Advanta Immuno-Oncology Gene Expression Assay

IMPORTANT Before using this quick reference, read and understand the detailed instructions and safety guidelines in the Advanta™ Immuno-Oncology Gene Expression Assay Protocol (PN 101-6108).

To ensure reliable results:

- Thaw reagents to room temperature unless directed to thaw them on ice.
- Protect assays from light and store in the refrigerator when not in use. Assays are light-sensitive.

Workflow Overview

	Workflow Step	Instrument
1	**Prepare the cDNA.*** Reverse transcription reaction	Standard thermal cycler
2	**Preamplify the cDNA.***	Standard thermal cycler
3	**Prime the IFC.**	Juno™ or IFC Controller HX
4	**Prepare assays and samples.**	—
5	**Load the IFC.**	Juno or IFC Controller HX
6	**Thermal-cycle and collect data.**	Biomark™ HD

* Potential stopping point

Prepare the cDNA

IMPORTANT
- The RNA samples must be DNA-free.
- Thaw RNA samples and Reverse Transcription Master Mix on ice and keep on ice.

Prepare RT Reactions

1 Briefly vortex and centrifuge the reagents before using.

2 On ice, prepare the RT pre-mix in a 1.5 mL PCR tube as shown in Table 1.

Table 1. RT pre-mix

Component	Volume per Reaction (µL)	Volume for 96 Reactions (µL)*
Reverse Transcription Master Mix (Fluidigm PN 100-6297)	1.0	105.6
PCR Water (Fluidigm PN 100-5941)	3.0	316.8
Total	**4.0**	**422.4**

* Includes 10% overage for ease of pipetting

3 Aliquot 51 µL of the RT pre-mix into each well of an 8-well PCR tube strip.

4 Prepare the RT sample mix as shown in Table 2.

Table 2. RT sample mix

Component	Volume per Reaction (µL)
RT pre-mix	4.0
RNA, 2–200 ng/µL	1.0
Total	**5.0**

a Using an 8-channel pipette, transfer RT pre-mix to individual wells of a 96-well PCR plate.

b Add RNA samples to each well containing RT pre-mix.

5 Properly seal plate and gently vortex to mix the RT reactions.

6 Centrifuge the RT reactions and then place in a standard thermal cycler.

7 Incubate the RT reactions using the thermal protocol:

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

STOPPING POINT The reverse-transcription reaction products (cDNA) can be used immediately for preamplification reactions with Preamp Master Mix or stored at −20 °C for later use.

Preamplify the cDNA

Pool the Gene Expression Assays

DANGER Advanta Immuno-Oncology Gene Expression Assay Panel A and B assay plates contain Formamide. For complete safety information, see the safety appendix in the Advanta Immuno-Oncology Gene Expression Assay Protocol (PN 101-6108).

IMPORTANT Pool the Panel A and Panel B assay plates separately to avoid cross-contamination or loss of traceability.

1 In a microcentrifuge tube, pool equal volumes from each well of one Advanta Immuno-Oncology Gene Expression Assay plate and dilute as shown in Table 3.

Table 3. Pooled Advanta Immuno-Oncology Gene Expression Assay

Component	Volume (µL)
Dilution Reagent (Fluidigm PN 100-9167)	12
Advanta Immuno-Oncology Gene Expression Assay (20X)	3 (x 96 wells = 288)
Panel A assay plate (Fluidigm PN 101-6145) or Panel B assay plate (Fluidigm PN 101 6146)	
Total	**300**

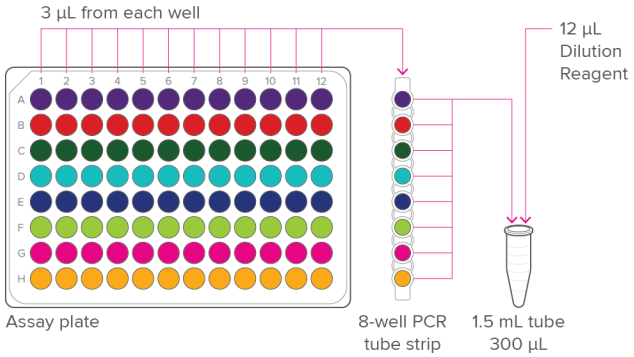
NOTE 300 µL of pooled assays is sufficient for preamplification of 192 samples (2 IFCs). Concentration of pooled assays is 0.2X.

