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iNtRON Biotechnology

Instruction for Use

e-Myco™ VALiD-Q Real-time PCR Kit (v3.0 PRO)

version; 1.0

Nov. 2025

Cat. No. **25247** **100 Tests**
for 20 µl reactions

Store the Kit below –20 °C.

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1. General Information

1) Intended Use

The e-Myco™ VALiD-Q Real-time PCR Kit (v3.0 PRO) is designed for the qualitative detection of microorganisms belonging to the Mollicutes class—including Mycoplasma, Acholeplasma, and Spiroplasma—in cell culture systems and a wide range of biological samples.

The assay utilizes real-time PCR technology to enable rapid and sensitive detection of Mollicutes contamination.

This kit targets a highly conserved genomic region within the Mollicutes class and is designed to cover the species listed in major pharmacopeial chapters, including European Pharmacopoeia 2.6.7 (EP 2.6.7), USP <63>, and Japanese Pharmacopoeia G3 (JP G3). Internal performance evaluations have confirmed compatibility with a broad range of real-time PCR instruments.

The kit is configured to support testing in accordance with EP 2.6.7, USP <63>, and JP G3 requirements across various sample matrices, such as cell-culture supernatants, serum-containing samples, and enriched cell-culture samples.

The e-Myco™ VALiD-Q Real-time PCR Kit (v3.0 PRO) has been developed to meet the analytical criteria specified in the above pharmacopeias, providing a reliable and reproducible solution for detecting Mycoplasma and related Mollicutes contamination across diverse laboratory settings.

2) Test Principle

The e-Myco™ VALiD-Q Real-time PCR Kit (v3.0 PRO) detects Mollicutes by amplifying a conserved region of the 16S rRNA gene, allowing specific identification of Mycoplasma and related organisms. Under the defined reaction conditions, eukaryotic DNA and non-Mollicutes bacterial genomes are not amplified. The Instructions for Use describe test procedures compliant with EP 2.6.7, USP <63>, and JP G3, along with a general screening method based on cell-culture supernatants. Guidance on DNA extraction methods and recommended sample volumes is also provided. The total assay time is less than 1 hours, and—unlike direct culture methods or indirect assays such as luminescence- or fluorescence-based techniques—the test does not require viable Mycoplasma cells. PCR-based detection provides superior analytical sensitivity and precision compared with biochemical or cell-based methods. The kit contains Hot-Start DNA Taq Polymerase, a primer/probe panel, and all required PCR reagents supplied as a 2X qPCR Mix, along with Internal Control (IC), External Control (EC), and DNase/RNase-free water. The Internal Control DNA is included to

identify potential false-negative results due to PCR inhibition or issues during DNA extraction. It may be added directly to the PCR Master Mix to monitor amplification performance, or mixed with raw samples during pre-processing to verify both extraction and amplification. The IC signal is detected in the HEX channel (Ex ~535–560 nm / Em ~556–580 nm, compatible with VIC or JOE channels), while Mycoplasma targets are detected in the FAM channel (Ex ~495 nm /Em ~520 nm). The External Control is intended to monitor false-positive results that may arise from contamination with frequently used positive controls. The EC is detected simultaneously in both the FAM channel and the Cy5 channel (Ex ~646 nm /Em ~667 nm). The reaction mixture also contains dUTP, enabling efficient removal of carryover contamination from previous PCR products during UNG (uracil-DNA glycosylase) treatment, thereby minimizing the risk of false-positive results.

3) Kit Contents

- *The e-Myco™ VALiD-Q Real-time PCR Kit (v3.0 PRO) is supplied as a 100-test kit, and contains the following components:*

Component	Volume per tube	Quantity	Tests per Kit
2X qPCR Mix	*520 µL*	*2 tubes*	*100 tests*
Detection Solution	*260 µL*	*2 tubes*	*100 tests*
Internal Control	*150 µL*	*2 tubes*	*100 tests*
External Control	*25 µL*	*3 tubes*	-
DNase/RNase Free Water	*1,000 µL*	*1 tube*	-

4) Storage

- *Storage condition : Store at –20 °C or below.*
- *Expiration : The kit is stable for up to 12 months when stored as recommended, and for up to 6 months after opening.*

5) Quality Control

- Each lot of the e-Myco™ VALiD-Q Real-time PCR Kit (v3.0 PRO) is tested to verify compliance with the established quality specifications.

Sample type	Quality Control (Ct Range)		
	FAM	HEX	Cy5
Mycoplasma	≤38	25 ± 2	NoCt
Non-Mycoplasma	NoCt or >38	25 ± 2	NoCt
External Control	22 ± 2	NoCt	22 ± 2

6) Technical Support

- For any inquiries during product use, please contact iNtRON or your local distributor. User feedback and comments are valuable resources that contribute to product improvement and future development. For technical support or additional information, please contact us.
- Korea (Domestic)
  T : +82-31-739-5733 E : info_dr@intron.co.kr
- International
  T : +82-31-739-5741 E : tech@intronbio.com

7) Application

- Monitoring Mycoplasma contamination in cell-culture systems
- Mycoplasma testing aligned with EP 2.6.7, USP <63>, and JP G3 guidelines
- Suitable for Mycoplasma testing performed under AQPA (Animal and Plant Quarantine Agency) and MFDS (Korean Ministry of Food and Drug Safety) guidelines.
- Detection of Mycoplasma in culture supernatants, media, and enrichment samples
- Verification of Mycoplasma-free status in biological samples and manufacturing processes
- Qualitative detection of Mollicutes DNA using real-time PCR

8) Safety Data Sheet

The Safety Data Sheet (SDS) is available on the product detail page of the official iNtRON website. Please follow all instructions provided in the SDS.

9) Explanations of Symbols

LOT	*Batch number*		*Expire date*
Σ	*Sufficient for tests*		*Attention*
	Manufacturing date	REF	*Product number*
	Manufactured by		*Storage temperature limitation (Upper limit)*
	Do not reuse		*Consult Instructions For Use*
RUO	*Research use only*	IVD	*In vitro Diagnostic*

10) Trademark

e-Myco™ is a registered trademark of iNtRON Biotechnology, Inc. Any other trademarks or product names appearing in this document are acknowledged as the property of their respective holders.

2. Sample Preparation and Handling

1) EP/USP/JP Compilant Testing Samples

During the "Mycoplasma Testing" required by EP, USP, and JP standards, PCR inhibitors such as cellular debris, proteins, and serum components present in the sample may affect the analytical result. To ensure accurate results, sample pre-processing and extraction/purification of mycoplasma nucleic acids are required after sample collection. Samples with a volume of 200 μL or less may proceed directly to the DNA extraction step without additional concentration.

The following sample types may inhibit PCR reactions and therefore require nucleic acid extraction and purification prior to testing:

- *Cell pellets: Direct use is not recommended due to potential PCR inhibition.*
- *Vaccines, cryopreserved stocks, paraffin-embedded materials, and other biological matrices*
- *Culture media with high serum content (FBS > 10%)*

According to the "Controls" section of EP 2.6.7, the Internal Control (IC) must be used during Mycoplasma Negative Testing to verify that all steps—from DNA extraction to PCR amplification—have been performed correctly. The IC included in this kit is designed to be added directly to the sample, enabling verification of extraction efficiency and detection of PCR inhibition.

The e-Myco™ VALiD-Q Real-time PCR Kit (v3.0 PRO) can be used with both automated and manual nucleic acid extraction methods. The extraction products listed below are suitable for sample pre-processing and nucleic acid recovery required for Mycoplasma Negative Tests and are recommended for use with this assay. The instructions for IC use for each extraction method (Auto Extraction Method / Manual Extraction Method) are provided below.

Auto Extraction Method

① *Add 16 µL of Internal Control (IC) to 200 µL of cell-culture supernatant.*

② *Load the sample-IC mixture into the first well containing the lysis buffer.*

③ *Add Protease K & RNase A to the designated wells, as instructed by the extraction kit.*

④ *Insert the prefilled extraction cartridge into the instrument and close the lid.*

⑤ *Run the extraction using the program recommended for gDNA (BACTERIA mode).*

⑥ *After completion, transfer 70-100 µL of the eluted DNA to a clean 1.5-mL tube.*

⑦ *Use 5 µL of the eluted DNA as the PCR template.*

Note : *For detailed procedures for the Auto Extraction Method, refer to the Instructions for Use of the Miracle-AutoXT Automated Nucleic Acid Extraction System (iNtRON, Cat. No. IMC-NC15PLUS) and the AutoXT Total gDNA Kit (iNtRON, Cat. No. 17188).*

Manual Extraction Method

(Optional) Centrifuge the cell-culture sample at 6,000 rpm for 1 minute.

