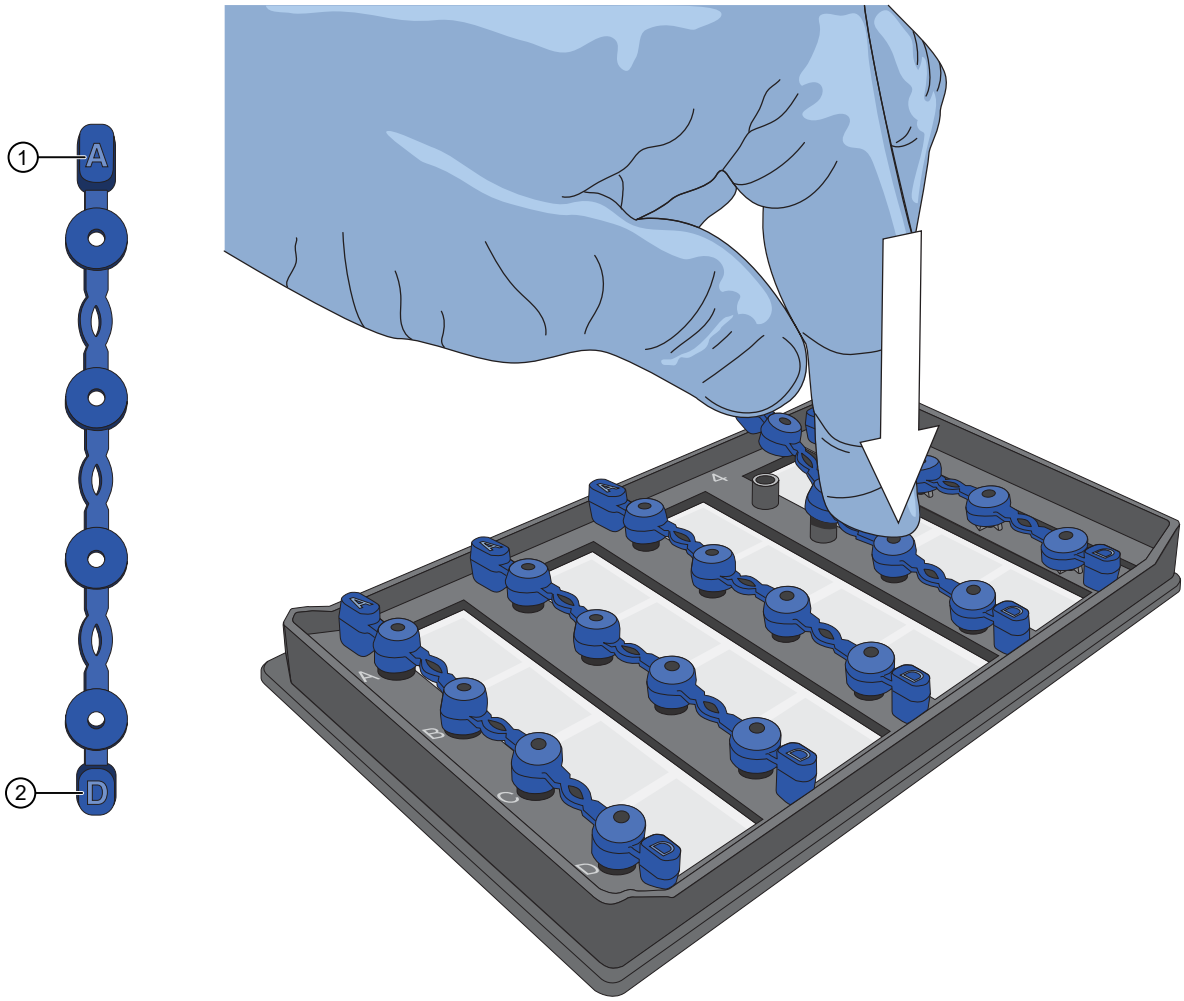


Figure 2 Place the MAP plate gasket strips firmly into place

1. Place this end of the MAP plate gasket strip on row A
2. Place this end of the MAP plate gasket strip on row D

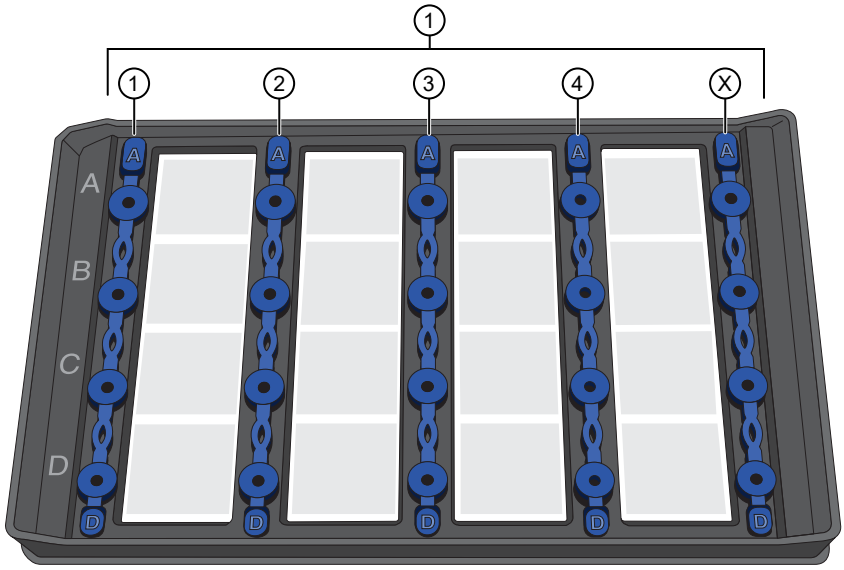


Figure 3 MAP plate with MAP plate gasket strips in place

① MAP plate gasket strips on columns 1–4 and column X

6. Move the MAP plate to the instrument.

IMPORTANT! Do not tip, invert, or shake the filled MAP plate.

Set up and run the experiment

1. In the left pane of the QuantStudio™ Absolute Q™ Digital PCR Software, select to access the **Instrument** page.
2. In the **PROTOCOL** area, select EDIT PROTOCOL.
3. In the thermal protocol settings area, enter the following settings:
   a. Set the **Preheat** setting to 96°C for 10 minutes.
   b. Set step one to 96°C for 5 seconds.
   c. Set step two to 60°C for 15 seconds.
   d. Set the **Cycles** to 40.
   e. Ensure that **Preheat** is selected.
4. In the **Channels** area, select the following optical channels:
   - **FAM™**
   - **VIC™**

Note: ROX™ should be selected by default for the QC channel.

5. Click **SAVE** to save the protocol settings.
6. Make sure that only the columns with samples are selected before beginning the experiment.
7. To load the MAP plate, click **Open** (next to the instrument icon) to open the plate tray.
   The instrument door opens to receive the loaded MAP plate.

   IMPORTANT! Confirm that gaskets are placed on all columns of the MAP plate, including unused columns. Failure to do so can produce poor results.
8. Click the **START** button under the instrument icon.
9. When prompted, verify that gaskets have been placed on all wells and on the column X posts on the far right as shown on the screen.

   Note: See callout 5 in the following figure for the location of column X.

10. Carefully load the MAP plate in the plate nest in the plate tray.

    IMPORTANT! Be sure to load the MAP plate gently to avoid damage to the plate nest.
11. Select **CLOSE DOOR**.
    The door closes and the MAP plate bar code is scanned and is displayed in the **Run name** dialog box.

    Note: If the instrument cannot scan the barcode, it can be manually added in the **Plate Barcode** field of **Run name** dialog box.
12. When prompted, enter a **Run name**.

13. Click **RUN**.
    - The run status displays in the left sidebar.
    - While processing the run, the instrument lights slowly pulse blue.
    - When the run is complete, the instrument lights are a steady blue.
    - Data populates the **ANALYSIS** tab on the **Runs** page as it becomes available.

