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Wisconsin Wastewater Monitoring Program's dPCR Quantification Workflow Using QIAGEN QIAcuity System

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We use this protocol and it's working

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Abstract

This protocol demonstrates how the Wisconsin State Laboratory of Hygiene quantifies nucleic acid of targeted pathogens in wastewater using the QIAcuity digital PCR (dPCR) system. The PCR parameters listed in this protocol are examples and may not represent our most current testing methods. Visit the following link to find most up-to-date dPCR information for our program's core monitoring targets: <https://doi.org/10.6084/m9.figshare.30836981>.

Image Attribution

Images created by the authors.



Materials

Equipment and Supplies:

QIAcuity Four Platform System FUL-2 (Cat. No. / ID: 911046)

Refrigerator storage (4°C)

Freezer storage (-80°C or below)

Ice box or a cooling block

Plate centrifuge or plate spinner

Biosafety level 2 biosafety cabinet or dead air box

QIAcuity Nanoplate 26k 24-well (Cat. No. / ID: 250001)

QIAcuity Nanoplate 8.5k 96-well (Cat. No. / ID: 250021)

Nanoplate Tray (Cat. No. / ID: 250098)

Nanoplate Seals (Cat. No. / ID: 250099)

Plate roller

Absorbent sheets

Sterile disposable strip tubes (eg.: 0.1mL)

Reagents and Standards:

QIAcuity OneStep Advanced Probe Kit; **Catalog #250132** [Includes: OneStep Advanced Probe Master Mix and OneStep RT (Reverse Transcriptase) Mix]

Resources:

Wisconsin State Laboratory of Hygiene PCR parameters: <https://doi.org/10.6084/m9.figshare.30836981>

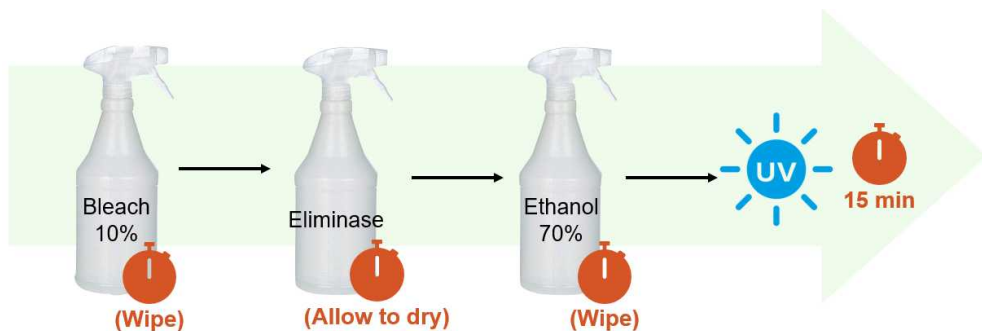
User manual for QIAcuity instruments and QIAcuity Software 2.5:  QIAcuity User Manual.pdf

Software:

QIAcuity Software Suite 2.5.0.1

Before start

Before and after using the dead air box (DAB) or biosafety cabinet (BSC), clean work area with 10% bleach, followed by ELIMINase™, and 70% ethanol. Then, turn on the interior ultraviolet (UV) light for at least 15 minutes.



QIAGEN QIAcuity software set-up

15m

- 1 Login to QIAGEN QIAcuity software.
- 2 Use the software to set up a QIAcuity Nanoplate with appropriate PCR parameters.

The screenshots provided throughout this workflow use SARS-CoV-2 N1 and N2 gene quantification settings as an example.

- 2.1 Click the "new plate" button.



Go to Step 2.2 if you do NOT have a plate template saved.

Go to Step 2.3 if you have a plate template saved from previous use.

- 2.2 Under the "**General Data**" tab fill in the **Plate name** field with a description for your batch of samples. In the **Plate type** drop-down menu choose your preferred Nanoplate such as a Nanoplate 26K 24-well. If desired, fill out the **Description** field with a description of the samples or system parameters. Leave the **Labels** field blank. The **Plate Ownership** field will autofill with the credentials that were used to login to the software. Leave the **Barcode** field blank.



General Data

Plate name *

Characters left: 96

Plate type

Description

Labels

Labels left: 10

Plate Ownership *

Type user name, surname or login to add user

Barcode

Note

There are several options to consider when choosing the type of Nanoplate to use for your desired disease target. QIAGEN recommends using the Nanoplate 8.5K 96-well for more abundant targets while the Nanoplate 26K 24-well is better suited for more rare disease targets.