① *Transfer 200 µL of the clarified supernatant into a 1.5 mL microtube.*

③ *Add 10 µL of Internal Control (IC) to 200 µL of cell-culture supernatant.*

④ *Add 20 µL of Proteinase K and 5 µl of RNase A Solution into sample tube, and then mix by pulse vortex.*

⑤ *Add 200 µl of Buffer BL into upper sample tube and mix thoroughly.*

⑥ *Place the mixture at Room Temperature for 2minutes.*

⑦ *Incubate the lysate at 56°C for 10 min.*

⑧ *Briefly centrifuge the 1.5 ml tube to remove drops from the inside of the lid.*

⑨ *Add 200 µL of absolute ethanol to the lysate and mix thoroughly by brief vortexing. Centrifuge briefly to collect any liquid from the tube lid.*

Manual Extraction Method

⑩ *Transfer the mixture to the Spin Column without wetting the rim. Centrifuge at 13,000 rpm for 1 min. Discard the filtrate and place the column into a fresh 2-mL Collection Tube.*

⑪ *Add 700 µL of Buffer WA (or Buffer WB for Protocol C) to the column, avoiding the rim, and centrifuge for 1 min at 13,000 rpm. Discard the flow-through and reuse the tube.*

⑫ *Add 700 µL of Buffer WB, avoiding the rim, and centrifuge for 1 min at 13,000 rpm. Discard the flow-through, transfer the column to a new 2-mL tube, and centrifuge again for 1 min to dry the membrane.*

⑬ *Place the column in a clean 1.5-mL tube and add 50 µL of Buffer CE directly onto the membrane. Incubate for 1 min at room temperature and centrifuge for 1 min at 13,000 rpm to elute.*

⑭ *Use 5 µL of the eluted DNA as the PCR template.*

Note : *For detailed procedures related to the Manual Extraction Method, refer to the Instructions for Use provided with the G-spin™ Total DNA Extraction Mini Kit (iNtRON, Cat. No. 17045/17046).*

2) General Cell Culture Samples

Cell-culture supernatants are an acceptable sample type for use with the e-Myco™ VALiD-Q Real-time PCR Kit (v3.0 PRO). The assay is designed to amplify Mycoplasma-specific genomic regions and has been optimized to allow reliable detection even in the presence of host-cell nucleic acids.

However, because cell-culture supernatants may contain residual host-cell materials or PCR inhibitors, DNA extraction and purification are required to ensure accurate results. When the host-cell concentration exceeds 1 × 10^7 cells/mL, excessive non-target DNA may reduce PCR amplification efficiency. In such cases, it is recommended to gently centrifuge the culture and use the clarified supernatant for DNA extraction (optional). Please prepare the sample according to the procedure described below.

Boiling Method

(Optional) Centrifuge the cell-culture sample at 6,000 rpm for 1 minute.

① *Transfer 100–500 µL of the clarified supernatant into a 1.5 mL microtube.*

① *Heat the sample at 95 °C for 10 minutes (minimum 5 minutes).*

① *Centrifuge the heated sample at 13,000 rpm for 1 minute to pellet residual debris.*

① *Use 5 µL of the resulting supernatant directly for the qPCR reaction.*

Note : *Use the extracted template promptly. If storage is necessary, keep it at +2 °C to +8 °C for up to 5 days or at –18 °C for up to 3 months.*

Note : *For the Boiling Method, add the Internal Control (IC) based on the initial sample volume if IC is included during the extraction step. However, for this method, it is recommended to add the IC at the PCR step.*

3) Important Notes before Starting

- *Use of Internal Control (IC)*

  The IC is used to verify that the DNA extraction process has been performed correctly. The amount of IC to be added should be based on the final elution volume, not on the volume of the original sample (e.g., add 2 µL of IC per 10 µL of final elution). The IC is added once during the extraction step and should not be added to the PCR reaction mixture. Because the IC is provided in a DNA form without a cell membrane, it cannot be used during sample concentration procedures.

- *Precautions for Positive Control (External Control)*

  The positive control is intended only for confirmation of positive amplification and is not suitable for evaluating extraction efficiency.

3. Protocol

1) Preparation of Reagents

- *Repeated freeze-thaw cycles may affect reagent performance; aliquoting is recommended when necessary.*
- *Before use, ensure that all reagents—including the negative and positive controls—are completely thawed. Do not leave thawed reagents at room temperature for more than 1 hour. After thawing, briefly spin down the reagents before use.*

2) Reaction Mix Preparation

- *For analysis, the qPCR reaction mix of the e-Myco™ VALiD-Q Real-time PCR Kit (v3.0 PRO) must be prepared by combining the 2X PCR Mix, Detection Solution, and Internal Control (IC). Reagents should be prepared in an amount equivalent to the number of tests plus two.*
- *The table below describes the preparation method for the qPCR reaction mix.*

Reagent	1 test	10 tests	20 tests
2X qPCR Mix	*10 µL*	*100 µL*	*200 µL*
Detection Solution	*5 µL*	*50 µL*	*100 µL*
Internal Control	*1 µL*	*10 µL*	*20 µL*
(qPCR Reaction Mix) Total volume	*16 µL*	*160 µL*	*320 µL*

3) Add Samples

- *After preparing the qPCR reaction mix, add the template, the positive control (EC), and the negative control (DNase/RNase-free water) to each reaction according to the composition described below. And then mix thoroughly and briefly spin down to remove any residual bubbles.*

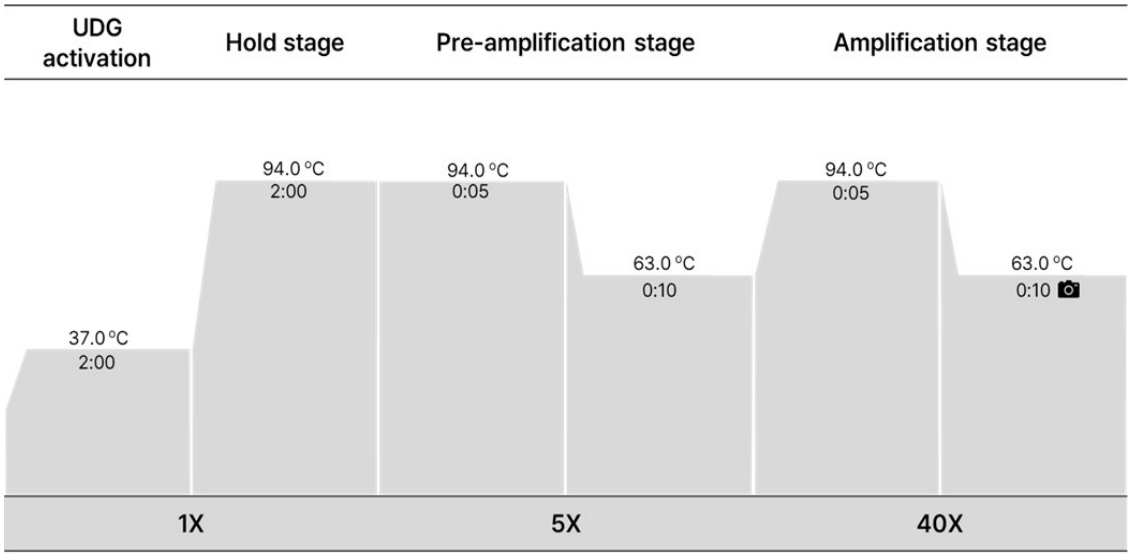
Reagent	Sample	Positive Control	Negative Control
qPCR Reaction Mix	*15 µL*	*15 µL*	*15 µL*
External Control	*- µL*	*5 µL*	*- µL*
Sample	*5 µL*	*- µL*	*- µL*
DNase/RNase free water	*- µL*	*- µL*	*5 µL*
Total reaction volume	*20 µL*	*20 µL*	*20 µL*

Note : *Real-time PCR is highly sensitive, and even trace contamination may produce detectable signals. Use dedicated filter tips and pipettes for positive controls and prepare them in a separate area.*

Note : *Because real-time PCR instruments detect fluorescence through the caps of PCR strip tubes, do not write on or attach labels to the cap surface.*

4) PCR Amplification

- *Perform the PCR reaction as described below.*



- *Set each detection channel according to the following configuration.*

Channel	FAM	HEX	Cy5
Target	*Mycoplasma*	*Internal control*	*External control*

Note : *The External Control is detected in both the FAM and Cy5 channels.*

- *Validated and Compatible Instruments*
  : This kit has been validated on the following real-time PCR platforms.

