TECHNICAL NOTE

Viral Pathogen Detection with Custom Assays Using the 48.48 IFC-X and the Biomark X9 System

About This Protocol

The protocol described in this technical note describes how to perform viral pathogen detection by adapting the Gene Expression Using the 48.48 IFC-X with TaqMan Assays chapter of the **Biomark™ X9 System Gene Expression and Genotyping User Guide (FLDM-01040)** for use with Advanta™ RT-Preamp and PCR Master Mixes from Standard BioTools and custom primers and probes from Integrated DNA Technologies (IDT). Review the Biomark X9 System User Guide for additional information, including best practices.

Please note that this protocol has not been fully validated by Standard BioTools and is intended for research use only. For a summary of a proof-of-concept study using this workflow, see the related application note: **Detection and Subtyping of Viral Pathogens Using Custom qPCR Assays on the Biomark X9 System (LAB-00015)**.

IMPORTANT Before using this kit, read and understand the detailed instructions and safety guidelines in this document. For complete safety information, see Appendix C.

The workflow can be used with purified nucleic acid samples (RNA or both DNA and RNA) or saliva specimens and allows detection of up to 48 pathogens (viral RNA) per sample using unique assays. Reverse transcription (RT) and preamplification of the samples is performed in PCR plates. The samples and assays are transferred to the 48.48 Dynamic Array™ IFC-X (integrated fluidic circuit for the Biomark X9 System for High-Throughput Genomics) for automated loading, thermal-cycling and data collection on the Biomark X9 System.

Materials

Product Name	Source	Part Number
48.48 Dynamic Array IFC-X – Real-Time PCR – 10 IFCs Contains: • 20X GE Sample Loading Reagent, 250 µL (85000736) • 2X Assay Loading Reagent, 1.5 mL (85000735) • Control Line Fluid 300, 300 µL x 20 (101-2344) • 48.48 Dynamic Array IFC-X – Real-Time PCR (102-1950)	Standard BioTools	102-1969
Advanta RT-Preamp Master Mix, 3.3 mL		102-0419
Advanta PCR Master Mix, 1.2 mL		102-0420
Biomark X9 System for High-Throughput Genomics		X9-X9
XC Interface Plate		102-1611
Custom primers and probes*	Major oligo supplier	Varies
1X TE Buffer (10 mM Tris-HCL, 1 mM EDTA, pH 8.0)	Major laboratory supplier (MLS)	Varies
PCR-certified water		
Disposable microcentrifuge tubes, polypropylene, 1.5 mL		
96-well PCR plates		
8-well PCR tube strips with caps		
Clear adhesive film for 96-well plates†		
2 centrifuges: 1 for microtubes, 1 for 96-well PCR plates		
Pipettes (P2–P1000) and appropriate filtered, low-retention tips		
Disposable microcentrifuge tubes, polypropylene, 1.5 mL		
8-channel pipettes and appropriate filtered, low-retention tips		
Vortexer		
Thermal cycler for 96-well plates (for example, Applied Biosystems Veriti 96-Well Thermal Cycler)		

* Probes should be labeled with 5′ FAM and 3′ Iowa Black FQ. ZEN internal quenchers are also compatible when paired with a 3′ Iowa Black FQ.
† Recommended: MicroAmp Clear Adhesive Film (Thermo Fisher Scientific, 4306311)

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

Pool and Dilute the Primers for Preamplification

IMPORTANT Prepare in a pre-PCR workspace of the lab.

1. Rehydrate primer stocks if necessary. Dilute primer stocks to 50 µM each primer.
2. After thawing, briefly vortex and centrifuge all primers and reagents before use.
3. Create a preamplification primer pool by combining all primers into a single tube where the final concentration of each is 108.9 nM. If using RNase P as a control, the final concentration of RNase P primers should be 21.4 nM due to high abundance of RNase P RNA in most sample types. See Table 1 for an example using 16 target assays and RNase P as a control.