Click the "Save changes" button.

On the left side of the screen, click the tab labeled "dPCR Parameters". Under the "Priming" tab, choose the preferred Priming Profile for your analysis.

QIAGEN

NEW PLATE CONFIGURATOR

date samples received# plate#

General Data

dPCR parameters

Reaction mixes

Samples & controls

Plate layout

dPCR parameters

Priming Cycling Imaging

Priming Profile Nanoplate 26K 24-well plate

☒ QIAGEN Priming Profile Probe (RT-) PCR
specific priming for QIAcuity Probe based (RT-) PCR Kits

☐ QIAGEN Priming Profile EvaGreen (RT-) PCR
specific priming for QIAcuity EvaGreen based (RT-) PCR Kits

Click the "Cycling" tab and set the cycling parameters for your disease target.

QIAGEN

NEW PLATE CONFIGURATOR

date samples received# plate#

General Data

dPCR parameters

Reaction mixes

Samples & controls

Plate layout

dPCR parameters

Priming Cycling Imaging

Temperature * °C Duration * min:ss Add step

Cycling profile

Start (room temperature)

	Temperature	Duration	Delete	Group	Ungroup
1 x	50 °C	40 min 00 s			
1 x	95 °C	02 min 00 s			
40 x	95 °C	00 min 15 s			
	60 °C	00 min 30 s			

End

Click the "Imaging" tab and set the imaging specifications for your target.

dPCR parameters

Priming Cycling Imaging

Channel

Exposure duration

Gain

Green 300 ms 6

Yellow 300 ms 6

On the left side of the screen, click the tab labeled "Reaction mixes".

- General Data
- dPCR parameters
- Reaction mixes**
- Samples & controls
- Plate layout

Click the "New Reaction" button.

⊕ New Reaction Mix

In the pop-out window, fill out the specifications for you reaction mix and click "Create".

New Reaction Mix

Reaction mix name *
 Characters left: 88
 Total volume

μl

 Change color

Color	Target Name *	Dye	Channel(s) *		
	N1	FAM	● Green	☐ Internal control	
	N2	VIC	● Yellow	☐ Internal control	
	Name	Select	Select	☐ Internal control	
	Name	Select	Select	☐ Internal control	
	Name	Select	Select	☐ Internal control	

Cancel

Create

Click "Save changes" at the bottom of the screen.

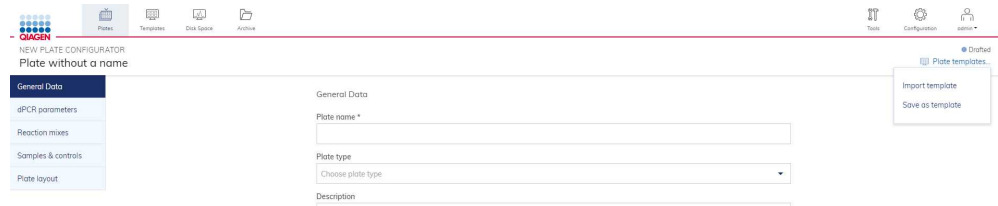
Note

Skip the tab labeled "Samples & Controls". The values in this tab will auto-populate after the data upload is completed in the next step (2.4).

Skip Step 2.3 and go to Step 2.4.

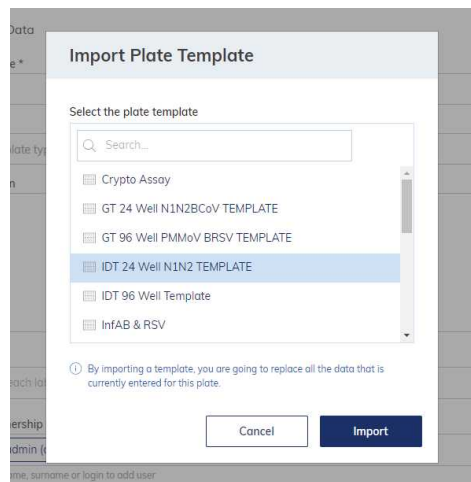
2.3 This step is for users that have a saved plate template.

Click on "plate templates" in the upper right corner of the screen. In the dropdown menu, click on "import template".



