

# Pathogen Detection Using the Advanta RT-PCR Kit

This quick reference describes how to perform pathogen detection using the Advanta™ RT-PCR Kit and 192.24 Dynamic Array™ IFC (integrated fluidic circuit) on Biomark™ HD or Biomark using probe-based assays (for example, TaqMan® probes).

**IMPORTANT** Before using this document, read and understand the safety guidelines in the Pathogen Detection Using the Advanta RT-PCR Kit Protocol (FLDM-00191). For detailed instructions on instrument and software operation, and complete instrument safety information, see the Juno™ System User Guide (100-7070) or IFC Controller RX User Guide (100-3385) and the Biomark HD Data Collection User Guide (100-2451) or Biomark/EP1™ Data Collection User Guide (68000127).

## Workflow

Workflow Step	Run Time*
1 Prepare the reverse transcription (RT) and preamplification reactions.	—
2 Perform the RT and preamplification reactions.	70 min
3 Prepare the final assay mixes and final sample mixes for real-time PCR.	—
4 Prepare the 192.24 IFC (integrated fluidic circuit) by injecting control line fluid	—
5 Pipet each final sample and assay mix, Actuation Fluid and Pressure Fluid into the IFC, then load the IFC on Juno or IFC Controller RX.	35 min
6 Thermal-cycle and collect data on Biomark HD or Biomark.	Biomark HD: 35 min Biomark: 45 min

\* Does not include hands-on time

## Prepare and Perform the 1-Step Reverse Transcription and Preamplification Reactions

**IMPORTANT** Assemble the 1-step pre-mix, sample mixes, and 1-step reactions in the pre-PCR area of the facility.

### Pool and Dilute the Primer Sets for Preamplification

**IMPORTANT** Prepare in the pre-PCR area of the facility.

- 1 Briefly vortex and centrifuge the reagents before use.
- 2 Pool and dilute the assays in a new 2 mL tube. The assay mix should be prepared and used immediately.

**NOTE** Volumes can be adjusted proportionally based on the number of samples to be amplified, up to 192 reactions.

- If using 2019-nCoV RUO assays (6.7 μM primers, forward and reverse; 1.7 μM probe), use components in [Table 1](#).
- If using custom assays (100 μM primers, forward and reverse; 100 μM probe), use components in [Table 2](#).
- If using 20X TaqMan® Gene Expression Assays (18 μM primers, forward and reverse; 5 μM probe), use components in [Table 3](#).

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Table 1. Pooled and diluted 2019-nCoV RUO Kit primer mix

Component	Vol for 3 Assays/ 1 Reaction (μL)*	Vol for 3 Assays/ 192 Reactions (μL)†
2019-nCoV RUO assays (102-0690 or 10006713)		
• 2019 nCoV_N1	0.112	25.0
• 2019 nCoV_N2	0.112	25.0
• RNase P	0.022	5.0
Dilution Reagent (100-8730)	6.754	1,509
<b>Total</b>	<b>7.0</b>	<b>1,564</b>

Concentration of pooled 2019-nCoV RUO assays:

- 2019 nCoV\_N1 and 2019 nCoV\_N2 assays: 107.10 nM primers; 27.17 nM probe
- RNase P assays: 21.42 nM primers; 5.43 nM probe

\* When preparing master mixes for less than 192 reactions, include an additional 10% to the volumes for overage.