Table 1. Preamp primer pool dilution

Component	Volume for N Assays for 96 Reactions (µL)	Volume for 16 Targets Plus RNase P (µL)
50 µM each primer (Target)	N x 1.7	32 (N) x 1.7 = 54.4
5 µM Each Primer (RNase P)	N x 3.3	2 (N) x 3.3 = 6.6
1X TE Buffer	782 – 61 (volume of primers combined)	721
Total	**—**	**782**

NOTE

- When preparing mixes for less than 96 reactions, include an additional 10% to the volumes for overage. Remaining volumes may not be stored and are discarded.
- Volumes can be adjusted proportionally based on the number of samples to be preamplified. Adjust the TE Buffer volume accordingly if more sample per reaction is desired.

Prepare the 1-Step Reverse Transcription and Preamplification Reactions

IMPORTANT Prepare in a pre-PCR workspace of the lab.

1. Thaw the Advanta RT-Preamp Master Mix and keep on ice or in a cold block. Briefly vortex and centrifuge them before use.
2. Combine the components shown in Table 2 to make the 1-step pre-mix and place on ice. Scale appropriately for multiple runs or if additional overage is needed based on pipette performance.

NOTE When preparing master mixes for fewer than 48 reactions, include an additional 10% in the volumes for overage.

Table 2. 1-step pre-mix

Component	Vol per Reaction (µL)	Vol for 48 Reactions (µL)*
Preamp primer pool (see Table 1)	7†	378
Advanta RT-Preamp Master Mix (102-0419)	3	162
Total	**10**	**540**

* Includes overage

† The preamp primer pool volume can be adjusted down to accommodate for more sample volume. The adjustment of the primer pool volume should be such that the final 1-step reaction volume in Step 4 is 15 µL.

3. Cap the tube, vortex and centrifuge the 1-step pre-mix.
4. Prepare the 1-step reaction plates:
   a. Dispense 10 µL of the 1-step pre-mix into respective wells of a 96 well plate.

      NOTE If reusing pipette tips for this dispense, be sure to pre-wet tips prior to first dispense.

   b. Add 5 µL RNA sample to the respective wells of the 96-well plates to bring the total volume of the reaction to 15 µL. Scale reaction volumes as appropriate.
5. Tightly seal the plates with clear adhesive film, gently vortex and then centrifuge them at 3,000 × *g* for 1 min to mix and collect contents at the bottom of each well.

Perform the 1-Step Reverse Transcription and Preamplification Reactions

1. Place each 96-well plate in a compatible thermal cycler and cycle using the program in Table 3:

Table 3. 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

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

Dilute the Preamplified DNA

IMPORTANT Perform the dilution in a post-PCR workspace of the lab.

After cycling, dilute the preamplified reactions in the 96-well plates in 1X TE Buffer as shown in Table 4 and described as follows:

IMPORTANT Briefly vortex the plates containing the preamplified DNA and then centrifuge them at 3,000 × *g* for 1 min to bring down the contents before removing the plate seals.

1. Transfer 60 µL of 1X TE Buffer into each well containing the preamplified DNA.
2. Tightly seal the plates with clear adhesive film, then gently vortex to mix and centrifuge at 3,000 × *g* for 1 min to bring down contents. Set aside until ready to prepare the final sample mixes.

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

Table 4. Diluted, preamplified cDNA

Component	Vol per Reaction (µL)
1X TE Buffer	60.0
Preamplified DNA (contained in the 96-well plates)	15.0
Total	**75.0**

Prepare and Perform Real-Time PCR Reactions on the IFC

IMPORTANT

- Open and handle preamplified diluted DNA only in a post-PCR workspace of the lab
- Final assay mixes and sample pre-mix can be prepared in a pre-PCR workspace of the lab, or in a DNA/template free hood in the post-PCR workspace in the lab

Prepare the Final Assay Mixes for Loading on the IFC

1. Thaw then briefly vortex and centrifuge all reagents before use.
2. Create diluted assays by combining appropriate primers and probes into wells of a 96-well plate. Final concentration per assay should be 18 µM each primer and 5 µM probe.
3. Prepare each final assay mix using the assays from Step 2 as shown in Table 5. Scale appropriately for multiple runs and/or use in replicate assay inlets if desired.