Instrument	Brand
FaSTAR96amp Real-time System	*iNtRON Biotechnology*
CXF96 Touch Real-Time PCR Detection System *CFX Opus 96 Real-Time PCR Detection System*	*Bio-Rad Laboratories*
QuantStudio 5 Real-Time PCR Instrument	*Applied Biosystems*

Note *: Other real-time PCR instruments with compatible optical channels (FAM ~520 nm, HEX ~560 nm, Cy5 ~670 nm) may be used. For detailed instrument setup and channel configuration, refer to the instrument manufacturer's instructions and the PCR Amplification section of this IFU.*

Note *: Other real-time PCR systems with equivalent optical channels may be used. Follow the instrument manufacturer's operating instructions.*

5) Instrument Setup

■ FaSTARamp96 Real-time PCR System

- *FaSTARamp96 is compatible with the following types of PCR strips/tubes.*

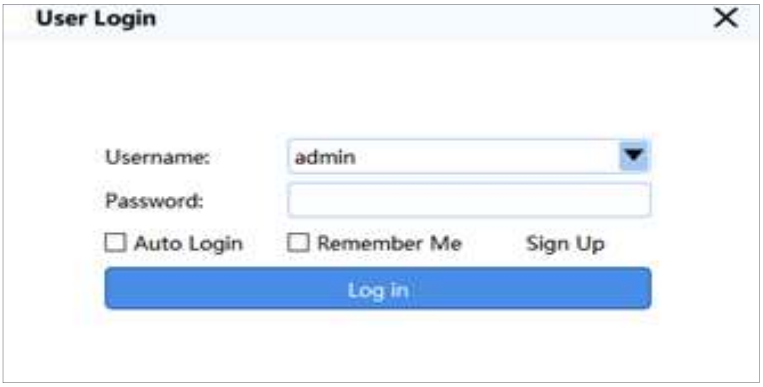
Suitable Consumables		
8-Strip PCR Tubes (qPCR-compatible)	*0.1 mL*	*Clear / White / Frosted*
	0.2 mL	*Clear / White / Frosted*
PCR Plates	*0.1 mL*	*Clear / White*
	0.2 mL	*Clear / White*
Single PCR tubes	*0.2 mL*	*Clear*

- ***Start Appication Software***

  To launch the instrument software, double-click the software icon <> on the desktop.

- ***User Login and Registration***

  The login interface of application software automatically pops up after starting the application software, as shown below.



Note : *If you are using the application for the first time, create a new account by selecting <Sign up>. After registration, enter your username and password and click <Log in> to access the application.*

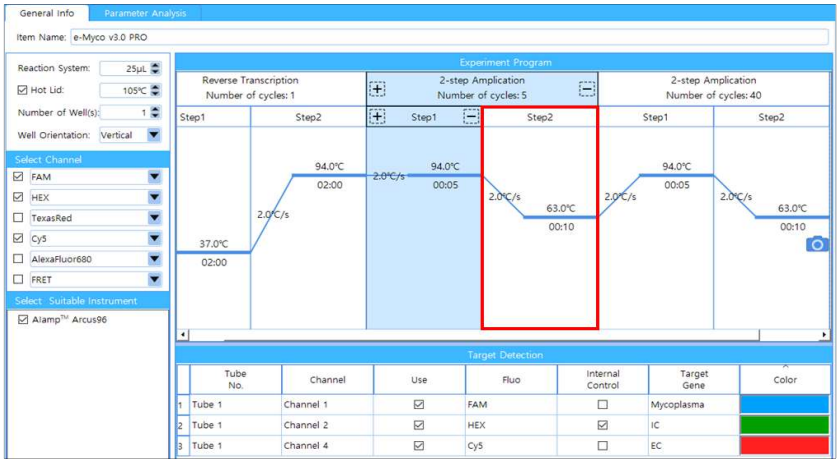
Note : *For instrument setup, account creation, and other system operations, refer to the user manual of the FaSTARamp96 Real-time PCR System. This IFU describes only the procedures required for kit operation.*

- ***Protocol Setup & Detection Channel***

① *From the main interface, verify that the instrument is connected in the "Instrument" panel (indicated by a green status light). Then, select "Management" in the "Item" panel.*

Note : *Click <Management> in the Instrument panel. The Instrument Management window will open automatically, allowing you to view and manage all instruments connected to the local network.*

② *From the Item popup window, select Management, and then click <Create> in the lower-left corner. This will open the "New Item" window. Specify the "Item Name" (e-Myco v3.0 PRO) and set the Reaction System (20 µL). Select the detection channels (FAM, HEX, Cy5), and enter the PCR cycling conditions (see "PCR Amplification", page 12). Refer to the image below.*

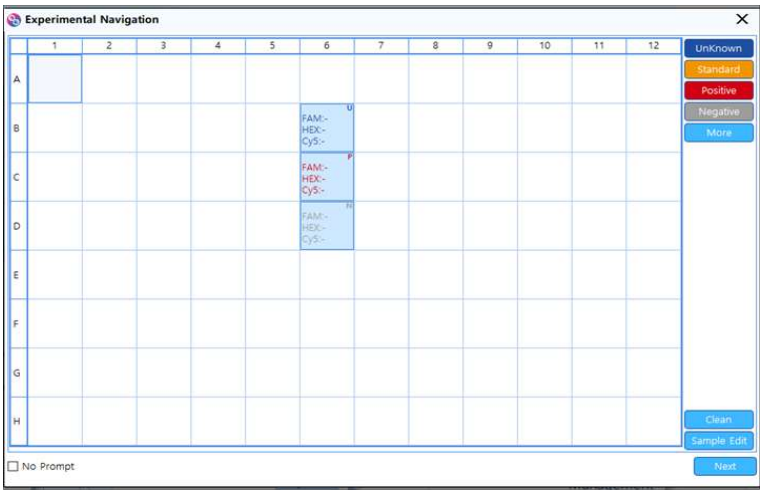


Note : *In the red-boxed section, ensure that the fluorescence detection mode is set to "Not Reading." Failure to apply this setting may result in incorrect data acquisition or invalid amplification curves.*

③ *Set the Analysis Method to "Absolute Quantitative (default setting)" in the Parameter Analysis window. All other parameters should remain at their default settings. Click <Save> and confirm that the "Saving Succeeded" message appears. The saved PCR condition file will then be available in the item list of the popup window.*

④ *From the Item list, double-click the saved PCR condition file (e-Myco v3.0 PRO). The "New Expt. from Item" window will appear, where you can edit the experiment name, specify the storage location for the result file, and check the instrument connection status. Select the connected instrument to enable the <Next> button, then click <Next> to proceed.*

⑤ *The "Experimental Navigation" window will appear. Select the appropriate sample information for each well (e.g., Sample – Unknown, Negative Control – Negative, External Control – Positive), then click <Next> to continue.*



Note : *The sample name for each well can be entered in the Sample List section at the bottom of the window.*

- ***Start PCR Run***

① *Press <* *Open/Close> function key on the touch screen, the instrument will eject the loading platform and the sample block will be present in front of the user.*

② *place the consumables that contain the sample and PCR reagent mixture into the sample block and press the <*  *Open/Close> function key again to withdraw the loading platform.*

③ *Select or edit an experiment program and then start running.*

- ***Reviewing Result***

① *For each selected well, the instrument displays the Ct value detected in each fluorescence channel in the following format. When amplification is detected, a numerical Ct value is shown. If no amplification is observed, the channel is displayed as "–". Interpret the results using the Ct values for each channel in accordance with the result interpretation table provided in this manual.*