14. When the **Run complete** dialog displays, select the run name to view the final data in the **Runs** page.

    For more information on analyzing experiment results, see Chapter 3, “Analyze data”.

3 Analyze data

Set up the plate for analysis

1. In the QuantStudio™ Absolute Q™ Digital PCR Software, when the **Run complete** dialog displays, select the run name to view the final data from the **Runs** page.
2. From the **SETUP** page in the upper-right corner of the sample plate area, click EDIT SETUP to enable editing.
3. Below the sample plate area, select **EDIT GROUPS** to open the groups dialog box.

   For information on managing groups, see the *QuantStudio™ Absolute Q™ Digital PCR System Installation, Use, and Maintenance Guide* (Pub No. MAN0025621).
4. Select **NEW GROUP** to create a group for an assay or a set of conditions for a group of samples.
5. In the **Group name** field, enter a name.
6. In the **Analysis** column, select **Signal** for the following channels.
   - **FAM™**
   - **VIC™**
7. For all other channels in the **Analysis** column, select **Not Used**.
8. In the **Samples** area, select the relevant option for your experiment, then select **SAVE** to save your changes and close the **EDIT GROUPS** dialog box.

   In this example, a group is created for the KRAS 554 assay.

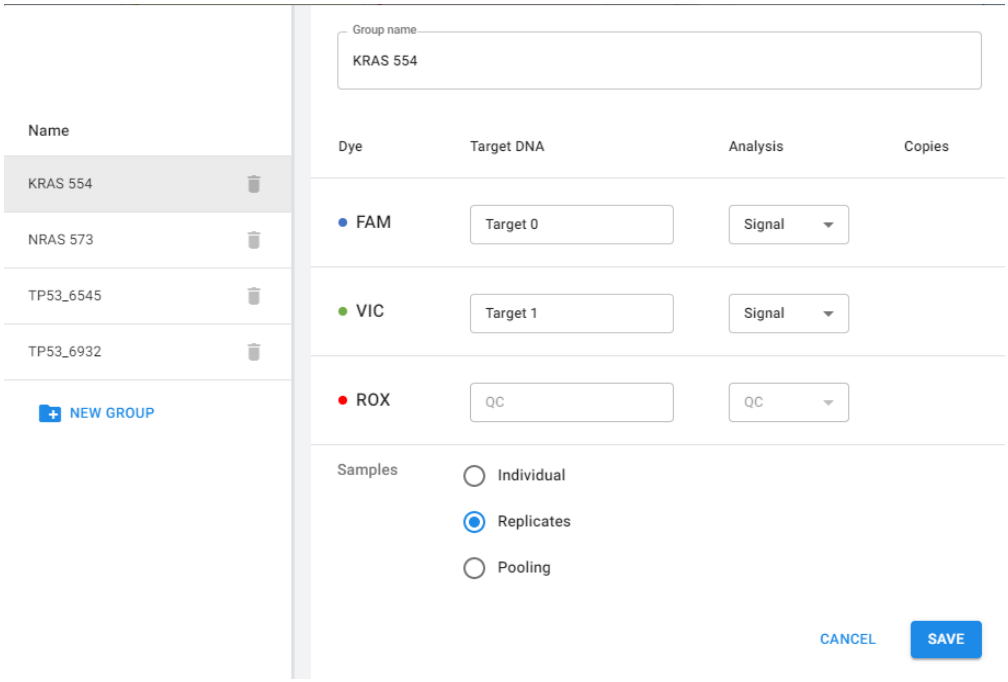


Figure 4 GROUP settings for the new group

9. On the **SETUP** page, in the sample plate area use the following options to select samples.

Option	Actions
Select a single sample.	1. Select the sample check box. 2. Click anywhere in the sample area to select it.
Select multiple samples.	1. Click and drag through samples to select multiple samples at once.