a Label a 1.5 mL microcentrifuge tube as either Pool A or Pool B, if necessary.

b Pipet Dilution Reagent into the 1.5 mL microcentrifuge tube.

c Using an 8-channel pipette, transfer 3 µL from each well of the Advanta Immuno-Oncology Gene Expression Assay plate into an 8-well PCR tube strip.

d Centrifuge the 8-well PCR tube strip, and then transfer the entire volume from each well of the 8-well PCR tube strip into the microcentrifuge tube with Dilution Reagent, making a total volume of 300 µL.



2 Mix the pooled assays by briefly vortexing and centrifuge.

3 Reseal the Advanta Immuno-Oncology Gene Expression Assay plate. If using the assay plate within 2 days, store at 4 °C. Otherwise store at −20 °C.

STOPPING POINT The pooled assays can be stored at 4 °C if used within 1 week, or at −20 °C for longer storage. Protect pooled assays from light.

Prepare the Preamplification Reactions

1 In a DNA-free hood, prepare the preamplification pre-mix into a 1.5 mL PCR tube as shown in Table 4.

Table 4. Preamplification pre-mix

Component	Volume per Reaction (µL)	Volume for 96 Reactions (µL)*
Preamp Master Mix (Fluidigm PN 100-5744)	1.00	105.6
Pooled Advanta Immuno-Oncology Gene Expression Assay (0.2X)	1.25	132.0
PCR Water (Fluidigm PN 100-5941)	1.50	158.4
Total	**3.75**	**396.0**

* Includes 10% overage

2 Aliquot 48 µL of the preamplification pre-mix into each tube of an 8-well PCR tube strip.

3 Prepare preamplification sample mix as shown in Table 5.

Table 5. Preamplification sample mix

Component	Volume per Reaction (µL)
Preamplification pre-mix	3.75
cDNA	1.25
Total	**5.00**

a Using an 8-channel pipette, transfer preamplification pre-mix into individual wells of a 96-well PCR plate.

b Remove the plate from the DNA-free hood and add cDNA samples to each well containing pre-mix.

4 Seal the plate using an adhesive seal.

5 Mix the reactions by briefly vortexing and then centrifuge.

6 Place the plate in the thermal cycler and cycle using the following thermal protocol:

Temperature	Time	Cycle	
95 °C	2 min	Hold	
95 °C	15 sec	Denaturation	16 cycles*
60 °C	4 min	Annealing/extension	
4 °C	∞	Hold	

* Recommended starting point (within a range of 10–20 cycles). The appropriate number of cycles may need to be determined empirically, as sample sets may vary with respect to sample amount and quality.

7 After cycling, dilute the reaction 1:5 by adding 20 µL of Dilution Reagent to the preamplification product.

STOPPING POINT The diluted preamplification products can be assayed immediately, stored at 2–8 °C if used within 1 week, or stored at −20 °C for later use.

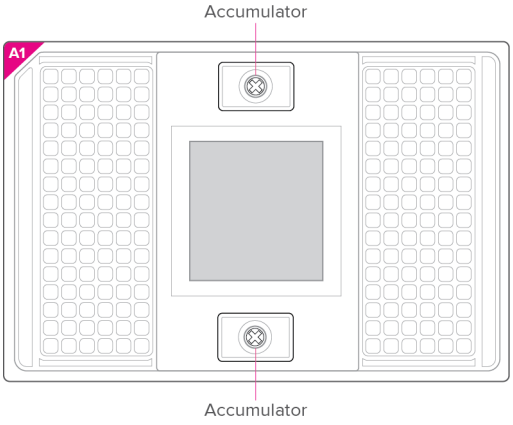
Prime the IFC

IMPORTANT

- Use the 96.96 Dynamic Array™ IFC within 24 hours of opening the package.
- Only use 96.96 syringes with 150 µL of control line fluid (Fluidigm PN 101-6334).
- Do not evacuate air from syringes prior to injecting control line fluid.
- Be careful not to bend the syringe tip.
- Be careful not to drip control line fluid in any inlet. Control line fluid in an inlet makes the inlet unusable.