Table 5. Final assay mixes

Component	Vol per Inlet (µL)*
2X Assay Loading Reagent (85000736)	3.0
Assay (18 µM primer, 5 µM probe)	3.0
Total	**6.0**

* Includes overage, but these volumes only reflect 1 assay per 48.48 IFC. If more replicates are desired, adjust volumes accordingly.

   a. Add 3.0 µL of 2X Assay Loading Reagent to each well of a new 96-well plate.

   b. Pipette 3.0 µL of each assay from Step 2 to respective wells in a new 96-plate for a total volume of 6.0 µL per well.

      IMPORTANT For IFC inlets that will not contain an assay, replace the assay with 3.0 µL of PCR-certified water to create 6.0 µL total.

4. Tightly seal the plate with clear adhesive film, vortex to mix, then centrifuge at 3,000 × g for 1 min to bring down the contents.

   STOPPING POINT Prepared final assay mixes can either be used immediately or stored at 4 °C for less than 2 weeks or −20 °C for at least 6 months.

Prepare the Final Sample Mixes

1. Thaw the Advanta PCR Master Mix and keep on ice.
2. Combine the components shown in Table 6 in a 1.5 mL tube to make the sample pre-mix and place on ice or in a cold block. Scale appropriately for multiple runs or if additional overage is needed based on pipette performance.

Table 6. Sample pre-mix for 1 IFC.

Component	Vol per Inlet (µL)*	Sample Pre-Mix for One 48.48 IFC (µL)†
Advanta PCR Master Mix (102-0420)	3.0	180
20X GE Sample Loading Reagent (85000735)	0.3	18
Total	**3.3**	**198**

* Includes overage

† 60 reactions for ease of pipetting

3. Prepare the final sample mixes:
   a. Briefly vortex and centrifuge the sample pre-mix from Table 6.
   b. Distribute 23 µL of sample pre-mix into each well of a new 8-well strip.
   c. Using an 8-channel pipette, transfer 3.3 µL of sample pre-mix into each well of a new 96-well plate.
   d. Remove the plate from the DNA/template free hood or to a DNA/template workspace in the lab.
   e. Add 2.7 µL of diluted, preamplified sample to the appropriate wells of the sample pre-mix plate, making a total volume of 6 µL per well.
   f. Tightly seal the plate with clear adhesive film, vortex to mix, then centrifuge at 3,000 × g for 1 min to bring down the contents.

   NOTE

   - Sample pre-mix and diluted preamplified sample volumes can be scaled as appropriate while keeping the ratio the same
   - For IFC inlets that will not contain a sample, replace the diluted, preamplified DNA in Step 3 with 2.7 µL of PCR-certified water

Prime the 48.48 IFC-X

Before pipetting assays and samples into the IFC, you need to prime the IFC with control line fluid.

Inject Control Line Fluid into the Accumulators

IMPORTANT When injecting control line fluid, only use Control Line Fluid 300 syringes, which are prefilled with 300 µL of control line fluid.

1. Remove the control line fluid syringes from the packaging and the IFC from the box and foil envelope.

   IMPORTANT Do not evacuate air from the syringe prior to injecting control line fluid (Step 4).

2. Actuate the check valves:
   a. Place the IFC on a flat surface.
   b. Use the syringe with the shipping cap in place to actuate the check valve in each accumulator with gentle pressure. Ensure that the poppet can move freely up and down.