† Includes overage for ease of pipetting

Table 2. Pooled and diluted custom primer mix

Component	Vol for N Assays/ 192 Reactions (μL)
Custom assays:	
• Forward primer	N x 1.7
• Reverse primer	N x 1.7
• Probe	N x (up to 0.42)
Dilution Reagent (100-8730)	1,564 – (volume of custom assays)
<b>Total</b>	<b>1,564</b>

Concentration of each assay: 108.70 nM primers; up to 26.85 nM probe

Table 3. Pooled and diluted TaqMan Gene Expression Assay primer mix

Component	Vol for N Assays/ 192 Reactions (μL)
20X TaqMan Gene Expression Assays	N x 9.3
Dilution Reagent (100-8730)	1,564 – (N x 9.3)
<b>Total</b>	<b>1,564</b>

Concentration of each assay: 107.03 nM primers; 29.73 nM probe

## Prepare the 1-Step Reverse Transcription and Preamplification Reactions

**IMPORTANT** Prepare in the pre-PCR area of the facility.

- 1 Thaw the Advanta RT-Preamp Master Mix and keep on ice. Briefly vortex and centrifuge the reagents before use.

- 2 In a DNA-free hood, combine the components shown in Table 4 in a new 5 mL tube to make the 1-step pre-mix and place on ice. Scale up appropriately for multiple runs.

Table 4. 1-step pre-mix

Component	Vol per Reaction (μL)*	Vol for 192 Reactions (μL)†
Pooled primer mix (see Table 1, Table 2, or Table 3)	7	1,484
Advanta RT-Preamp Master Mix (102-0419)	3	636
<b>Total</b>	<b>10</b>	<b>2,120</b>

\* When preparing master mixes for less than 192 reactions, include an additional 10% to the volumes for overage

† Includes overage

- 3 Cap the tube, vortex, and centrifuge the 1-step pre-mix.
  - 4 Aliquot 128 μL of 1-step pre-mix into each well of two 0.2 mL 8-well strips (see Figure 1).
  - 5 Use an 8-channel pipette to combine the 1-step pre-mix and the samples in 2 new 96-well plates as shown in Figure 1.
    - Transfer 10.0 μL of 1-step pre-mix into each well of 2 new 96-well plates.
    - Add 5.0 μL of sample to each well of the 96-well plates.
- NOTE** Only one preamplification reaction is prepared for each sample.
- 6 Tightly seal the plates with clear adhesive film, then gently vortex and centrifuge them at 3,000 × g for 60 sec to mix the reactions.

### Perform the 1-Step Reverse Transcription and Preamplification Reactions

- 1 Place each plate in a 96-well plate thermal cycler and cycle using the program in Table 5:

Table 5. 1-step reverse transcription and preamplification

Temperature	Time	Condition
+50 °C	15 min	RT
+95 °C	2 min	Hot start
+95 °C	15 sec	20 cycles
+60 °C	2 min	
+4 °C	∞	Hold

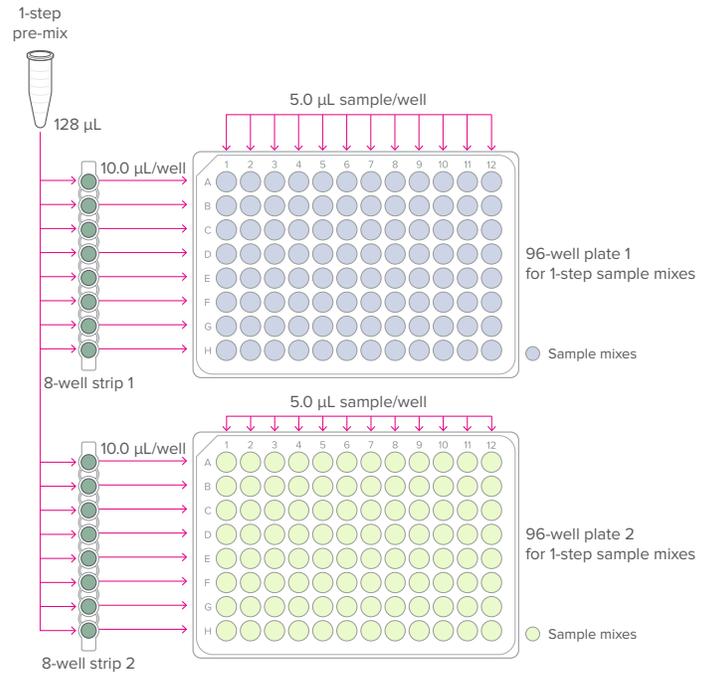


Figure 1. 1-step reaction plates (per-well transfer volumes)

### Dilute the Preamplified cDNA

**IMPORTANT** Prepare in the post-PCR area of the facility.

After cycling, dilute the preamplified reactions in the 96-well plates in Dilution Reagent as shown in Table 6 and described as follows:

- 1 Transfer 13 mL of Dilution Reagent into a new 25 mL reagent reservoir.
 