1 Turn on the Biomark HD system (computer and instrument), if necessary, and launch the Data Collection software to allow the instrument to start up and the camera to cool to the appropriate temperature.

2 Inject control line fluid into each accumulator on the IFC. Use the entire contents of one syringe in each accumulator.



3 Remove and discard the protective film from bottom of IFC.

4 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**.

  NOTE You may be prompted to switch the interface plate. Use the HX interface plate.

- IFC Controller HX: Press **EJECT** to open the instrument tray and align the notched corner of the IFC to the A1 mark. Press **Load Chip**.

5 Run the prime script:

- Juno: Tap **Prime 96.96 GE**, and then tap **Run**.
- HX: Select **Prime (136x)** and press **Run** Script.

Priming the IFC takes approximately 20 minutes. During that time, prepare the assays and samples.

IMPORTANT Load the IFC within 60 minutes of completing the prime step.

Prepare Assays and Samples

Prepare 10X Assays

1 In a DNA-free hood, prepare aliquots of Advanta Immuno-Oncology Gene Expression Assays in a PCR plate using volumes shown in Table 6. Scale up appropriately for multiple runs. Follow the assay plate map on page 5.

Table 6. 10X assay mix

Component	Volume per Inlet with Overage (µL)*	Volume for 2 IFCs (µL)
Advanta Immuno-Oncology Gene Expression Assay) Panel A assay plate (Fluidigm PN 101-6145) or Panel B assay plate (Fluidigm PN 101 6146)	3	6
2X Assay Loading Reagent (Fluidigm PN 85000736)	3	6
Total	**6**	**12**

* Includes 20% overage

a For each IFC, aliquot 41 µL of 2X Assay Loading Reagent into each well of an 8-well PCR strip tube.

b Using an 8-channel pipette, transfer 2X Assay Loading Reagent to all wells of a 96-well PCR plate.

c Add assay (or PCR water) to each well containing Assay Loading Reagent.

NOTE Wells in the assay plate that do not contain assay have been pre-filled with PCR water for ease of preparation.

IMPORTANT All inlets of the IFC must be filled with reagent for proper loading.

2 Reseal the Advanta Immuno-Oncology Gene Expression Assay plate. If using the assay plate within 2 days, store at 4 °C. Otherwise, store at −20 °C.

STOPPING POINT The 10X assay mix can be stored at 4 °C if used within 1 week, or at −20 °C for longer storage. Protect assay mix from light.

Prepare the Sample Pre-Mix and Sample Mix

IMPORTANT The preamplified cDNA must be diluted 1:5 (see Prepare the Preamplification Reactions on page 2).

The sample pre-mix and sample mix volumes can be scaled based on the number of runs.

1 In a DNA-free hood, prepare the sample pre-mix in a 1.5 mL sterile tube using volumes shown in Table 7 (sufficient volume to fill an entire IFC).

Table 7. Sample pre-mix

Component	Volume per Inlet with Overage (µL)*	Sample Pre-Mix for One 96.96 IFC (µL)†
Gene Expression Master Mix (2X) (Fluidigm PN 101-5852)	3.0	360.0
20X GE Sample Loading Reagent (Fluidigm PN 100-6311)	0.3	36.0
Total	**3.3**	**396.0**

* Includes 20% overage.

† 120 reactions for ease of pipetting

2 Vortex to mix and centrifuge briefly.

3 For each IFC, aliquot 45 µL of sample pre-mix into each well of 8-well PCR tube strip.

4 Prepare sample mix as shown in Table 8.

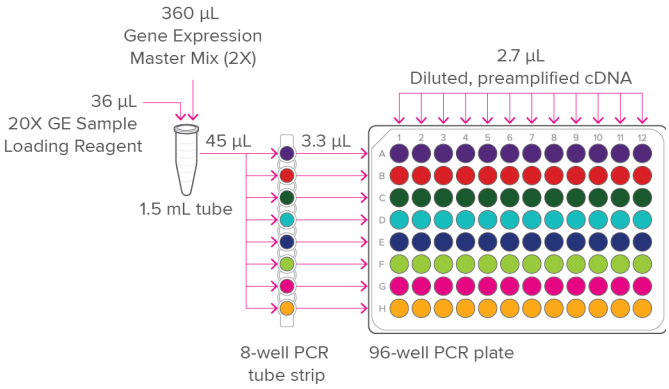
Table 8. Sample mix

Component	Volume per Inlet with Overage (µL)*
Sample pre-mix	3.3
Diluted, preamplified cDNA (see Prepare the Preamplification Reactions on page 2)	2.7
Total	**6.0**