② *You can review the amplification curves for each selected well by opening the Amplification Curve tab in the experiment file.Each channel (FAM, HEX, Cy5) provides its measured Ct value, which reflects the fluorescence signal generated during the reaction.*

Note *: If an amplification curve appears abnormal, manual adjustment of the baseline or fluorescence threshold may be required. If the issue persists or proper adjustment is unclear, contact the manufacturer for technical assistance.*

■ CFX96 Touch Real-time PCR Detection System

- *The e-Myco™ VALiD-Q Real-time PCR Kit (v3.0 PRO) is compatible with both the CFX96 Touch Real-Time PCR Detection System and the CFX Opus Real-Time PCR System.*

Note : *The instrument setup described in this section is based on the CFX Maestro software. This software is compatible with both Bio-Rad CFX96 and CFX Opus real-time PCR systems. Settings may vary depending on instrument configuration; refer to the instrument manufacturer's documentation for hardware-specific details.*

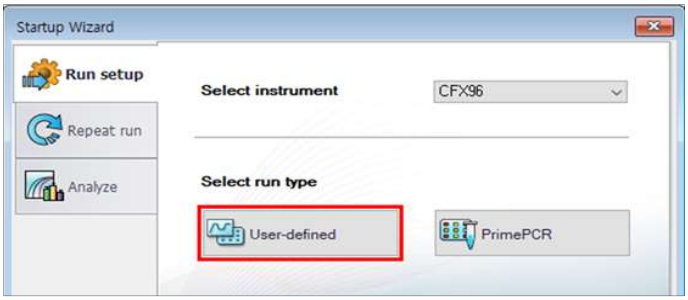
Note : *Recommended consumables for this kit include 0.1 mL 8-strip PCR tubes (white, optical). For information on compatible consumables specific to the Bio-Rad CFX96™ or CFX Opus™ systems, refer to the instrument manual or the manufacturer's website.*

- ***Start Appication Software***

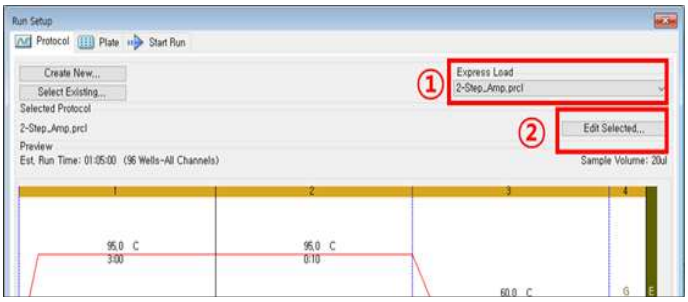
To launch the instrument software, double-click the software icon <> on the desktop.

- ***Protocol Setup & Detection Channel***

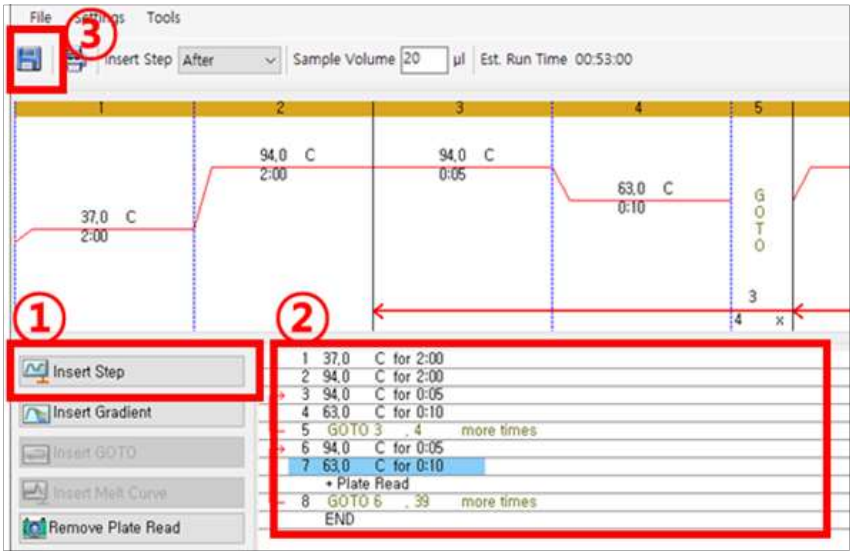
① *From the main interface, click <User-defined> in the "Run setup" panel.*



② *From the Run Setup window, select "2-Step-Amp.prcl" in Express Load, and then click <Edit Selected>.*



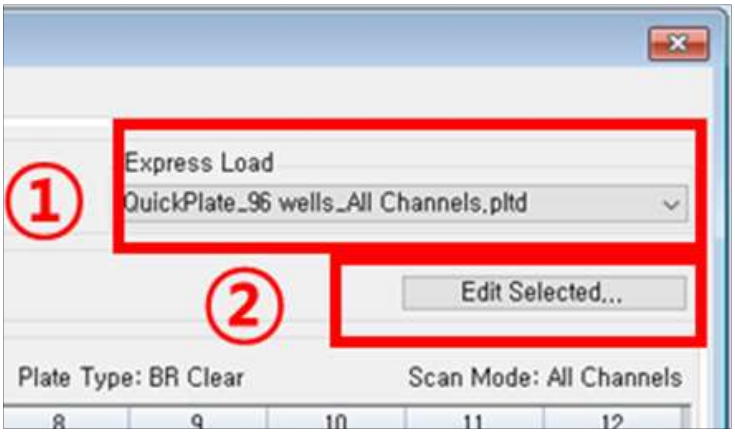
③ *In the Protocol Editor window, configure the PCR cycling conditions (see "PCR Amplification", page 12). Use the <Insert Step> button at the lower left to add cycling steps, and set the temperature and time parameters in the fields located in the center of the window. After confirming all entries, click <Save>, followed by <OK>, to store the PCR protocol.*



Note : *The sample name for each well can be entered in the Sample List section at the bottom of the window. Saved protocols can be loaded from the Run Setup window using <Select Existing>.*

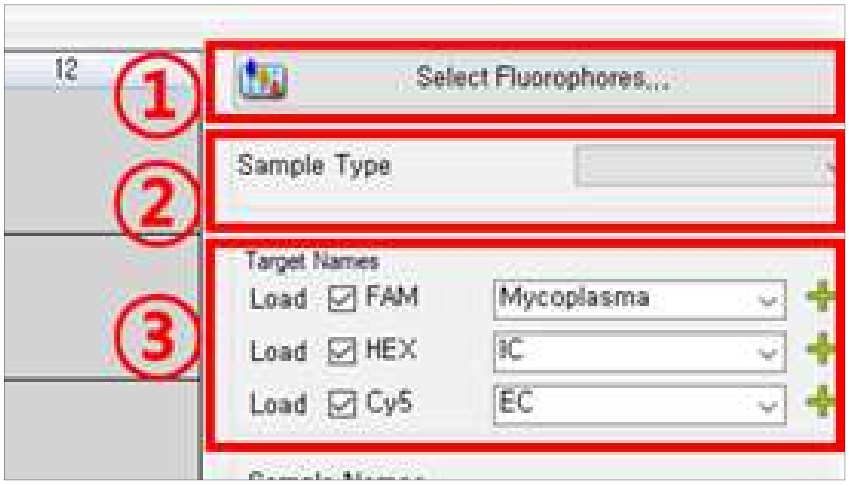
Note : *Enter 4 cycles for the Pre-amplification stage and 39 cycles for the Amplification stage when configuring the cycling parameters in this software.*

④ *After completing the PCR protocol setup, the software returns to the Run Setup window. Click <Next> to proceed to the Plate Setup page. For plate configuration, select "QuickPlate_96 wells_All Channel.pltd" under Express Load, then click <Edit Selected> to open the plate layout for editing.*



⑤ *In the Plate Editor window, click the selector located between column A and row 1 to highlight all wells. Select Clear Wells to remove any pre-assigned values. Click Select Fluorophores and enable FAM, HEX, and Cy5. If any other channels are selected, deselect them. Click an empty area to deselect the entire plate.*