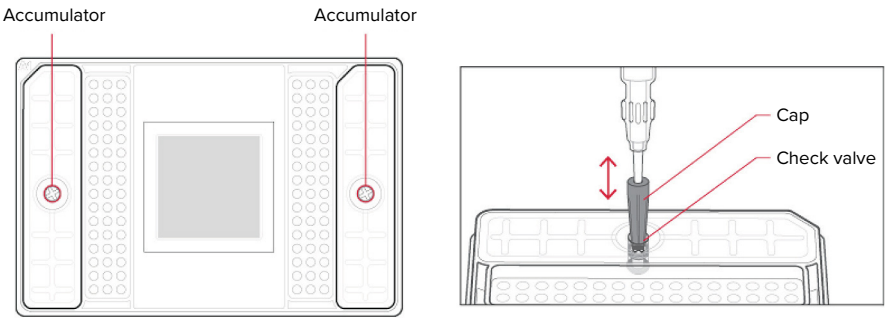

Figure 1. Actuate the check valves

3. Hold the syringe firmly in one hand with the tip facing up and away from the IFC, pull back on the plunger slightly to create negative pressure and remove the shipping cap with the other hand. Be careful when removing the control line fluid syringe cap to prevent drips.

4. Holding the IFC at a 45° angle, insert the syringe tip into an accumulator.

   IMPORTANT

   - Avoid bending the syringe tip. Be careful when removing the syringe cap to prevent drips.
   - 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.

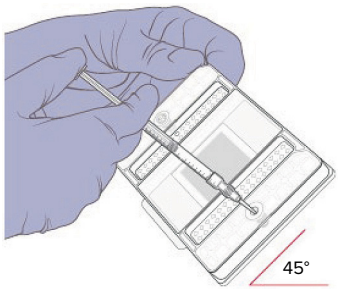

Figure 2. Inserting the syringe into an accumulator

5. Insert the syringe tip into one of the spaces between the arms of the X at the top of the valve and then press down gently to move the black O-ring to the side. Visually confirm that the O-ring has moved.
6. Release the control line fluid:
   a. Press the syringe plunger to release the control line fluid into the accumulator while maintaining the 45° angle to allow the fluid to flow away from the O-ring.
   b. Slowly inject the control line fluid by pushing down on the syringe plunger. The control line fluid flows into the accumulator through the open check valve. Use the entire contents of the syringe.
   c. After fully depressing the plunger, wait approximately 5 sec before withdrawing the syringe. Before removing the syringe from the accumulator, ensure that all the control line fluid and air are purged from the syringe to avoid dripping fluid on the surface of the IFC. Control line fluid or air remaining in the syringe tip is normal.

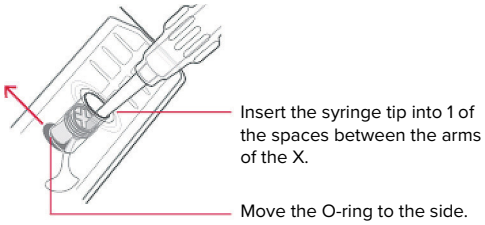

Figure 3. Injecting control line fluid into the IFC

7. Check to ensure that the O-ring returns to its normal position after the syringe is removed.
8. Repeat Steps 3 through 7 for the other accumulator.
9. Pull the protective film down and away from the bottom of the IFC. Discard the film.
10. Place the XC Interface Plate over the IFC. Align the barcoded edges.

Prime the 48.48 IFC-X on the Biomark X9 System

1. Tap the Biomark X9 System touchscreen to unlock it, and, if required, log in to the instrument.
2. Tap **Open** to eject the tray.
3. Place the IFC containing control line fluid and the interface plate on the tray. Align the notched corner of the IFC to the notch on the tray and face the barcoded edges of the IFC and interface plate forward.