10. Click on the group name above the sample plate to assign these samples to the group.

    In this example, samples in the first column of the plate are assigned to the KRAS 554 assay group.

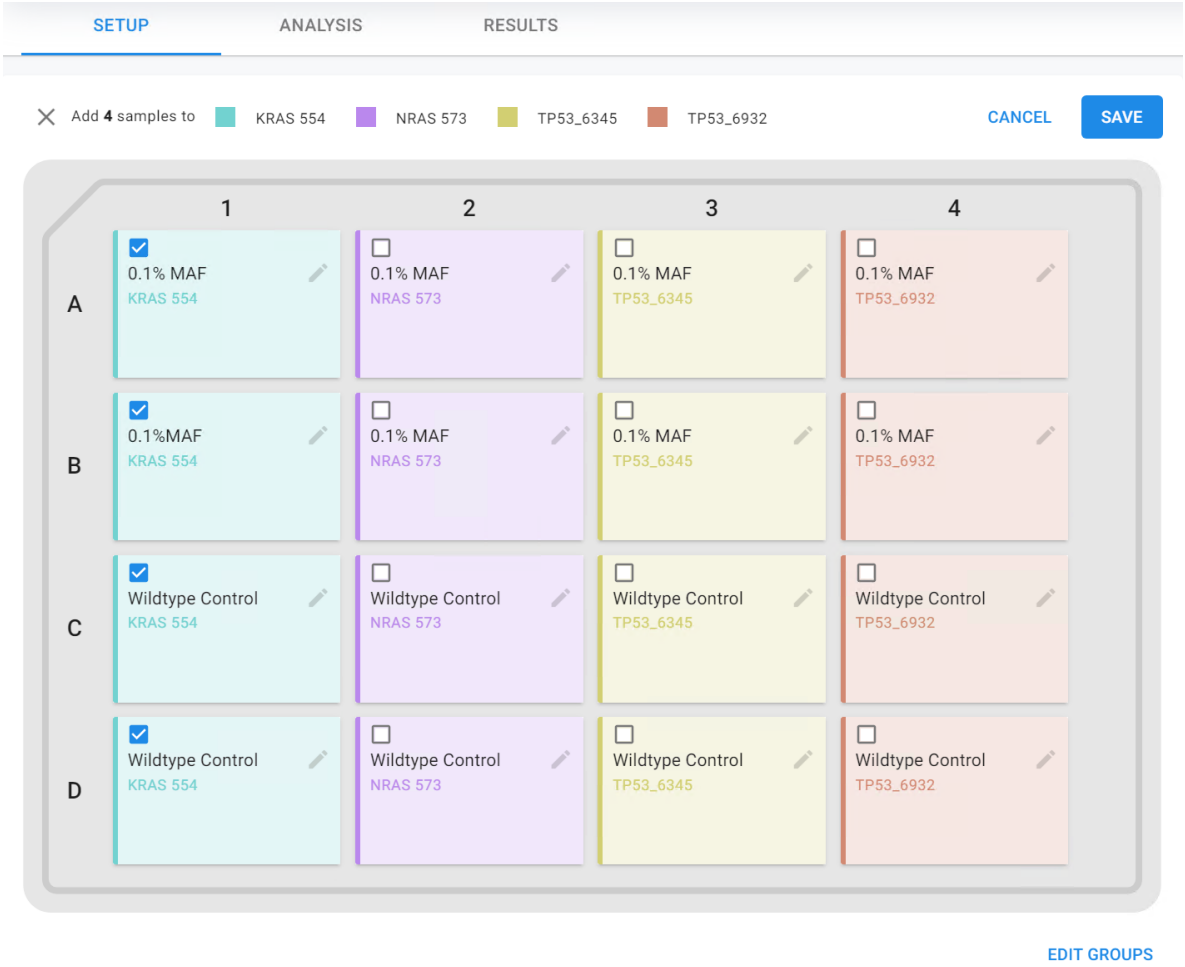


Figure 5 Assign samples to the group

11. To add information that identifies the sample, click the ✎ on the sample well, then enter a description in the label field.