⑤ *Select the wells containing samples. Under "Sample Type", choose the appropriate type (e.g., Unknown, Negative Control, Positive Control). For each selected well, assign the corresponding target name(s) for each active fluorescence channel. Review all entries and click <OK> to apply the settings. Save the plate configuration file. Click <Next> after completing the plate setup. The software will automatically open the Start Run tab.*



Note : *The sample name for each well can be entered in the Sample List section at the bottom of the window. Saved protocols can be loaded from the Run Setup window using <Select Existing>.*

Note : *Before saving the plate layout, ensure that the Scan Mode ( ) in the Plate Editor window is set to "All channels." If this setting is not enabled, the instrument cannot acquire fluorescence signals. When All channels mode is active, you may analyze or assign detection channels (FAM, HEX, Cy5) after the run is completed.*

Note : *The Plate Type may be left at the default value, as it does not affect data analysis. For all other panel settings in the Plate Editor, please refer to the instrument manufacturer's user manual.*

- ***Start PCR Run***

① *In the center panel, confirm that the instrument is properly connected under "Start Run on Selected Block(s)".*

② *When the connection is confirmed, click <Open Lid> and place the PCR tubes/strips into the block according to the configured plate layout. Click <Close Lid> to close the instrument lid. Click the activated <Start Run> button to begin the assay. Save the experiment file when prompted and verify that the run has successfully started.*

Note : *If the instrument is not properly connected to the software, the Open Lid, Close Lid, and Start Run buttons may not be activated. Verify the connection status in the Start Run tab before proceeding*

- ***Reviewing Result***

  When the run is complete, the "Data Analysis" window will open automatically. In the "Quantification" tab, review the amplification curves. Below the graph, verify the information and Cq values for each well.

  Note *: If an amplification curve appears abnormal, manual adjustment of the baseline or fluorescence threshold may be required. If the issue persists or appropriate adjustment cannot be determined, please contact iNtRON Technical Support for assistance.*

■ QuantStudio 5 Real-Time PCR Instrument

- *The e-Myco™ VALiD-Q Real-time PCR Kit (v3.0 PRO) is compatible with QuantStudio 5 Real-time PCR Instrument.*

  Note *: The instrument setup described in this section is based on the QuantStudio™ 5 Dx Develpoment Software.*

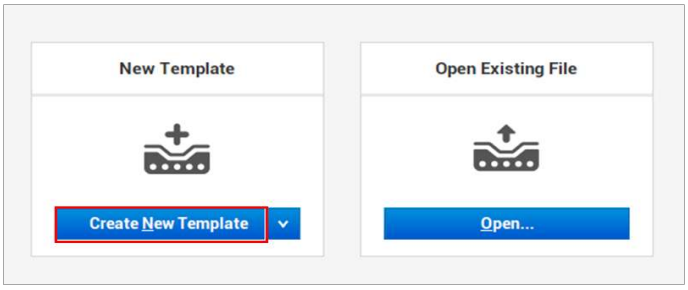
  Note *: Recommended consumables for this kit include 0.2 mL 8-strip PCR tubes (Clear, optical). For information on compatible consumables specific to the QuantStudio 5 Real-time PCR Instrument, refer to the instrument manual or the manufacturer's website.*

- ***Start Appication Software***

  To launch the instrument software, double-click the software icon <> on the desktop.

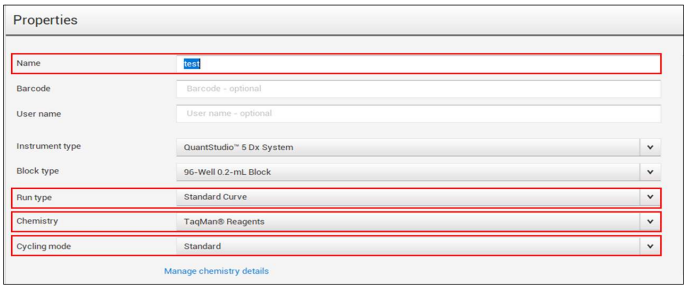
- ***Protocol Setup & Detection Channel***

  ① From the main interface, click <Create New Template> in the "New Template" panel.

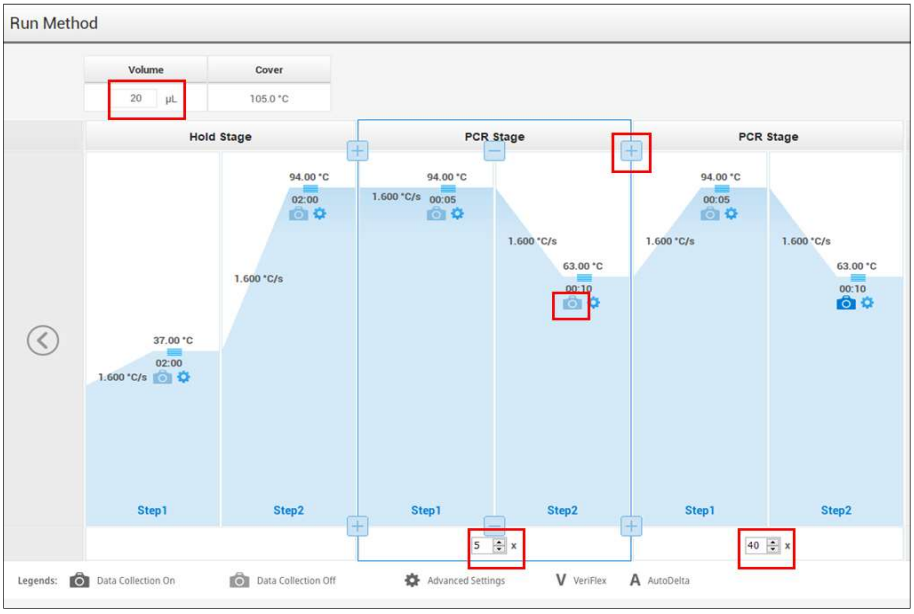


Note *: You can load a previously saved PCR protocol by clicking the <> button. For all other panel settings in the main interface, please refer to the instrument manufacturer's user manual.*

② *In the "Properties" tab, enter the experiment name, set the Run Type to "Standard Curve," select "TaqMan® Reagents" for the Chemistry option, and choose "Standard" as the Cycling Mode. Click <Next> to proceed to the following tab.*



③ *In the "Run Method" tab, configure the PCR cycling conditions (see "PCR Amplification", page 12). Hover the cursor over the "PCR Stage" section to display the option to add a new stage. Click the "+" icon to insert the stage and enter the required PCR conditions. For the Pre-amplification stage, disable fluorescence acquisition by clicking the camera icon. Set the reaction volume to 20 µL, then click the Save button in the upper-right corner to store the PCR protocol. After completing the configuration, click <Next> to proceed to the following tab.*

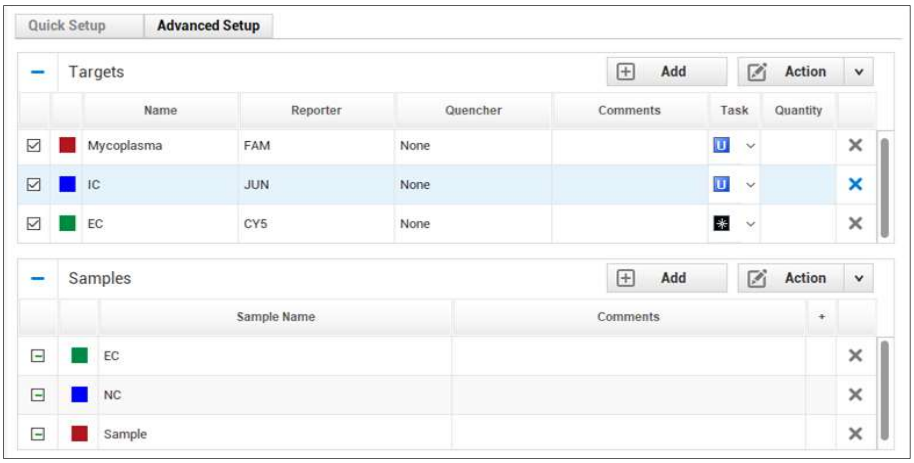


Note : *Ramp time may be left at the default setting; no additional adjustment is required. For all other panel settings in the Run Method, please refer to the instrument manufacturer's user manual.*

④ *In the "Quick Step" tab, Select "None" for the Passive Reference option in the "Plate Attributes" window.*

Note : *If a Passive Reference is enabled, the instrument may fail to display proper RFU values and amplification curves.*

⑤ *In the "Advanced Setup" tab, select FAM, JUN (as the HEX equivalent), and Cy5 as the active detection channels. Set the quencher to "None." Assign each sample, IC, and EC to the appropriate well positions on the plate.*



Note : *For all other panel settings in the Advanced Setup, please refer to the instrument manufacturer's user manual.*

- ***Start PCR Run***

① *In the "Advanced Setup" tab, select FAM, JUN (as the HEX equivalent), and Cy5 as the active detection channels. Set the quencher to "None." Assign each sample, IC, and EC to the appropriate well positions on the plate.*

- ***Reviewing Result***

When the run is complete, the "Data Analysis" window will open automatically. In the "Quantification" tab, review the amplification curves. Below the graph, verify the information and Ct values for each well.