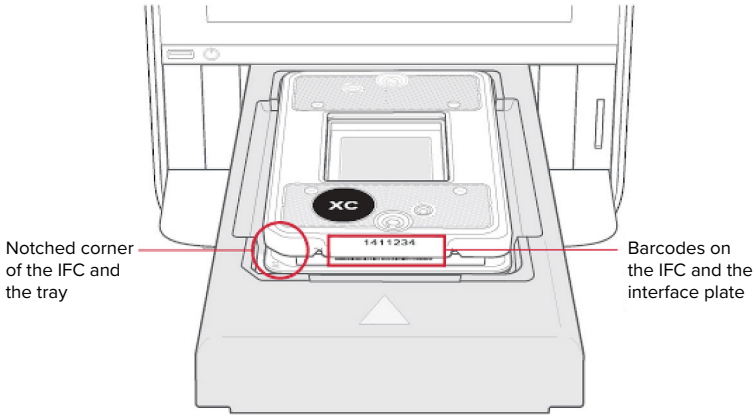

Figure 4. 48.48 IFC-X and interface plate on the instrument tray for priming

4. Tap **Start**. The system scans the IFC barcode and closes the tray.

   NOTE While the IFC is being primed on the Biomark X9 System, you can prepare the assay and sample mixes.

5. When priming is complete, tap **Next**, tap **Open**, then remove the IFC and interface plate from the instrument. Tap **Close**.

   IMPORTANT

   - Do not leave the tray open while you are pipetting samples and assays into the IFC
   - Run the IFC on the Biomark X9 System within 60 min of completing IFC priming

Pipette Assays and Samples into the IFC

IMPORTANT

- Vortex all assays, samples and control reagents thoroughly, then centrifuge them to bring down contents before removing the plate seals and pipetting the mixes into the IFC inlets. Failure to do so may result in compromised data.
- While pipetting, do not go past the first stop on the pipette. Doing so may introduce air bubbles into inlets.
- Follow the best practices in the Biomark X9 System User Guide for preventing bubbles

Refer to Figure 5 when pipetting final assay and sample mixes into the 48.48 IFC-X. Note the orientation of the notched A1 corner. The barcoded edge is on the left side.

1. Remove the interface plate from the primed IFC.
2. Pipette 5 µL of each final assay mix into the respective assay inlets on the IFC.
3. Pipette 5 µL of each final sample mix into the respective sample inlets on the IFC.
4. Use clear tape to remove any dust particles or debris from the surface of the center of the IFC, if necessary.
5. Replace the XC Interface Plate over the IFC, aligning the barcoded edges.

   IMPORTANT Run the IFC on the Biomark X9 System immediately after pipetting assay and sample mixes.