The screenshot shows the 'NEW PLATE CONFIGURATOR' interface. On the left, there's a sidebar with 'General Data' selected. The main area has a form with fields for 'Plate name *', 'Plate type' (with a dropdown menu), and 'Description'. In the top right corner, a dropdown menu for 'Plate templates' is open, showing 'Import template' and 'Save as template' options.

In the pop-up window, select the saved plate template for quantification of your desired disease target and click "Import".



The screenshot shows a pop-up window titled 'Import Plate Template'. It contains a search bar and a list of templates. The 'IDT 24 Well N1N2 TEMPLATE' is selected. Below the list, there's a warning message: 'By importing a template, you are going to replace all the data that is currently entered for this plate.' At the bottom, there are 'Cancel' and 'Import' buttons.

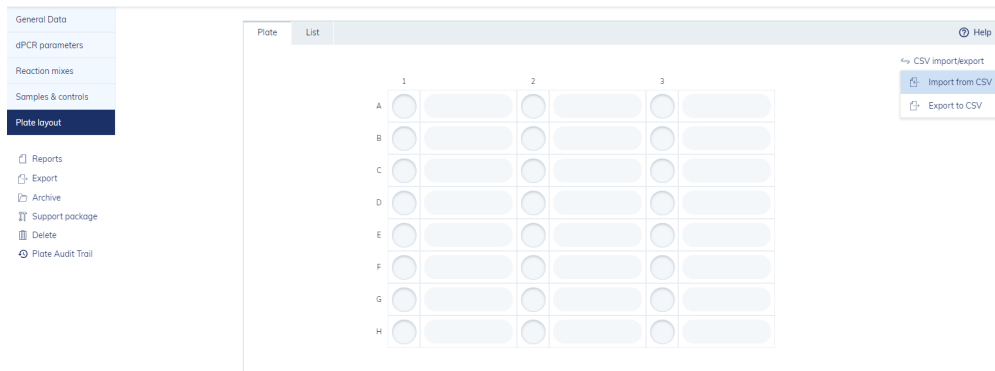
Fill in the "Plate name" field with a description for your batch of samples. Click the "Save changes" button.



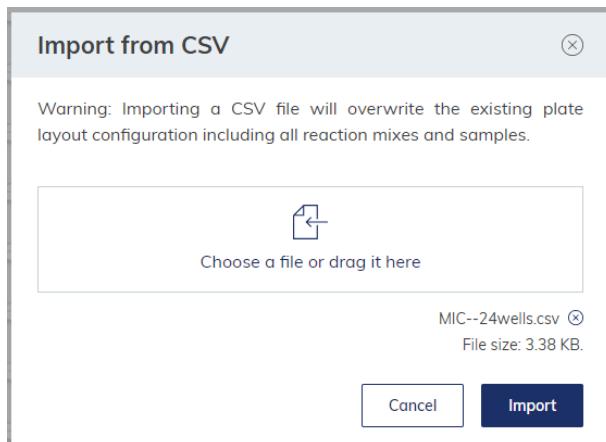
The screenshot shows the 'General Data' section of the form. The 'Plate name *' field is highlighted, containing the text 'date samples received# plate#'. To the right of the field, it says 'Characters left: 96'.



- 2.4 On the left side of the screen, click the tab labeled "Plate Layout". Then click on "CSV import/export" in the top right of the screen. (If "Import from CSV" is grayed out, you need to save the plate.)



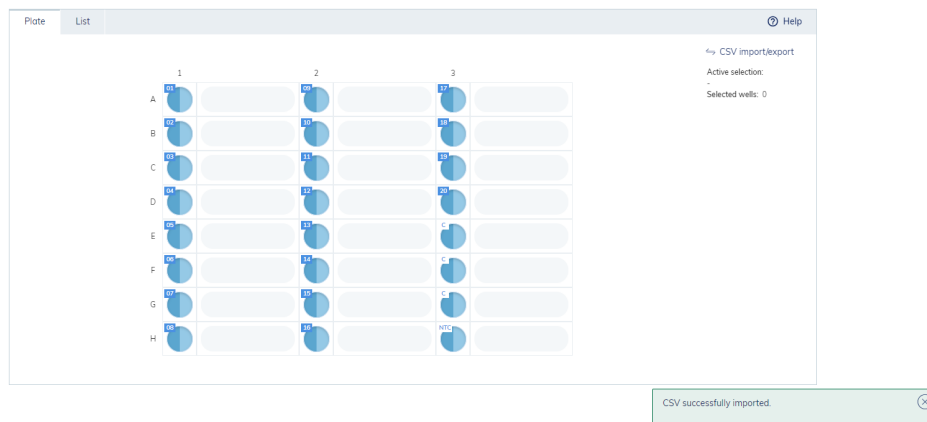
In the pop-up window, click on the file button to open file explorer. Choose your comma-separated values (CSV) file containing the sample specifications for the sample batch, and click "Import" to upload the file.