6) Data Interpretation

- *Mycoplasma detection is determined by the fluorescence signal in the FAM channel. Because Ct calculation may vary depending on the Real-time PCR instrument and software used, please follow the guidance provided by the instrument manufacturer.*
- *This kit includes an External Control. To verify proper assay performance, the negative control (DNase/RNase-free water) and the positive control (External Control) must each be tested individually to confirm valid control results. Refer to the table below when evaluating assay validity.*

Channel	FAM	HEX	Cy5
External Control	*22.5 ± 2.5*	*22.5 ± 2.5*	*22.5 ± 2.5*
Negative control	-	*22.5 ± 2.5*	-

Note : *If abnormal results occur under proper storage and within the expiration period, you may request product replacement from the manufacturer.*

- *Evaluate each sample by checking its Ct value. A result of Ct ≤ 38 is interpreted as positive, and Ct > 38 is interpreted as negative. An example of result interpretation is provided in the table below.*

Case	FAM	HEX	Cy5	Interpretation
Sample 1	+	+	-	*Positive*
Sample 2	-	+	-	*Negative*
Sample 3	+	-	-	*Positive (IC check required*)*
Sample 4	-	-	-	*Invalid (Retest required*)*
Sample 5	+	+	-	*Carry-over contamination suspected*
External Control	+	+	+	*Valid*
	+	-	+	*Valid (IC check required*)*
	-	-	-	*Test failure**
Negative control	-	+	-	*Valid*
	-	-	-	*Test failure**

Note : *"IC check required" indicates that the Internal Control (IC) was not detected. For Mycoplasma negative testing, IC must confirm the validity of both the extraction and PCR steps. If IC is not detected, the test must be repeated starting from the extraction.*

Note : *For "Invalid (Retest required)" results where both FAM and IC (HEX) are absent: If IC was added during extraction → extraction validity cannot be confirmed; retest from extraction. If IC was added only at the PCR step → excessive template may suppress IC; dilute and repeat PCR.*

Note : *If the Internal Control (IC) signal is not detected in the External Control, first confirm that IC was added according to the procedure. If IC was added correctly and the IC signal is still absent, the control result may be invalid. Repeat the assay, and if the issue persists, contact the manufacturer.*

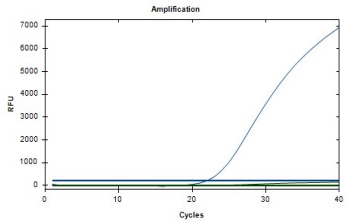
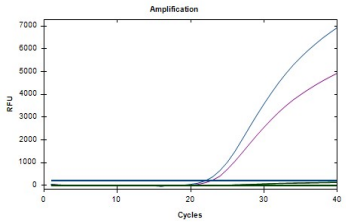
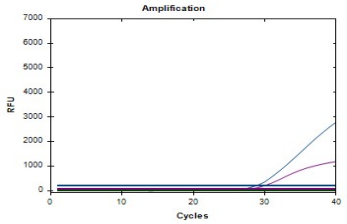
Note : *A "Test failure" result indicates invalid controls. Repeat the assay with fresh reagents. If the issue persists, contact the manufacturer.*

7) Control of Contamination

- ***Identifiaction of Carry-Over Contamination (External Control)***

The External Control included in this kit is designed to generate amplification signals in both the FAM and Cy5 channels. The Cy5 signal enables identification of carry-over contamination when trace amounts of positive-control material enter the reaction.

Therefore, if a sample shows fluorescence in both the FAM and Cy5 channels, contamination introduced during PCR setup is suspected, and the reaction must be repeated. An example graph illustrating carry-over contamination is provided below.

Positive (Sample)	Positive (External control)	False Positive (PC carry-over)
		

When such false-positive data are observed, contamination originating from the External Control is assumed, and the reaction is considered invalid. Prepare a fresh PCR reaction mix and repeat the assay.

- ***General Precautions***

To ensure accuracy and reproducibility in real-time PCR experiments, general precautions related to reagent handling, workspace management, and contamination control must be followed. The following precautions should be observed when using this product:

- *This product is for Research Use Only and must not be used for diagnostic purposes.*
- *Prepare the reaction mix, samples, and positive control in a clean bench whenever possible, and always in physically separated areas. After testing, clean the bench surface with alcohol.*
- *Laboratory personnel must wear protective equipment such as lab coats, gloves, and masks, and must exercise caution to prevent contamination.*
- *Use dedicated pipettes for reaction-mix preparation and employ filter tips (aerosol-resistant) to avoid contamination.*
- *When contamination is suspected or during routine maintenance, clean laboratory surfaces, instruments, and pipettes with a 1:10 diluted bleach solution or MycoClean™ Plus Mycoplasma Prevention Spray (iNtRON, Cat. No. 21084), then remove residues with distilled water.*
- *Add the External Control at the final step of reaction setup.*
- *Do not mix reagents from different lot numbers.*

8) Trouble Shooting

Observation	Possible Cause	Recommendation
ΔRn ≤ No Template control ΔRn, and no amplification plot	Incorrect detection channel selected	• Check dye component prior to data analysis
	Reaction component omitted	• Check that all the correct reagents were added
	Degraded template or no template added	• Repeat with fresh template
	Reaction inhibitor present	• Repeat with purified template
ΔRn ≤ No Template control ΔRn, and both reaction show an amplification plot	Template contamination of reagents	• Review workflow to prevent contamination. Use fresh reagents. • Repeat using aerosol-resistant filter tips after decontaminating the workspace.
Amplification plot dips downwards	Ct Value less than 15, amplification signal detected too early	• Verify baseline settings (adjust if necessary) or repeat with diluted sample
Amplification plots is not within the log phase	PCR efficiency is poor	• Re-optimize reaction conditions
Ct value is higher than expected	Less template added than expected	• Use a freshly prepared sample (avoid repeated freeze-thaw cycles) and repeat the assay.
	Sample is degraded	• Evaluate sample integrity
Ct value is lower than expected	More template added than expected	• Reduce sample amount
	Template contamination of reagents	• Check technique and equipment to confine contamination. Use fresh reagents. • Repeat with aerosol barrier pipette tip after space cleaning

4. Performance

1) Performance Summary

The performance characteristics of the e-Myco™ VALiD-Q Real-time PCR Kit (v3.0 PRO) were evaluated in accordance with relevant assay guidelines.

The following table summarizes key analytical performance parameters—including limit of detection, specificity, Reproducibility, and robustness—validated under defined test conditions.

