Real-Time PCR for Viral RNA Detection

This quick reference describes how to perform viral RNA detection using the 192.24 Dynamic Array™ IFC (integrated fluidic circuit) on Biomark™ HD or Biomark using 2019-nCoV RUO assays manufactured by Integrated DNA Technologies (IDT™). To prepare custom assays with this workflow, see the appendix on page 4.

IMPORTANT Before using this quick reference, read and understand the detailed instructions and safety guidelines in the Real-Time PCR for Viral RNA Detection Protocol (FLDM-00103).

Workflow

	Workflow Step		Run Time*
1	Prepare and perform the RT reactions.		40 min
2	Preamplify the cDNA.		1 hr 40 min
3	Prepare the 2019-nCoV RUO assays and sample mixes.		—
4	Prepare the 192.24 IFC.		—
5	Load the IFC on Juno™ or IFC Controller RX.		30 min
6	Thermal-cycle and collect data on Biomark HD or Biomark.	Fast: Standard:	30 min 1 hr 30 min

* Does not include hands-on time

Prepare and Perform the RT Reactions

☐ **1** Thaw all reagents on ice. Briefly vortex and centrifuge the reagents before using.

☐ **2** On ice, prepare the reverse transcription (RT) reactions:

Table 1. RT reactions

Component	Vol per Reaction (µL)	Vol for 192 Reactions (µL)*
RNA template	5.0	—
Reverse Transcription Master Mix (100-6297)	1.25	264
Total	**6.25**	**–**

* Includes overage

a Add 5.0 µL of RNA template into each well of 2 new 96-well PCR plates (on ice).

b Aliquot 33 µL of Reverse Transcription Master Mix into each well of an 8-well strip.

c Use an 8-channel pipette to transfer 1.25 µL of Reverse Transcription Master Mix into each well containing RNA in the 96-well plates from Step a.

☐ **3** Properly seal and gently vortex to mix the RT reactions.

☐ **4** Centrifuge the reactions at 3,000 × *g* for 60 sec, then place the plates in a 96-well plate thermal cycler and incubate using the following protocol:

Temperature	Time	Condition
25 °C	5 min	Hold
42 °C	30 min	Hold
85 °C	5 min	Hold
4 °C	∞	Hold

STOPPING POINT After the RT reaction is complete, the reactions can be stored at −20 °C or used immediately for preamplification reactions with Preamp Master Mix.

Preamplify the cDNA

NOTE If you are using custom assays, see the appendix on page 4 for the assay preparation and pooling procedure.

Pool the 2019-nCoV RUO Assays

☐ **1** Briefly vortex and centrifuge the reagents before use.

☐ **2** Dilute the pooled 2019-nCoV RUO assays as shown in Table 2.

Table 2. Pooled 2019-nCoV RUO assays

Component		Vol for 3 Assays/ 192 Reactions (µL)
2019-nCoV RUO Kit (10006713) (6.7 µM primer, forward and reverse; 1.7 µM probe) (N1, N2, and RNase P)	10.5 µL × each assay	31.5
Dilution Reagent (100-8730)		668.5
Total		**700**

Final concentration of pooled 2019-nCoV RUO assays: 100.5 nM primer; 25.5 nM probe

NOTE Volume can be adjusted proportionally based on the number of samples to be amplified.

Prepare the Preamplification Sample Mix

☐ **1** Thaw the Preamp Master Mix and keep on ice. Briefly vortex and centrifuge the reagents before use.

☐ **2** In a DNA-free hood, combine the components (Table 3) in a new 1.5 mL tube to make the preamplification pre-mix and place on ice. Scale up appropriately for multiple runs.

Table 3. Preamplification pre-mix

Component	Vol per Reaction (µL)	Vol for 192 Reactions/ 1 IFC (µL)†
Preamp Master Mix (100-5744)	2.5	528
Pooled 2019-nCoV RUO assays (see Table 2)* (100.5 nM primer, forward and reverse; 25.5 nM probe)	3.125	660
PCR Water (100-5941)	0.625	132
Total	**6.25**	**1,320**

Final concentration of 2019-nCoV RUO assays in preamplification pre-mix: 25.125 nM primer; 6.375 nM probe

* If using custom assays, see Table 9 on page 4.
† Includes overage

☐ **3** Cap tube, vortex and centrifuge the preamplification pre-mix.

☐ **4** Add preamplification pre-mix to the RT reactions to prepare the preamplification sample mix (Table 4).