    In this example, the sample in column 1, row A is selected for edit. Samples A and B are labelled as **0.1% MAF**, while samples C and D are labelled as **Wildtype Control** for the KRAS 554 assay.

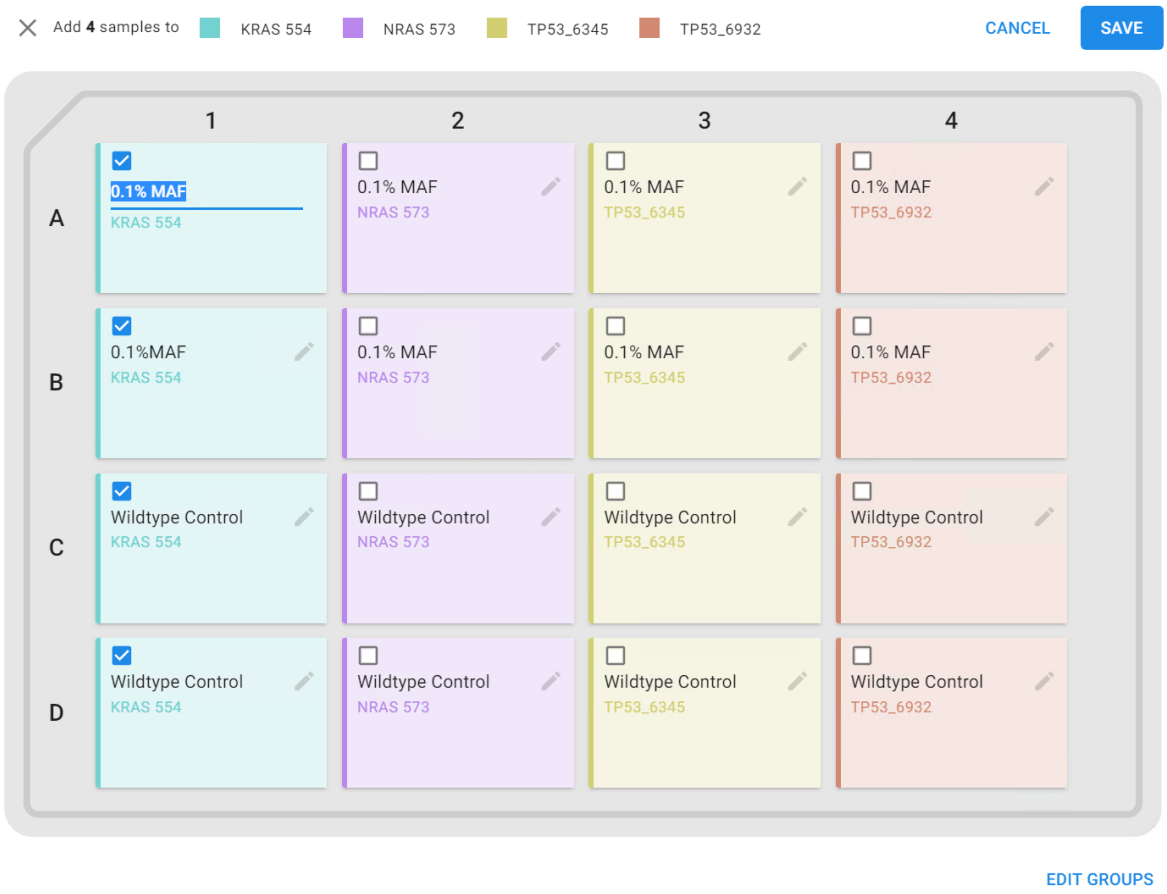


Figure 6 Label samples

12. Perform one of the following actions to save the sample name.
    - Click away from the sample name field.
    - Press enter to save the edit and open to the next sample name in the same column for editing.
13. Click **SAVE** to save your changes and exit the editing mode.