Note

For assistance creating the CSV file go to chapter 7.5.6 in the QIAcuity User Manual which can be found in the Materials section of this protocol.

The sample wells in the plate will populate with color representing the reaction mix(es) that were selected for your desired disease target. Verify the plate layout in the QIAcuity software matches the sample layout on your plate map.



2.5 Save the plate by clicking "Done".



Sample processing: making master mix

45m

- 3 Perform this section in a clean molecular area that is designated for reagent prep and separate from sample handling and analysis areas. Remove the One-Step Advanced Master Mix, One-Step Advanced RT Mix, and oligonucleotides for your desired disease target from the storage freezer to thaw.

Note

Do not leave reagents at room temperature longer than necessary. The One-Step Advanced Master Mix and RT Mix should be kept on ice or in a cooling block as much as possible, while thawed and outside of the freezer.

- 4 Combine the appropriate amount of each reagent (e.g., sterile water, primers, probes, One-Step Advanced Master Mix, and One-Step Advanced RT Mix) for the number of samples in the batch plus 2-4 samples of overage to account for potential pipetting errors. The final mixture is the working master mix.

Note

The list of oligonucleotide sequences, their final concentrations in the PCR reaction, the dPCR cycling and imaging settings, as well as the positive controls used in the core wastewater surveillance conducted by the Wisconsin Wastewater Monitoring Program can be found at this [website](#).

- 4.1 Return One-Step Advanced Master Mix, One-Step Advanced RT Mix, and all oligonucleotides to the freezer for storage.
- 4.2 Take your completed working master mix and move to the next molecular level, i.e. template addition area to perform the remaining sample analysis steps.

Sample processing: preparing QIAcuity Nanoplates

1h

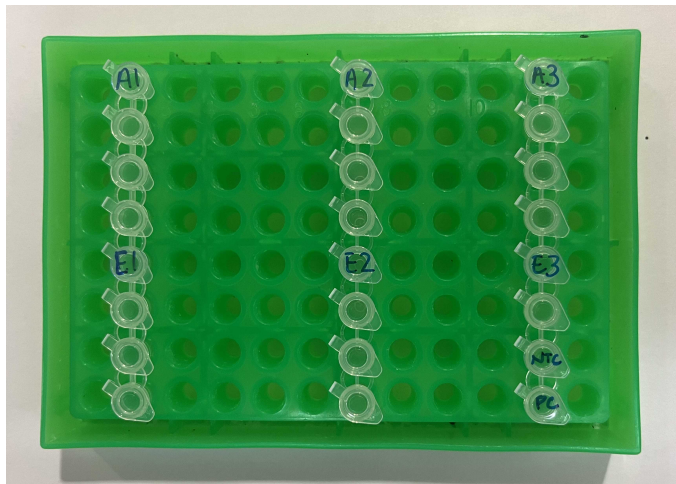
- 5 Inside a decontaminated dead air box or biosafety cabinet, obtain one set of 8-well strip tubes for every column on your plate map that contain samples. Label the strip tubes corresponding to the plate layout.

Pictured below is an example of a plate map for a Nanoplate 26K 24-well with corresponding labeled strip tubes.

Example plate map for a Nanoplate 26K 24-well

	1	2	3
A	8690	8698	8706
B	8691	8699	8707
C	8692	8700	8708
D	8693	8701	8709
E	8694	8702	8710
F	8695	8703	8711
G	8696	8704	Negative Control
H	8697	8705	Positive Control

An example of a 24-well plate map



- 6 Pipette the working master mix into the strip tubes following the plate map layout.

Note

The volume of working master mix varies depending on the Nanoplate being used in the procedure.