* Includes 20% overage.

a Using an 8-channel pipette, transfer sample pre-mix from the tube strip into each well of a 96-well PCR plate. **Avoid creating bubbles.** Remove the aliquots of sample pre-mix from the DNA-free hood.

b Add diluted, preamplified cDNA (or PCR water if <96 samples) to each aliquot of sample pre-mix, making a total volume of 6.0 µL.



IMPORTANT All inlets of the IFC must be filled with reagent for proper loading.

5 Seal the plate well using an adhesive seal.

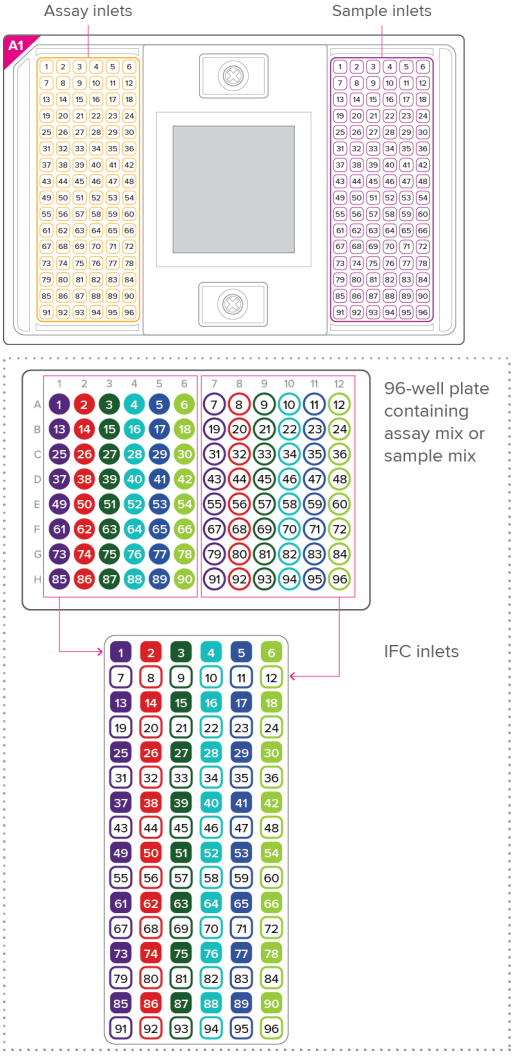
6 Vortex to mix and centrifuge.

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.
- Note orientation of the A1 corner before you start pipetting.

1 When the prime script is complete, remove the primed IFC from the controller and pipet 5 µL of each 10X assay mix and 5 µL of each sample mix into their respective inlets on the IFC as shown in the pipetting scheme. Refer to the assay plate maps in Appendix A to ensure traceability.



2 Return the IFC to the controller and run the load mix script:

- Juno: Tap **Load Mix 96.96 GE**, and then tap **Run**.
- HX: Select **Load Mix (136x)** and press **Run Script**.

Loading the IFC takes approximately 90 minutes.

3 After loading is complete, transfer the IFC to Biomark HD and collect real-time PCR data.

IMPORTANT Start the IFC run within 15 minutes of completing the load script.

Collect Real-Time PCR Data on Biomark HD

1. Use clear tape to remove any dust particles or debris from the IFC surface.
2. If necessary, double-click the **Data Collection** icon on the desktop of the Biomark HD system computer to launch the software.
3. Click **Start a New Run**.
4. Confirm that the camera status indicator at the bottom of the window is green.
5. Place the IFC on the instrument tray, aligning the notched A1 corner on the IFC with the A1 on the tray, and click **Load**.
6. Complete the Chip Barcode and Type section:
   a. Verify IFC barcode and IFC type.
   b. Choose project settings (if applicable) and click **Next**.
7. Complete the Chip Run section by selecting either a new or pre-defined run.
8. 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**.
9. 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**

10. Complete the Thermal Protocol section:
    a. Browse to and select the thermal protocol: **GE 96x96 Standard v2.pcl**.
    b. Confirm that Auto Exposure is selected and click **Next**.
11. Verify the IFC run information and click **Start Run**.