Post-analysis procedure

Adjust the threshold

When the run has completed, it is critical to inspect the wild-type control that was run with the sample.

1. In the left pane, click to access the **Runs** page.
2. Select the run, then select the **ANALYSIS** page.
3. Select **GROUPS**.

   In this example, each column is a unique liquid biopsy assay. The KRAS 554 assay samples in the first column are selected to be analyzed as a group.

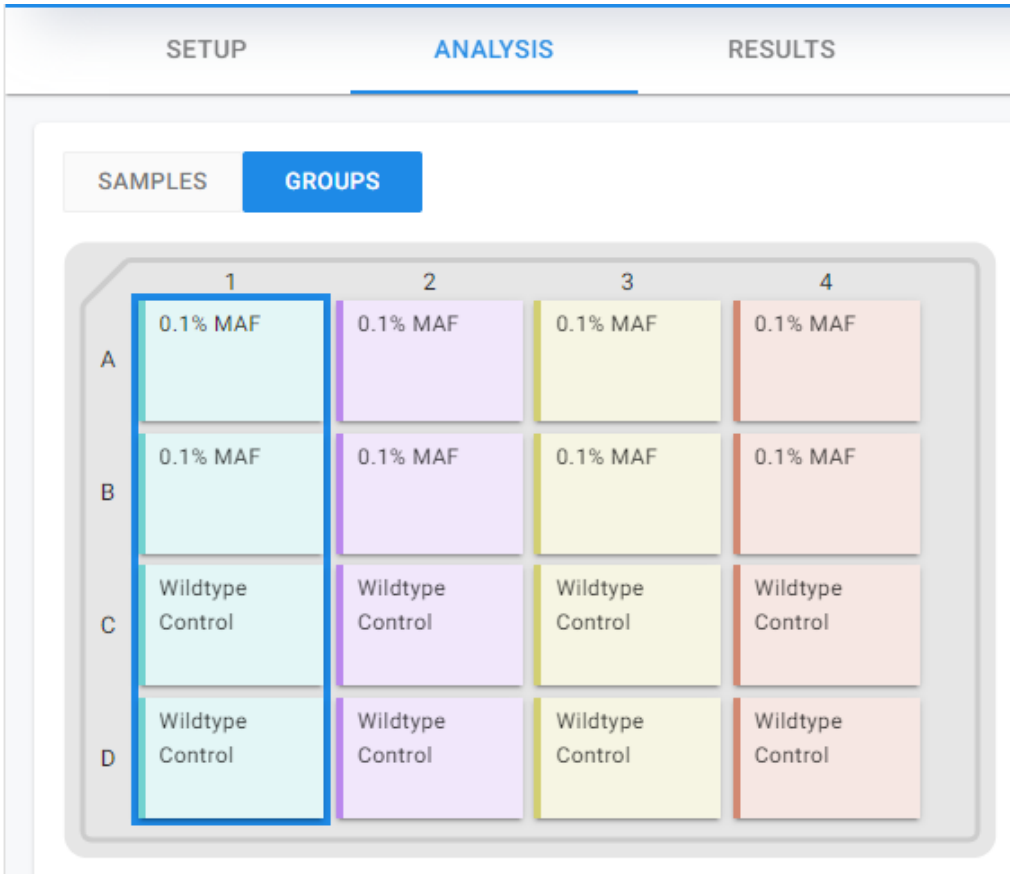


Figure 7 Analyze samples as group

4. In the plot area, select to view the 2D scatter plot of each sample in the group.

5. To enable editing in the plot area, click EDIT ANALYSIS.

6. Gate the wild-type population in VIC™ and the mutant population in FAM™ respectively by adjusting their thresholds

   a. Hover over the threshold line until the threshold value appears.

   b. Click on the threshold value, then drag the threshold bar up or down to adjust the value.

   Note: Clicking on the threshold line results in a zoom action.

   c. Click **SAVE**.