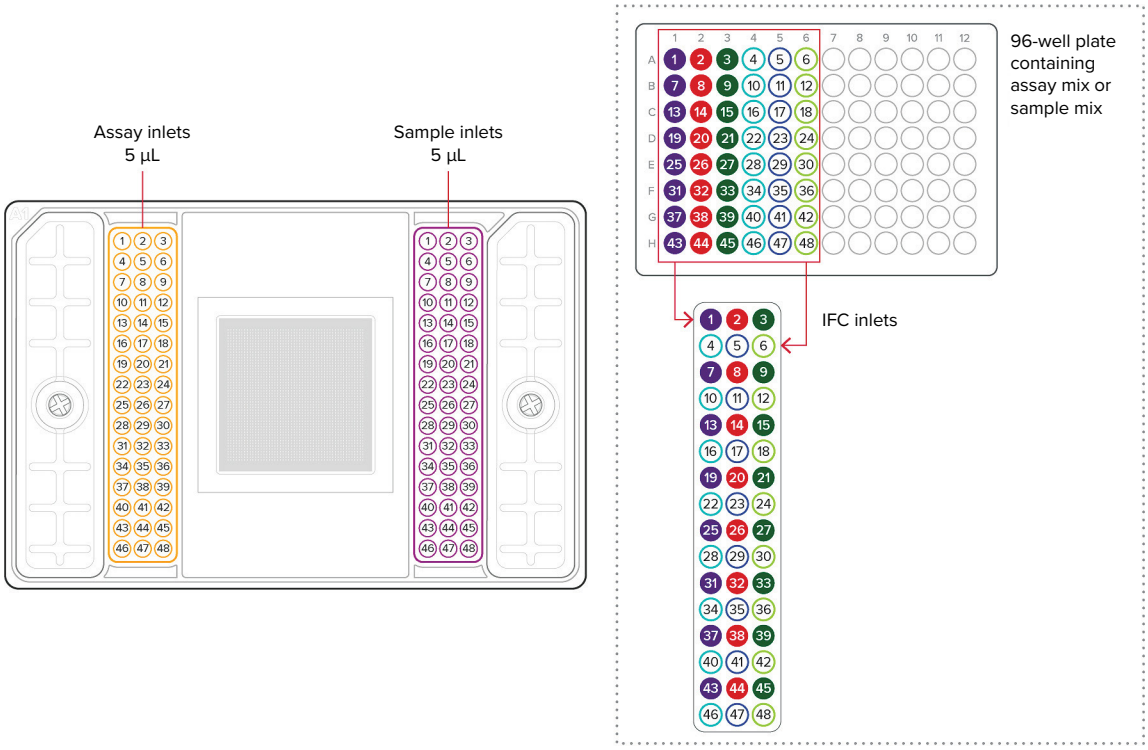

Figure 5. Pipetting map for the 48.48 IFC-X

Run the 48.48 IFC-X on the Biomark X9 System

1. Tap the Biomark X9 System touchscreen to unlock it, and, if required, log in to the instrument.
2. Tap **Open** to eject the tray.
3. Place the IFC and interface plate on the tray. Align the notched corner of the IFC to the notch on the tray and face the barcoded edges of the IFC and interface plate forward.
4. Tap **Start**. The system scans the IFC and interface plate barcodes, closes the tray and then starts the run.
5. When the run is complete, export your data to a USB drive or network location.

   NOTE If you turned on Network Export in Settings, the data is automatically exported to the designated server. See the Biomark X9 System User Guide for more information about setting up network export.

   To export data using a USB drive:

   a. Insert a USB drive into the USB port on the front of the instrument.

   b. Tap **Export** to copy the IFC run data to the USB drive.

   NOTE You can export the data files later from the History screen. See the Biomark X9 System User Guide for more information.

   c. Tap **Next**.
6. Tap **Open** to eject the tray, then remove the IFC and interface plate from the instrument.
7. Tap **Close**.

   IMPORTANT Close the tray after you remove the IFC and interface plate.
8. Return the interface plate to the storage container. Discard the used IFC.
9. Annotate and analyze the exported data using Standard BioTools™ Real-Time PCR Analysis Software. For more information about using the software, see the Standard BioTools Real-Time PCR Analysis Software User Guide (FLDM-01051).

Appendix A: Biomark X9 Cycler Protocols

GE 48x48 Fast v1 Thermal Protocol

Temperature	Time	Cycles	Description	
95 °C	60 sec	1	Hot start	
96 °C	5 sec	35	PCR	Denaturation
60 °C	20 sec			Annealing

Appendix B: Related Documents

Title	Document Number
Biomark X9 System Gene Expression and Genotyping User Guide	FLDM-01040
Standard BioTools Real-Time PCR Analysis Software User Guide	FLDM-01051
Detection and Subtyping of Viral Pathogens Using Custom qPCR Assays on the Biomark X9 System	LAB-00015

Appendix C: Safety

Safety Alert Conventions

Standard BioTools documentation uses specific conventions for presenting information that may require your attention. Refer to the following safety alert conventions.