Test	Acceptance Criteria	Result
Sensitivity	*≤10 CFU/mL (per EP 2.6.7, USP <63>, JP G3)*	*Complies with pharmacopoeial requirements (10 Mycoplasma species)*
Specificity	*No amplification of non-Mycoplasma organisms (Ct > 38)*	*No detectable amplification in non-Mycoplasma species.*
Reproducibility	*CV ≤ 5% ; consistent qualitative interpretation*	*Meets Reproducibility requirements across operators, days, and reagent lots.*
Robustness	*CV ≤ 5% ; consistent qualitative results across varied conditions.*	*Consistent across instruments, extraction methods, primer vendors, matrices, and reagent compositions.*

2) Sensitivity

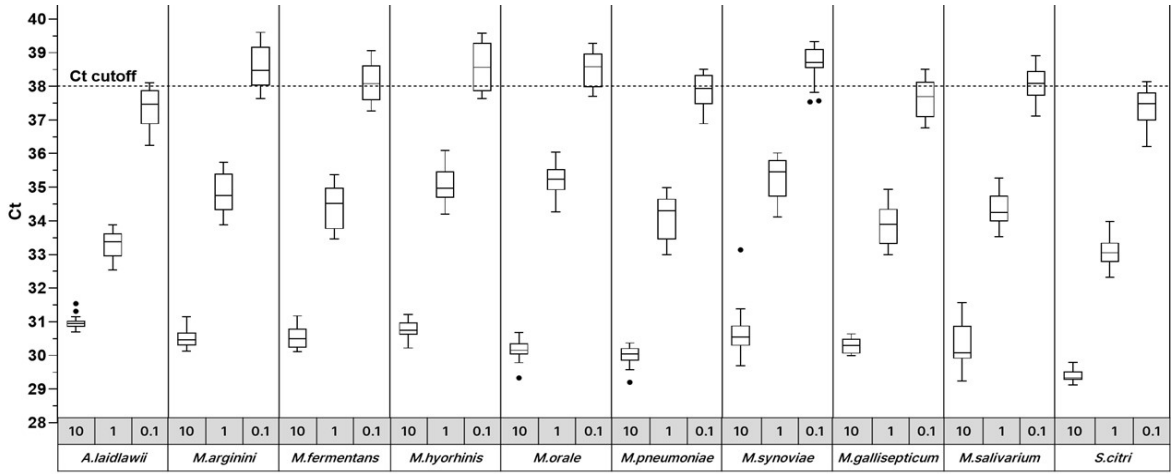
The analytical sensitivity of the e-Myco™ VALiD-Q Real-time PCR Kit (v3.0 PRO) was evaluated using a Mollicutes reference panel. Experimental LoD values were determined by testing serial dilutions of each species, and Probit analysis was performed to calculate the concentration corresponding to a 95% detection probability.

Across all Mollicutes species tested (A. laidlawii, M. arginini, M. fermentans, M. hyorhinis, M. orale, M. pneumoniae, M. synoviae, M. gallisepticum, M. salivarium, S. citri), the kit demonstrated an LoD range of 0.15–0.46 CFU/mL (Probit LoD95%), meeting the acceptance criterion of ≤10 CFU/mL for Mycoplasma testing.

Mycoplasma	Experiment LoD (CFU/ml)	Probit LoD (CFU/ml)	Ct cutoff value
Acholeplasma laidlawii	0.50	0.23	
Mycoplama arginini	0.50	0.42	
Mycoplasma fermentans	0.50	0.37	
Mycoplasma hyorhinis	0.50	0.40	
Mycoplasma orale	0.50	0.41	*Positive : Ct ≤ 38*
Mycoplasma pneumoniae	0.50	0.34	*Negative : Ct > 38*
Mycoplasma synoviae	0.50	0.46	
Mycoplasma gallisepticum	0.50	0.27	
Mycoplasma salivarium	0.50	0.39	
Spiroplasma citri	0.50	0.15	

The Ct cutoff for result interpretation was established as: Positive: Ct ≤ 38 / Negative: Ct > 38

The box-plot below summarizes Ct distributions for each species at the experimentally determined LoD, confirming consistent amplification performance within the established cutoff range.



Raw Ct distributions used for Probit-based LoD95% analysis.

Box-plots show the Ct values obtained from serial dilution testing of ten Mollicutes species at concentrations near the detection limit. These data served as the input for Probit analysis to estimate the LoD95% (95% detection probability).

3) Specificity

- ***Exclusivity (Wet tested)***

The specificity of the e-Myco™ VALiD-Q Real-time PCR Kit (v3.0 PRO) was evaluated through inclusivity testing of Mollicutes species and exclusivity testing against non-target microorganisms.

Inclusivity was assessed using a Mollicutes reference panel consisting of Acholeplasma laidlawii, Mycoplasma arginini, M. fermentans, M. hyorhinis, M. orale, M. pneumoniae, M. synoviae, M. gallisepticum, M. salivarium, and Spiroplasma citri. All listed species were detected as positive, confirming that the assay meets inclusivity requirements for Mollicutes.

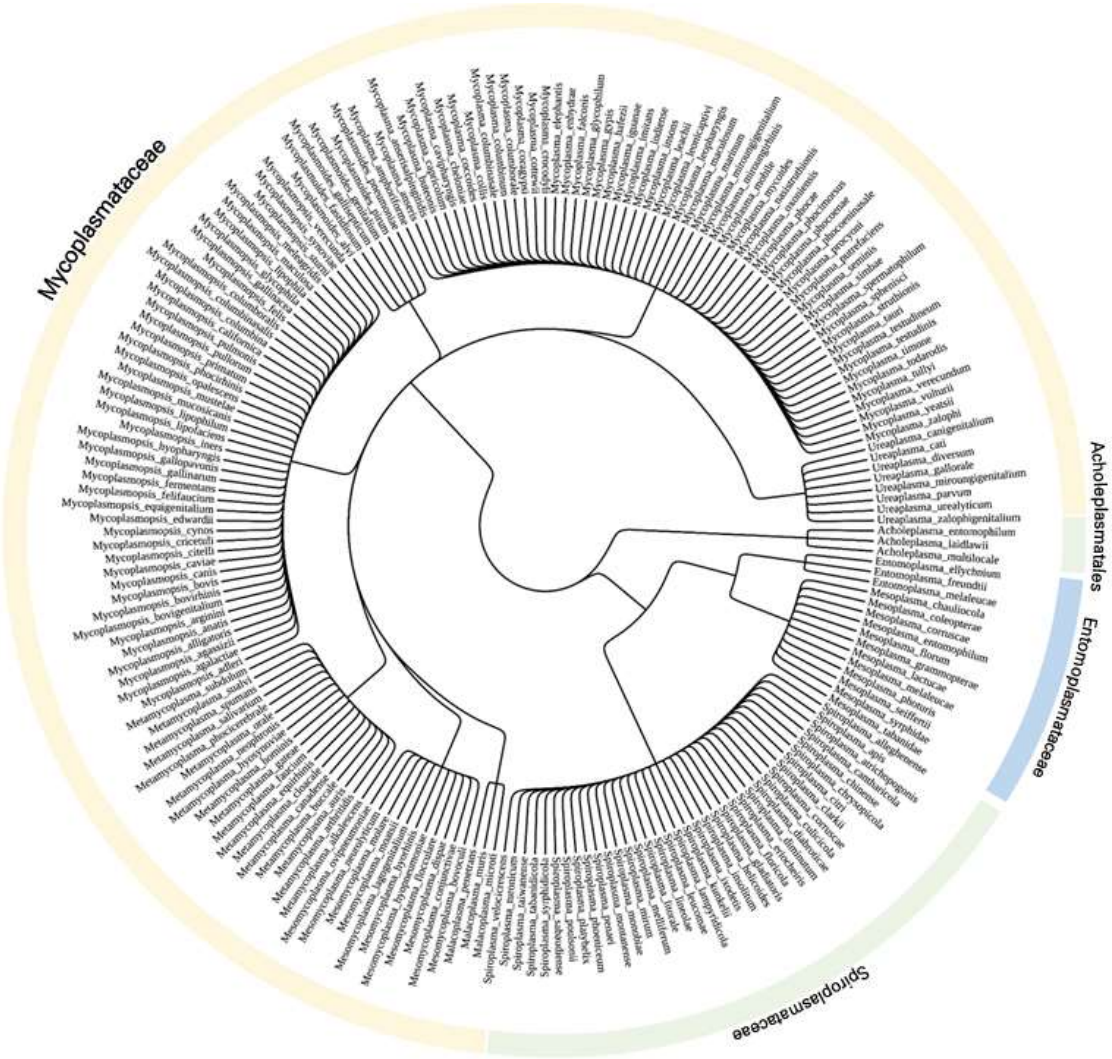
Exclusivity was verified by testing non-target organisms, including species required by EP/JP guidelines (e.g., Clostridium, Lactobacillus, Streptococcus spp.), common environmental microorganisms (E. coli, Bacillus subtilis), and a swine pathogen (Erysipelothrix rhusiopathiae).