In the example below, the wild-type control samples in C1 and D1 only have cluster of wild-type population (orange) and no background positives in the mutant population in FAM™ (i.e., nothing above the FAM™ threshold line of approximately 8,600).

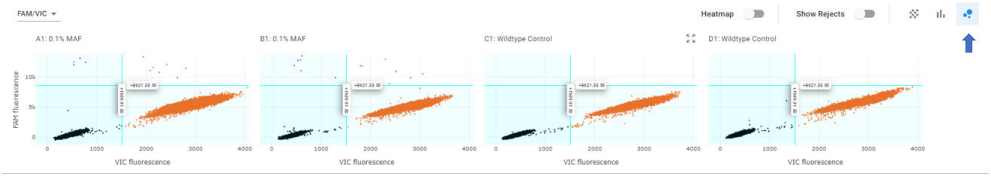


Figure 8 Adjust thresholds on wild-type control samples

Normalize the background and calculate the mutant allele frequency percentage (%MAF)

To detect a target mutant allele at low abundance against wild-type background, use the wild-type control (CEPH gDNA control) to establish the background signal in the FAM™ channel, as described in the following procedure.

1. From the concentration table on the **RESULTS** page in the QuantStudio™ Absolute Q™ Digital PCR Software, record the concentrations of the unknown sample (units = cp/µL) in the FAM™ and VIC™ channels, and the concentration of the wild-type control in the FAM™ channel.

			FAM						VIC					
Name	Well	Total	Target	Conc. cp/µL	SD	CV%	95%CI	Positives	Target	Conc. cp/µL	SD	CV%	95%CI	Positives
	Average	**20456.50**	**Target 1**	**1.08**	**0.86**	**79.20**	**0.27**	**9**	**Target 2**	**2050.76**	**23.72**	**1.16**	**18.93**	**11758**
0.1% MAF	A3	20469		2.11			0.78	18		2079.22			38.24	11868
0.1% MAF	B3	20460		1.76			0.70	15		2051.54			37.86	11763
Wildtype Control	C3	20443		0.23			0.18	2		2058.67			37.98	11779
Wildtype Control	D3	20454		0.23			0.18	2		2013.63			37.35	11621

Figure 9 Concentration table example

2. Correct the FAM™ concentration (units = cp/µL) of the unknown sample using the following equation:

$$FAM_{corrected} = FAM_{unknown} - FAM_{wildtype} \qquad (Eq. 1)$$

where $FAM_{corrected}$ is the background-corrected FAM™ concentration, $FAM_{unknown}$ is the FAM™ concentration of the unknown sample, and $FAM_{wildtype}$ is the FAM™ concentration of the wild-type control sample. $FAM_{corrected}$ corresponds to the mutant template concentration.

3. Calculate the percentage mutant concentration of the unknown sample using the following equation:

$$\%MAF = \frac{FAM_{corrected}}{VIC_{unknown} + FAM_{corrected}} \times 100\% \qquad (Eq. 2)$$

where *%MAF* is the mutant allele frequency and $VIC_{unknown}$ is the VIC™ concentration of the unknown sample.

A Documentation and support

Related documentation

Document	Publication number	Description
QuantStudio™ Absolute Q™ Digital PCR System Installation, Use, and Maintenance Guide	MAN0025621	Detailed instructions for using the QuantStudio™ Absolute Q™ Digital PCR System to prepare and run digital PCR experiments, and analyze results.

Note: For additional documentation, see "Customer and technical support" on page 20.

Customer and technical support

Visit **thermofisher.com/support** for the latest service and support information.

- Worldwide contact telephone numbers
- Product support information
  - Product FAQs
  - Software, patches, and updates
  - Training for many applications and instruments
- Order and web support
- Product documentation
  - User guides, manuals, and protocols
  - Certificates of Analysis
  - Safety Data Sheets (SDSs; also known as MSDSs)

  Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale at **www.thermofisher.com/us/en/home/global/terms-and-conditions.html**. If you have any questions, please contact Life Technologies at **www.thermofisher.com/support**.