Safety Alerts for Chemicals

For hazards associated with chemicals, this document follows the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (GHS) and uses indicators that include a pictogram and a signal word that indicates the severity level:

Indicator	Description
	Pictogram (see example) consisting of a symbol on a white background within a red diamond-shaped frame. Refer to the individual safety data sheet (SDS) for the applicable pictograms and hazards pertaining to the chemicals being used.
DANGER	Signal word that indicates more severe hazards.
WARNING	Signal word that indicates less severe hazards.

Safety Alerts for Instruments

For hazards associated with instruments, this document uses indicators that include a pictogram and signal words that indicate the severity level:

Indicator	Description
	Pictogram (see example) consisting of a symbol on a white background within a black triangle-shaped frame. Refer to the instrument user guide for the applicable pictograms and hazards pertaining to instrument usage.
DANGER	Signal word that indicates an imminent hazard that will result in severe injury or death if not avoided.
WARNING	Signal word that indicates a potentially hazardous situation that could result in serious injury or death if not avoided.
CAUTION	Signal word that indicates a potentially hazardous situation that could result in minor or moderate personal injury if not avoided.
IMPORTANT	Signal word that indicates information necessary for proper use of products or successful outcome of experiments.

Safety Datasheets

Read and understand the safety datasheets (SDSs) before handling chemicals. To obtain SDSs for chemicals ordered from Standard BioTools, either alone or as part of this kit, go to standardbio.com and search for the SDS using either the product name or the part number.

Some chemicals referred to in this user guide may not have been provided with your system. Obtain the SDSs for chemicals provided by other manufacturers from those manufacturers.

General Safety

In addition to your site-specific safety requirements, Standard BioTools recommends the following general safety guidelines in all laboratory and manufacturing areas:

- Use the appropriate personal protective equipment (PPE): safety glasses, fully enclosed shoes, lab coats and gloves, according to your laboratory safety practices
- Know the locations of all safety equipment (fire extinguishers, spill kits, eyewashes/ showers, first-aid kits, safety datasheets, etc.), emergency exit locations and emergency/injury reporting procedures
- Do not eat, drink, smoke or apply cosmetics in lab areas
- Maintain clean work areas
- Wash hands before leaving the lab

Instrument Safety

For complete instrument safety information, including a full list of the symbols on the instrument, refer to the Biomark X9 System Gene Expression and Genotyping User Guide (FLDM-01040).

WARNING POTENTIAL BIOHAZARD. When handling biohazardous materials or when using biohazardous material on the instrument, use appropriate personal protective equipment and adhere to *Biosafety in Microbiological and Biomedical Laboratories* (BMBL), a publication from the Centers for Disease Control and Prevention, and to your lab's safety protocol to limit biohazard risks. If biohazardous materials are used, properly label the equipment as a biohazard. For more information, see the BMBL guidelines online at **cdc.gov/labs/bmbl/index.html**.

Chemical Safety

The responsible individuals must take the necessary precautions to ensure that the surrounding workplace is safe and that instrument operators are not exposed to hazardous levels of toxic substances. When working with any chemicals, refer to the applicable SDSs provided by the manufacturer or supplier. When handling any chemical, the following safe-handling guidelines should be strictly observed:

- Do not inhale fumes from chemicals. Use adequate ventilation and return caps to bottles immediately after use.
- Use, store and dispose of chemicals according to manufacturer recommendations and to regulations applicable to the locality, state, province and/or country
- When preparing chemical solutions, always work in a fume hood that is suitable for those chemicals
- Conduct sample preparation away from the system to minimize corrosion and contamination
- Store solvents in an approved cabinet (with the appropriate ventilation) away from the system

Disposal of Products

Used slides and reagents should be handled and disposed of in accordance with federal, state, regional and local laws for hazardous waste management and disposal.

For technical support visit
standardbio.com/tech-support

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LAB-00014 Rev 02 072025
Viral Pathogen Detection Using the Biomark X9 System Tech Note
For Research Use Only. Not for use in diagnostic procedures.