    The IFC run takes approximately 2 hours.
12. After the run is complete, process your data using the Real-Time PCR Analysis software. For information about using the analysis software, see the Real-Time PCR Analysis User Guide (PN 68000088).

Advanta Immuno-Oncology Gene Expression Assay Map

Panel A (PN 101-6145)

	1	2	3	4	5	6	7	8	9	10	11	12
A	GAPDH*	TFRC	VEGFA	EOMES	GZMA	GZMB	CCL22	ITGAM	TMEM55B	TNF	CD1C	TNFRSF4
B	ACTB	GUSB	MS4A1	LAG3	PTPRC	IDO1	CCL28	ITGAX	VPS33B	FOXP3	CD70	ARG1
C	B2M	CCL2	CD244	PDCD1LG2	KLRK1	IL7R	CCR5	LGALS9	RORC	IL10	CLEC4C	CSF2
D	HLA-C	HMOX1	CD274	TNFRSF14	PRF1	CXCL9	CD40	MAP4K1	**MICA**†	CCR7	IL2RA	IL17A
E	HLA-B	NCAM1	CD276	VTCN1	CD3E	CX3CL1	CD40LG	PTGER2	BTLA	CD27	TNFRSF9	IL17F
F	HLA-A	EPCAM	CD69	CD86	CD4	CXCL10	ITGB2	PTGER4	CD28	CXCR3	IL7	IL2
G	CXCL8 (IL8)	SDHA	CD80	CD48	IL12A	ICAM1	IL13	PTGS2	ICOS	IFNG	**MICB**†	IL4
H	HAVCR2**	VCAM1	CTLA4	TGFB1	CD8A	IL1B	IL6	SP2	TNFSF4	TBX21	PDCD1	TNFSF9

Panel B (PN 101-6146)

	1	2	3	4	5	6	7	8	9	10	11	12
A	GAPDH*	TFRC	LAPTM5	STAT1	CD22	CD2	IL2RG	PYGL	STAT2	EBI3		
B	ACTB	GUSB	NKG7*	CYBB	IGLJ3*	CD37	SLAMF7	LCK	STAT5A	FASLG		
C	B2M	IGKC*	DGAT2	IRF9	CD160	CD53	SLAMF8	GZMH	STAT6	IFNA2*		
D	IGHA1	HLA-DPB1	CD63	STAT3	NT5E	CXCR4	TNFAIP8	ISG15*	TLR7	CD19		
E	HLA-DRB1	CCL21	GNLY	STAT5B	CCL3	GZMK	FYB	IFIT2	APOBEC3B	TNFRSF18		
F	NRAS	CD52	IL15	ARG2	CCL4	HLA-DMB	CA4	CD1D	FCRLA	TLR8		
G	IGHM	CTSS	CCL5	ERBB2	HLA-DQB1	IGSF6	KREMEN1	GATA3	IGHG1	IL12B		
H	JCHAIN (IGJ)	FCER1G	IFI27	APOBEC3A	CCL18	IL10RA	LRG1	JAK2	TNFSF18			

Reference genes

Open wells for user customization (prefilled with PCR water)

* Assays that can detect gDNA

** Assays that may also detect with gDNA when gDNA is present in the RT-preamplification reaction

† The **MICA** assay can also detect the MICB gene.

For technical support visit fluidigm.com/support.

North America +1 650 266 6100 | Toll-free (US/CAN): 866 358 4354 | techsupport@fluidigm.com **Latin America** +1 650 266 6100 | techsupportlatam@fluidigm.com
Europe/Middle East/Africa/Russia +44 1223 598100 | techsupporteurope@fluidigm.com **China (excluding Hong Kong)** +86 21 3255 8368 | techsupportchina@fluidigm.com
Japan +81 3 3662 2150 | techsupportjapan@fluidigm.com **All other Asian countries/India/Australia** +1 650 266 6100 | techsupportasia@fluidigm.com