**NOTE** This is sufficient for the dilution of two 96-well plates of preamplified samples.
- 2 Use an 8-channel pipette to transfer 60 μL of Dilution Reagent into each well containing the preamplified cDNA.
 

**NOTE** Any unused Dilution Reagent dispensed in Step 1 should be discarded.
- 3 Tightly seal the plates with clear adhesive film, then gently vortex to mix dilutions and centrifuge them at 3,000 × g for 60 sec. Set aside until ready to prepare the final sample mixes.

**STOPPING POINT** The diluted, preamplified cDNA can either be assayed immediately or stored at -15 °C to -25 °C for later use.

Table 6. Diluted, preamplified cDNA

Component	Vol per Reaction (μL)
Dilution Reagent (100-8730)	60.0
Preamplified cDNA (contained in the 96-well plates)	15.0
<b>Total</b>	<b>75.0</b>

## Prepare and Perform the Real-Time PCR Reactions on the IFC

### Prepare the Final Assay Mixes for Loading on the IFC

- 1 Briefly vortex and centrifuge the reagents before use.
- 2 In a DNA-free hood, prepare each final assay mix in a new 1.5 mL tube using the components in Table 7, Table 8, or Table 9, as appropriate. The tables show examples for preparing a 50 µL stock, which is sufficient for 10 IFCs. Scale appropriately for multiple runs.
  - If using 2019-nCoV RUO assays (6.7 µM primers, forward and reverse; 1.7 µM probe), use components in Table 7 as shown in Figure 2.
  - If using custom assays (100 µM primers, forward and reverse; 100 µM probe), use components in Table 8 and shown in Figure 3.
  - If using 20X TaqMan® Gene Expression Assays (18 µM primers, forward and reverse; 5 µM probe), use components in Table 9 and shown in Figure 3.

**NOTE** Unused assay mixes can be stored at -20 °C for at least 6 months.

Table 7. Final 2019-nCoV RUO assay mixes

Component		Vol per Inlet (µL)*	Vol for 50 µL Stock for Each Assay (µL)*
2019-nCoV RUO assays (102-0690 or 10006713)		3.0	37.5
4X Assay Loading Reagent (102-0114)	●	1.0	12.5
<b>Total</b>		<b>4.0</b>	<b>50.0</b>

Final concentration: 5 µM primers; 1.28 µM probe

\* Includes overage

Table 8. Final custom assay mixes

Component		Vol per Inlet (µL)*	Vol for 50 µL Stock for Each Assay (µL)*
Custom assays:			
• Forward primer		0.36	4.50
• Reverse primer		0.36	4.50
• Probe		0.10	1.25
Dilution Reagent (100-8730)	●	1.18	14.75
PCR Water (100-5941)	●	1.0	12.50
4X Assay Loading Reagent (102-0114)	●	1.0	12.50
<b>Total</b>		<b>4.0</b>	<b>50.00</b>

Final concentration: 9 µM primers; 2.5 µM probe

\* Includes overage

Table 9. Final TaqMan Gene Expression Assay mixes

Component		Vol per Inlet (µL)*	Vol for 50 µL Stock for Each Assay (µL)*
20X TaqMan® Gene Expression Assays		2.0	25.0
PCR Water (100-5941)	●	1.0	12.5
4X Assay Loading Reagent (102-0114)	●	1.0	12.5
<b>Total</b>		<b>4.0</b>	<b>50.0</b>

Final concentration: 9 µM primers; 2.5 µM probe

\* Includes overage

- 3 Pipet 4.0 µL of each assay stock into the respective wells in a new 96-well plate as shown in Figure 2 (for 2019-nCoV RUO assays) or Figure 3 (for custom or TaqMan assays).