Nanoplate 26K 24-well: 38.5uL of master mix

Nanoplate 8.5K 96-well: 8uL of master mix

- 7 Obtain the freshly extracted nucleic acids (for best results avoid freeze-thaws) of all the samples for this PCR analysis batch. Mix the samples well by pipetting up and down and



then pipette the appropriate amount (equal to the PCR template for a given assay) into the strip tubes on top of the working master mix.

Note

The volume of nucleic acid material varies depending on the Nanoplate being used in the procedure.

Nanoplate 26K 24-well: 5.5uL of extracted nucleic acid sample

Nanoplate 8.5K 96-well: 8uL of extracted nucleic acid sample

8 Vortex the strip tubes for 2 minutes.

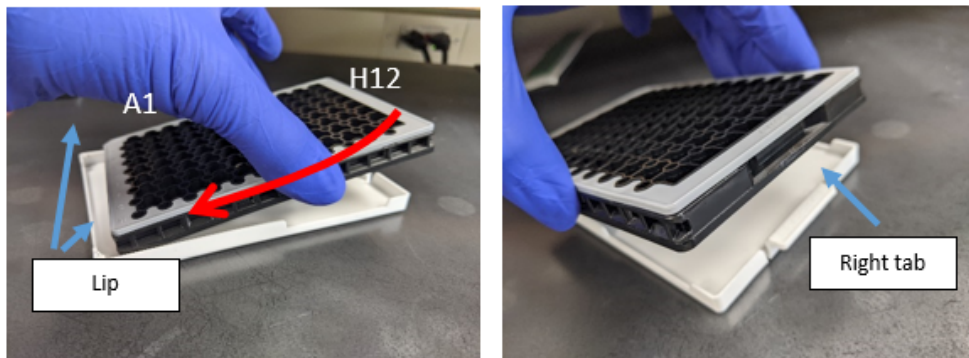
8.1 Centrifuge strip tubes thoroughly until there are no bubbles in the mixture.

Note

Bubbles transferred into the final plate may impact how the sample is spread over the partitions and prevent proper imaging.

9 Obtain the QIAcuity Nanoplate needed to quantify your disease target. Do not touch the bottom of the Nanoplate.

- 9.1 Insert the Nanoplate into a clean, dust-free Nanoplate Tray. Slide the left edge (Column A wells) of the Nanoplate into the Nanoplate Tray until it is pushed under the Tray lip opposite from the tab. While pushing down on the tab, gently lay the Nanoplate down into the Tray.



- 10 Transfer the master mix/sample mixture from the strip tubes into the Nanoplate following your plate map layout.

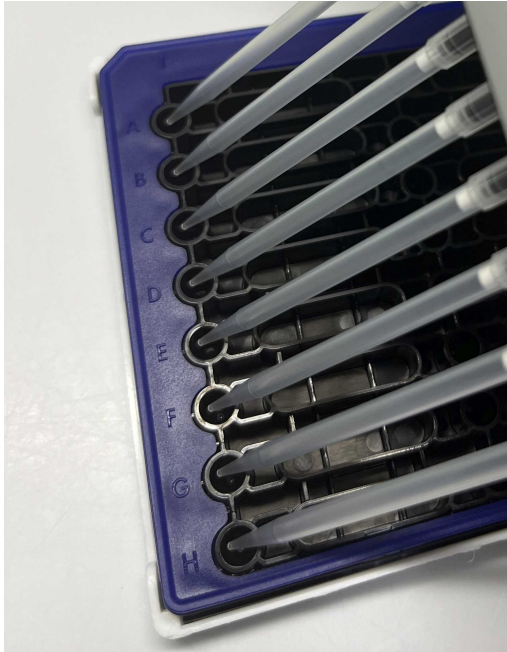
Note

The volume of master mix/sample mixture transferred to the Nanoplate varies depending on the Nanoplate being used in the procedure.

Nanoplate 26K 24-well: 40uL of master mix

Nanoplate 8.5K 96-well: 12uL of master mix

Do not touch the bottom of the Nanoplate well with the pipette tip as it may damage the Nanoplate channels and prevent proper filling of the plate wells.



- 11 Obtain a Nanoplate Seal. Press down on a short edge of the seal to adhere it to the plastic. Peel off the white plastic side of the seal.

Note

Sealing the Nanoplate is a critical step in this process. Ineffective plate sealing techniques may prevent imaging of the Nanoplate wells. Watch the QIAGEN plate sealing video tutorial [here](#) (minute 10:40).