Table 4. Preamplification sample mix

Component	Vol per Inlet (µL)
Preamplification sample pre-mix (see Table 3)	6.25
RT reactions (see Prepare and Perform the RT Reactions on page 1)	6.25
Total	**12.5**

a Aliquot 165 µL of preamplification sample pre-mix into each well of an 8-well strip.

b Use an 8-channel pipette to transfer 6.25 µL of pre-mix into each well containing the RT reactions from Prepare and Perform the RT Reactions on page 1.

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

Thermal-Cycle the Preamplification Sample Mix

☐ **1** Place the plates in a 96-well plate thermal cycler and cycle using the following table as a guide:

Temperature	Time	Condition	
95 °C	2 min	Hold	
95 °C	15 sec	20 cycles	Denaturation
60 °C	4 min		Annealing/extension
4 °C	∞	Hold	

NOTE The appropriate number of cycles may need to be determined empirically.

Dilute the Preamplified cDNA

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

☐ **1** After cycling, dilute the preamplified reactions in the 96-well plates in Dilution Reagent as shown in Table 5:

Table 5. Diluted, preamplified cDNA

Component	Vol per Reaction (µL)
Dilution Reagent (100-8730)	50.0
Preamplified cDNA (contained in the 96-well plates)	12.5
Total	**62.5**

☐ **2** Tightly seal the plates with clear adhesive film, then gently vortex to mix the 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.

Prepare the 2019-nCoV RUO Assay Mixes

NOTE If you are using custom assays, see the appendix on page 4 for the assay mixes preparation procedure.

☐ **1** Briefly vortex and centrifuge the reagents before use.

☐ **2** In a DNA-free hood, prepare aliquots of 10X 2019-nCoV RUO assay mixes using components in Table 6. Scale up appropriately for multiple runs.

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

Table 6. 2019-nCoV RUO assay mixes

Component	Vol per Inlet (µL)*	Vol for *N* Assays (µL)
2019-nCoV RUO assays (10006713)	3.0	3.0 x *N*
4X Assay Loading Reagent (102-0114)	1.0	1.0 x *N*
Total	**4.0**	4.0 x *N*

Final concentration (at 10X): 5 µM primers; 1.25 µM probe

* Includes overage

Prepare the Sample Mix

☐ **1** Briefly vortex and centrifuge the reagents before use.

☐ **2** In a DNA-free hood, combine the components (Table 7) in a sterile 1.5 mL tube to make the sample pre-mix. Scale up appropriately for multiple runs.

NOTE This is enough volume to fill the entire IFC.

Table 7. Sample pre-mix

Component	Vol per Inlet (µL)*	Sample Pre-Mix for 192.24 IFC (µL)†
Master Mix • **For standard:** TaqMan Gene Expression Master Mix (Thermo Fisher Scientific, 4369016) • **For fast:** TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific, 4444557)	2.0	440.0
20X GE Sample Loading Reagent (85000735)	0.2	44.0
Total	**2.2**	**484.0**

* Includes overage
† 220 reactions for ease of pipetting

☐ **3** Prepare the final sample mixes:

a Aliquot 60.5 µL of sample pre-mix into each well of a new 8-well strip

b 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.

c 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 5 on page 2 to each well.

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

☐ **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

IMPORTANT

- Use the IFC within 24 hr of opening the package.
- Only use a 192.24 syringe (100-4058). The syringe is prefilled 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 (Figure 1). Use the entire contents of the syringe.

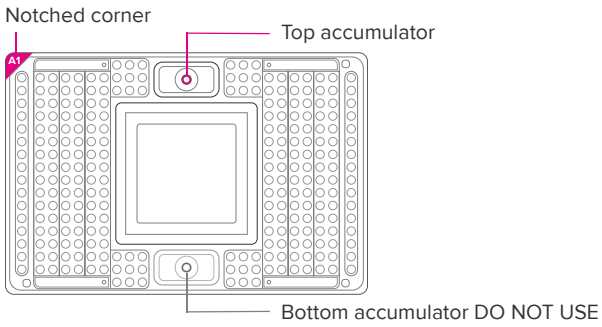

Figure 1. Top accumulator on 192.24 IFC

☐ **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.

☐ **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.

☐ **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 the notched corner of the IFC to the white notch on the 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.