**NOTE** For each unused assay inlet, combine 3.0 µL of PCR Water (100-5941) with 1.0 µL 4X Assay Loading Reagent (102-0114) in the respective wells.

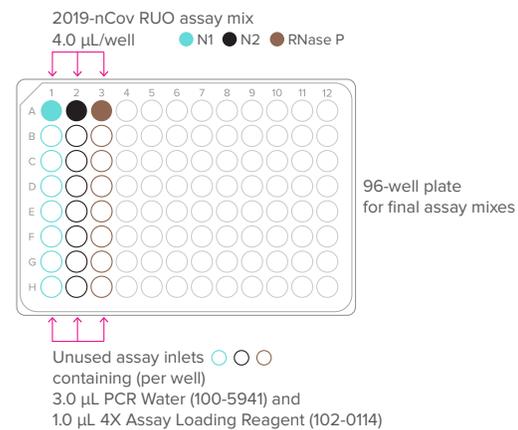


Figure 2. Final 2019-nCoV RUO assay mixes plate (per-well transfer volumes)

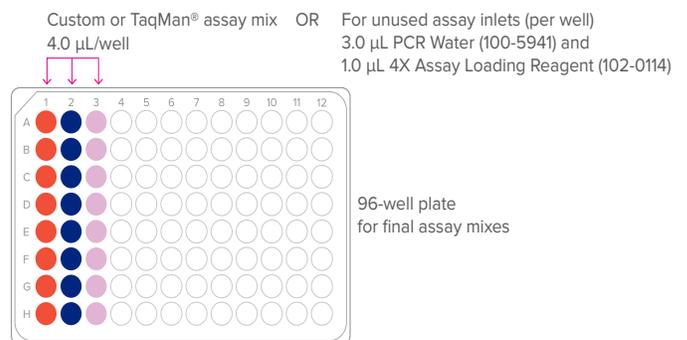


Figure 3. Final custom and TaqMan Gene Expression assay mixes plate (per-well transfer volumes)

### Prepare the Final Sample Mixes

- 1 Thaw the Advanta PCR MM and keep on ice. Briefly vortex and centrifuge the reagents before use.
- 2 In a DNA-free hood, combine the components shown in Table 10 in a new 1.5 mL tube to make the sample pre-mix and place on ice.

Table 10. Sample pre-mix

Component		Vol per Inlet (μL)*	Sample Pre-Mix for One 192.24 IFC (μL)†
Advanta PCR MM (102-0420)	●	2.0	460.0
20X GE Sample Loading Reagent (85000735)	●	0.2	46.0
<b>Total</b>		<b>2.2</b>	<b>506.0</b>

\* Includes overage

† 230 reactions for ease of pipetting

- 3 Prepare the final sample mixes as shown in Figure 4.
    - a Briefly vortex and centrifuge sample pre-mix from Table 10.
    - b Aliquot 60 μL of pre-mix into each well of a new 8-well strip.
    - c Use an 8-channel pipette to transfer 2.2 μL of sample pre-mix from the 8-well strip into each well of 2 new 96-well plates.
    - d Remove the plates from the DNA-free hood and prepare the final sample mix by adding 1.8 μL of each diluted, preamplified sample from Table 6 on page 2 to each well.
- IMPORTANT** Before use, briefly vortex and centrifuge the plates containing the diluted, preamplified cDNA.