- 11.1 While holding the clear plastic tabs, align the sticky blue side of the seal with the left edge of the Nanoplate. Slowly lay the seal down from left to right to prevent bubbles from being trapped under the seal. Be sure that all of the wells are fully covered by the blue rubber seal.
- 11.2 To secure the seal to the surface of the Nanoplate use the plate roller and make unidirectional movements across the seal from each edge of the plate.
- 11.3 Slowly peel the clear plastic off of the Nanoplate making sure the blue seal is not removed with it.
- 11.4 Take the plate roller and roll over the blue seal again from every direction. Firmly roll over the edges of the seal. Gently fold over any excess blue seal, sticking it to the side of the

plate, if needed .

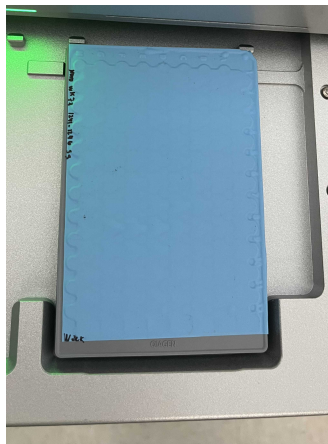
QIAcuity dPCR System: loading nanoplates

5m

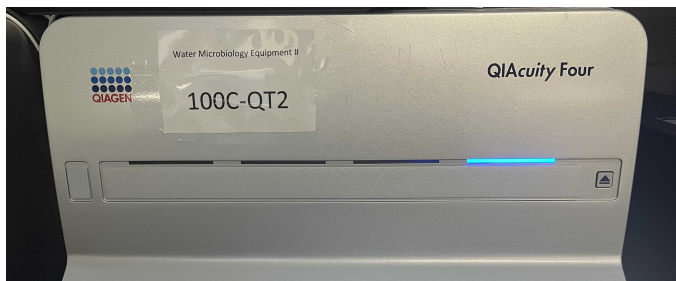
12 Take your sealed Nanoplate to the QIAcuity instrument.

12.1 Login to the software. Click the eject tray button.

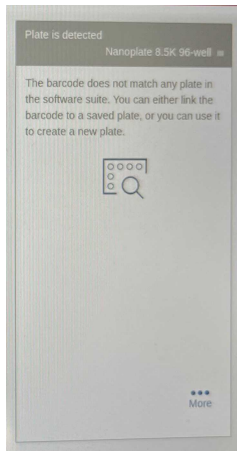
12.2 Remove the Nanoplate from its white Nanoplate Tray by pressing down on the tab and gently sliding the Nanoplate out from the lip of the Tray. Place the Nanoplate into the loading slot taking care to match the A1 corner of the Nanoplate to the A1 corner in the loading slot.



12.3 Click the eject button to close the loading tray. The QIAcuity instrument will read the Nanoplate barcode and a blue light will illuminate if the instrument approves the Nanoplate.



- 12.4 On the Qiacuity touch screen, click the magnifying glass icon to assign your previously set up Qiacuity Plate software file to each Nanoplate you want to run. Select the Plate software file that you completed in Step 2 of this procedure. Alternatively, the Nanoplate's barcode can be scanned before placing it into the tray and the instrument will automatically recognize which plate belongs to what Plate software file.

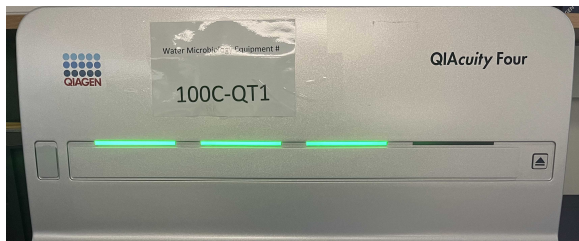


- 12.5 Press the "Run Program" button. The program will run for about three hours.

QIacuity dPCR System: unloading nanoplates

2m

- 13 When the program is finished running, the light on the machine will turn green.



- 13.1 Login to the software. Click the eject tray button.



- 13.2 Remove the Nanoplates from the instrument and dispose of the plates according to your biosafety protocol.

Acknowledgements

Many people have contributed to making of this protocol, including the entire wastewater department at the Wisconsin State Laboratory of Hygiene, but the authors would like to especially thank Devin Everett, Evelyn Doolittle, Griffin Knuth and Leah Fannin for their contribution.