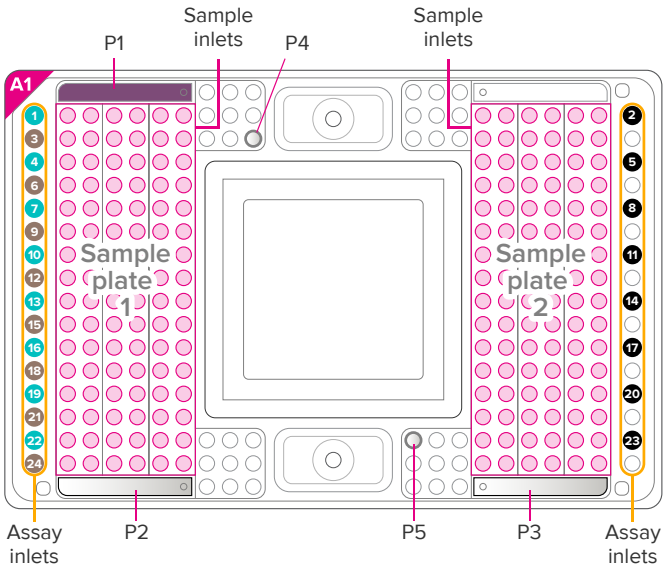

Figure 2. Pipetting map for the 192.24 IFC

Collect 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 on the desktop of the Biomark HD or Biomark system 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** Confirm that 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	**Gene Expression**
Passive reference	**ROX™**
Assay	**Single probe**
Probes	**FAM-MGB**

☐ **11** Browse to and select the thermal protocol:

For	Select
Standard	**GE 192x24 Standard v1.pcl**
Fast (Biomark HD only)	**GE 192x24 Fast v1.pcl**

☐ **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: Custom Assays

Prepare the 20X Custom Assays

Based on the final reaction concentration of primers and probes, prepare the 20X custom assays. For example, Table 8 shows the preparation for a reaction with a final target of concentration for forward and reverse primers each at 500 nM and probe at 125 nM.

Table 8. 20X custom assays

Component	Vol per Inlet (µL)	Final 20X Concentration (µM)
Forward primer (100 µM)	10	10
Reverse primer (100 µM)	10	10
TaqMan probe (FAM-MGB) (100 µM)	2.5	2.5
Dilution Reagent (100-8730)	77.5	—
Total	**100**	—

Pool the 20X Custom Assays for Preamplification

☐ **1** Briefly vortex and centrifuge the reagents before use.

☐ **2** In a microcentrifuge tube, combine equal volumes of each 20X custom assay for preamplification, up to a total of 24 assays.

☐ **3** Dilute the pooled custom assays as shown in Table 9. Table 9 provides an example using 24 assays.

NOTE The primer concentration in the preamplification reaction may need optimization.

Table 9. Pooled custom assays for preamplification

Component		Vol for 24 Assays/ 192 Reactions (µL)*
20X assays	7 µL × each assay	168
Dilution Reagent (100-8730)	700 – (7 µL × each assay)	532
Total		**700**

* Includes overage

☐ **4** Go to Prepare the Preamplification Sample Mix on page 1.

Prepare the 10X Custom Assay Mixes

The 10X custom assay mixes require 2X Assay Loading Reagent, included in the 192.24 GE Dynamic Array Reagent Kit (100-6267). If you are using the 192.24 GE Dynamic Array 4X Reagent Kit (102-0166), obtain 2X Assay Loading Reagent by either:

- Ordering from fluidigm.com (100-7611), or
- Preparing by combining components (Table 10) in a sterile tube.

Table 10. 2X Assay Loading Reagent

Component	Vol (µL)
4X Assay Loading Reagent (102-0114)	30
PCR Water (100-5941)	30
Total	**60**

☐ **1** Briefly vortex and centrifuge reagents before use.

☐ **2** In a DNA-free hood, prepare aliquots of 10X custom assay mixes using the components in Table 11. Scale up appropriately for multiple runs.

Table 11. 10X custom assay mixes

Component	Vol per Inlet (µL)†	Vol for 24 Assays (µL)
20X custom assays (see Table 8)*	2.0	48.0
2X Assay Loading Reagent	2.0	48.0
Total	**4.0**	**96.0**

* For each unused assay inlet, combine 2.0 µL of PCR Water (100-5941) with 2.0 µL of 2X Assay Loading Reagent (102-0114) in the respective wells.
† Includes overage

NOTE Volume can be adjusted proportionally based on the number of samples to be amplified.

☐ **3** Go to Prepare the Sample Mix on page 2.

For technical support visit techsupport.fluidigm.com.

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