**NOTE** For each unused sample inlet, add 1.8 μL of PCR Water (100-5941) to the 2.2 μL sample pre-mix in the plate.

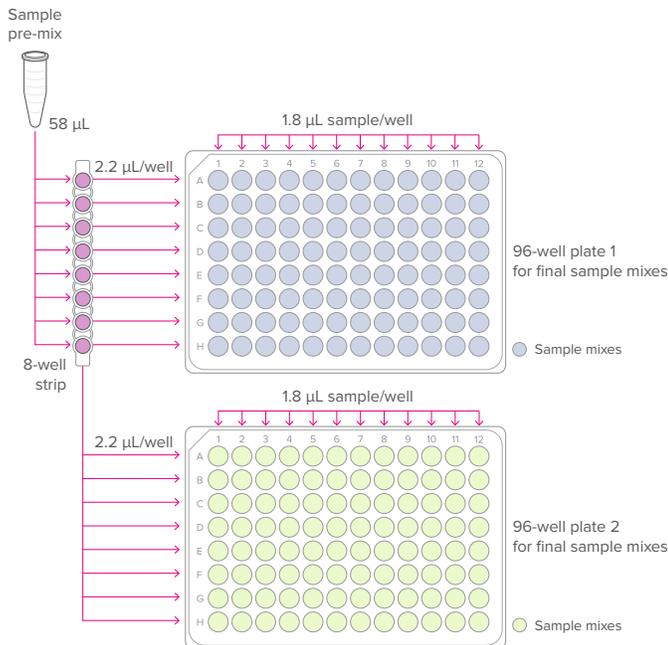


Figure 4. Final sample plates (per-well transfer volumes)

- 4 Tightly seal the plates with clear adhesive film, then vortex and centrifuge them at 3,000 × g for 60 sec.

### Prepare the 192.24 IFC

For detailed instructions about injecting control line fluid, see the Control Line Fluid Loading Procedure (68000132).

### IMPORTANT

- Use the IFC within 24 hr of opening the package.
- Only use a 192.24 syringe (100-4058). The syringe is pre-filled with 150 μL of control line fluid.
- Do not evacuate air from the syringe prior to injecting control line fluid (Step 3).
- Avoid getting control line fluid on the exterior of the IFC or in the inlets because this makes the IFC unusable. If this occurs, use a new IFC.

- 1 Remove the 192.24 Control Line Fluid syringe (100-4058) and the 192.24 Dynamic Array IFC (100-6266) from the packaging.

- 2 Place the IFC on a flat surface and actuate the check valve for the top accumulator by pressing gently with the syringe cap.

**IMPORTANT** The bottom accumulator is not used.

- 3 Inject control line fluid into the top accumulator on the IFC (see Figure 5). Use the entire contents of the syringe.

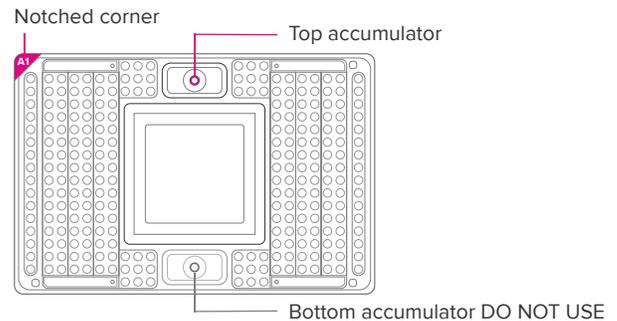


Figure 5. Top accumulator on the 192.24 IFC

- 4 Check to ensure that the O-ring returns to its normal position after the syringe is removed.
- 5 Remove and discard the protective film from bottom of IFC.

### Load the IFC

#### IMPORTANT

- Vortex thoroughly and centrifuge all assay and sample solutions before pipetting into the IFC inlets. Failure to do so may result in a decrease in data quality.
- While pipetting, do not go past the first stop on the pipette. Doing so may introduce air bubbles into inlets.

Refer to Figure 6 (for 2019-nCoV RUO assays) or Figure 7 (for custom or TaqMan assays) on page 5 when pipetting final sample and assay mixes, Actuation Fluid, and Pressure Fluid into the IFC.

- 1 If using Juno, ensure that the RX Interface Plate is installed in the Juno instrument.
- 2 Pipet 3 μL of each final sample mix into the respective sample inlets on the IFC.
- 3 Pipet 3 μL of each final assay mix into the respective assay inlets on the IFC
- 4 Pipet 150 μL of Actuation Fluid (100-6250) into the P1 reservoir (■) on the IFC.
- 5 Pipet 150 μL of Pressure Fluid (100-6249) into each of the P2 and P3 reservoirs (□) on the IFC.
- 6 Pipet 20 μL of Pressure Fluid into each of the P4 and P5 inlets (○) on the IFC.

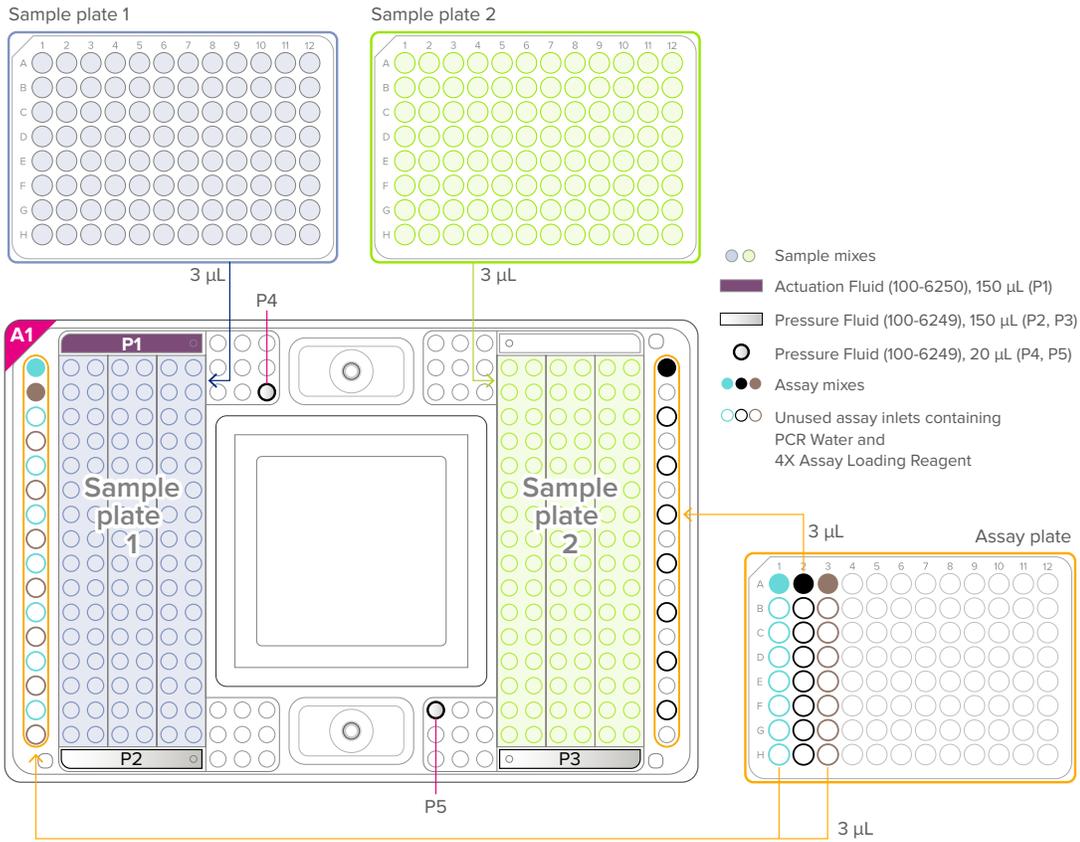


Figure 6. Pipetting map for the 192.24 IFC for 2019-nCoV RUO assays

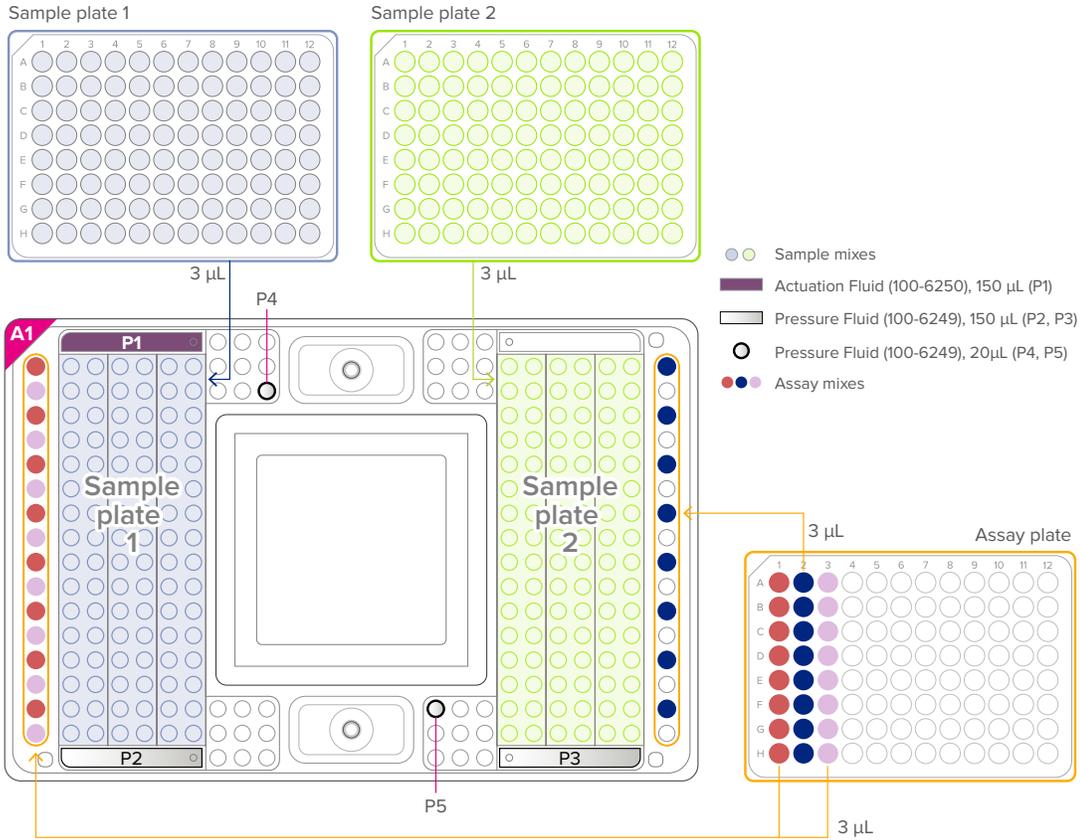


Figure 7. Pipetting map for the 192.24 IFC for custom and TaqMan assays

- 7 Blot the IFC surface with a dry, lint-free cloth.
- 8 Place the IFC into the controller:
  - Juno: Tap **OPEN** to open the instrument tray and align notched corner of IFC to white notch on tray. Tap **LOAD**.
  - RX: Press **EJECT** to open the instrument tray and align the notched corner of the IFC to the A1 mark. Press **Load Chip**.
- 9 Run the Load Mix script:
  - Juno: Tap **Load Mix 192.24 GE**, then tap **Run**.
  - RX: Select **Load Mix (169x)** and press **Run Script**.

**IMPORTANT** Start the IFC run on the Biomark HD or Biomark instrument within 1 hr of completing the Load Mix script.
- 10 If necessary, turn on the Biomark HD or Biomark system (computer and instrument). For Biomark, also launch the Data Collection software, and turn on the lamp. The lamp takes 20 min to warm up.

### Thermal-Cycle and Collect Real-Time PCR Data

- 1 Remove the loaded IFC from Juno or IFC Controller RX.
- 2 Use clear tape to remove any dust particles or debris from the IFC surface, if necessary.
- 3 If necessary, double-click the **Data Collection** icon (  or  ) on the desktop of the Biomark HD or Biomark computer to launch the software.
- 4 Click **Start a New Run**.
- 5 Confirm that the camera status indicator and the lamp status indicator (Biomark only) at the bottom of the window are green.
- 6 Place the loaded IFC on the instrument tray and align the notched A1 corner on the IFC with the A1 label on the tray. In the Data Collection software, click **Load**.
- 7 In the Data Collection software, confirm the IFC barcode and IFC type and then click **Next**.
- 8 Complete the Chip Run section by selecting either a new or a pre-defined run.
- 9 Complete the Chip Run Name and Location section:
  - a Enter a run name or select the checkbox to use the IFC barcode as the run name.
  - b Select a file storage location for a new IFC run or browse to select a pre-defined run file and click **Next**.
- 10 Complete the Application, Reference and Probes section and then click **Next**.

For	Select
Application	<b>Gene Expression</b>
Passive reference	<b>ROX™</b>
Assay	<b>Single probe</b>
Probes	<b>FAM-MGB</b>

- 11 Browse to and select the thermal protocol:

For	Select
Biomark HD	<b>GE 192x24 Fast v1.pcl</b>
Biomark*	<b>GE 192x24 Quick v1.pcl</b>

\* See the Pathogen Detection Using the Advanta RT-PCR Kit Protocol (FLDM-00191) for more information about the GE 192x24 Quick thermal protocol.

- 12 Confirm that **Auto Exposure** is selected. Click **Next**.
- 13 Confirm that IFC run information is correct and click **Start Run**.
- 14 After the run is complete, analyze your data using the Real-Time PCR Analysis software.

## Appendix: Saliva Preparation

### Collect the Saliva Specimens

**IMPORTANT** Use universal precautions when handling biological samples.

Collect saliva specimen in a sterile container. Store specimens at  $-20\text{ }^{\circ}\text{C}$  to  $-80\text{ }^{\circ}\text{C}$  and ship on dry ice. Transport and test specimens as soon as possible after collection. Specimens are stable for up to 120 hr at ambient temperature.

### Process the Saliva Specimens

#### IMPORTANT

- Prepare in the pre-PCR area of the facility.
  - Use universal precautions when handling biological samples. Prior to heat inactivation, the saliva specimens should be handled in a BSL-2 environment.
- 1 Mix each saliva specimen with an equal volume of nuclease-free PBS (Thermo Fisher Scientific, 10010023). For example, if 15  $\mu\text{L}$  of saliva specimen was collected, add 15  $\mu\text{L}$  of PBS.
  - 2 Aliquot 24  $\mu\text{L}$  of each saliva/PBS mix into a tube or plate and add 1  $\mu\text{L}$  of RNasecure (Thermo Fisher Scientific, AM7005) to the mix, then briefly vortex and centrifuge.
  - 3 Heat-inactivate the prepared saliva specimens in a thermal cycler using the program in [Table 11](#):

Table 11. Heat-inactivation of saliva samples

Temperature	Time
+90 $^{\circ}\text{C}$	10 min
+4 $^{\circ}\text{C}$	2 min
+4 $^{\circ}\text{C}$	$\infty$

- 4 After 2 min at +4  $^{\circ}\text{C}$ , you can place the samples on ice until ready to use.
- 5 Use 5  $\mu\text{L}$  of each heat-inactivated saliva sample in the 1-step reverse transcription and preamplification reactions on [page 1](#).

### For technical support visit [techsupport.fluidigm.com](https://techsupport.fluidigm.com).

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