All non-target species showed no amplification (Not Detected), demonstrating that the assay meets exclusivity criteria.

Inclusivity (Wet tested Mollicutes)	Exclusivity (Wet tested)		
	Species	Interpretation	Note
A.laidlawii	*Escherichia coli*	Not Detected	-
M.arginini	*Baciilus subtilis*	Not Detected	-
M.fermentans	*Clostridium chauvoei*	Not Detected	EP/JP listed
M.hyorhinis	*Lactobacillus acidophilus*	Not Detected	EP/JP listed
M.orale	*Sterptococcus salivarius*	Not Detected	EP/JP listed
M.pneumoniae	*Erysipelothrix rhusiopathuae*	Not Detected	-
M.synoviae	-	-	-
M.gallisepticum	-	-	-
M.salivarium	-	-	-
S.citri	-	-	-

- ***Inclusivity (in-silico analysis)***

An in-silico analysis was performed for the primers and probe used in the e-Myco™ VALiD-Q Real-time PCR Kit (v3.0 PRO) against representative 16S rRNA gene sequences of Mollicutes. The analysis demonstrated high sequence concordance, with more than 200 Mollicutes species exhibiting ≥90% coverage and fewer than 3 bp mismatches within the binding regions. These results indicate that the kit provides broad inclusivity across diverse Mollicutes species and is expected to reliably detect organisms exhibiting similar sequence homology.



***In-silico* Inclusivity Mapping of Mollicutes Species.**

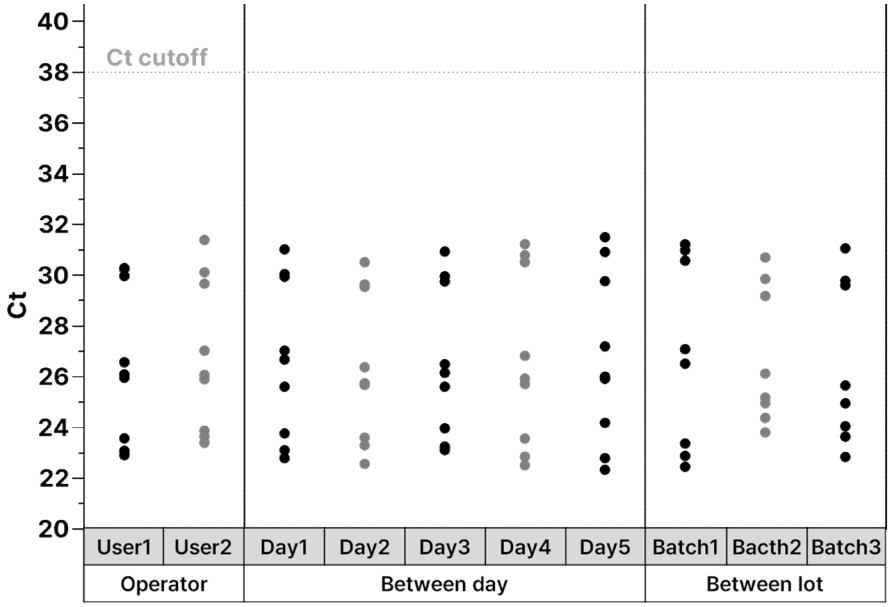
The circular phylogenetic diagram illustrates Mollicutes species that exhibited ≥90% sequence concordance with the primers and probe used in the e-Myco™ VALiD-Q Real-time PCR Kit (v3.0 PRO). Each leaf represents a species meeting this homology threshold, indicating that these organisms are expected to be detectable based on in-silico analysis.

4) Reproducibility

Reproducibility was assessed using A. laidlawii genomic DNA at concentrations of 1,000, 100, and 10 CFU/mL. Each concentration was tested in triplicate across different operators, different days, and different reagent lots. Ct values showed consistent trends according to the dilution series, and all concentrations produced 100% detection.

Standard deviations ranged from 0.24 to 0.96, corresponding to coefficients of variation (CV) between 0.57% and 4.17%, meeting the acceptance criteria (SD ≤ 1.0; CV < 5%).

The highest CV value (4.17%) was observed at 1,000 CFU/mL on Day 5 and was attributed to normal experimental variation.



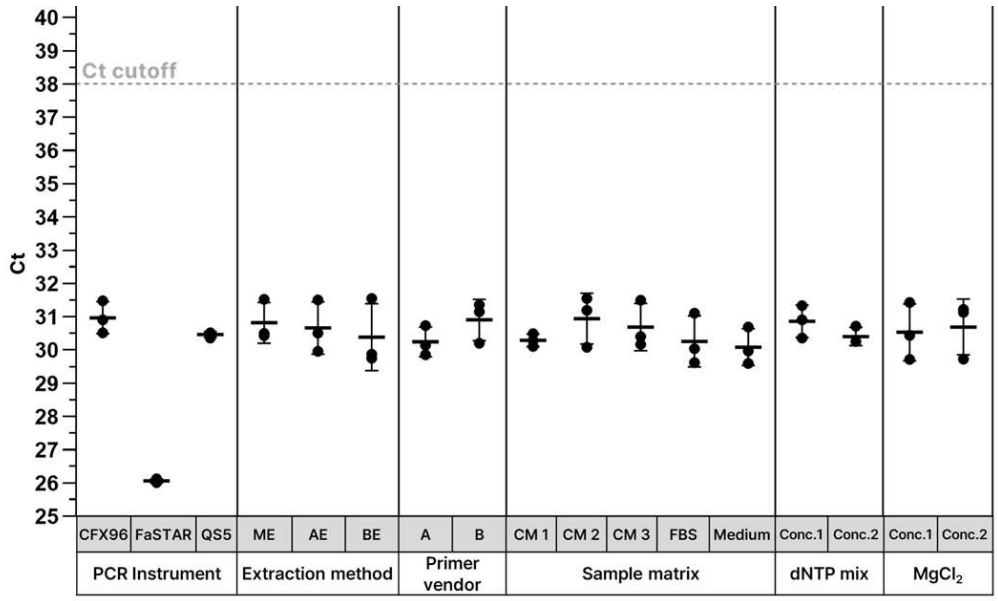
Reproducibility and intermediate precision of the assay.
A. laidlawii genomic DNA was tested at 1,000, 100, and 10 CFU/mL (n=3 per level) across variations in operator, test day, and reagent lot. Ct values showed SD ≤ 1.0 and CV < 5%, meeting the acceptance criteria. All concentrations yielded 100% detection.

5) Robustness

Robustness was evaluated to verify assay performance under minor variations that may occur during routine use. The following parameters were tested using Acholeplasma laidlawii genomic DNA at 10 CFU/mL

Across all tested conditions (n=51), the assay showed consistent positive detection (100%), with mean Ct values ranging from 26.06 to 30.97, standard deviation below 1.0 (SD 0.06–0.86), and coefficient of variation within 5% (0.21–2.81%).

These results confirm that assay performance remains stable under the evaluated conditions.
The lowest Ct value (26.06) was observed on the FaSTARamp96 system and reflects the inherent Ct shift between PCR instruments.



Robustness testing was performed under the following variable conditions:
Ct values across instrument types, extraction methods, primer vendors, sample matrices, and reagent composition variations. Dots represent replicates; bars indicate mean.

- *PCR Instrument: CFX96, FaSTARamp96, QS5*
- *Nucleic Acid Extraction Method: Manual, Automated, Boiling*
- *Primer Vendor: Supplier A, Supplier B*
- *Sample Matrix: FBS; cell-culture supernatants at 1×10^5, 1×10^6, and 1×10^7 cells/mL*
- *Reagent Composition: dNTP, $MgCl_2$ concentration variations*

5. Ordering Information

Product	Cat. No.	Q'ty
e-Myco™ VALiD-Q Real-time PCR Kit (v3.0 PRO)	25247	100 tests
e-Myco™ VALiD-Q Real-time PCR Kit (ver.2.0)	25246	50 tests
e-Myco™ VALiD-Q Mycoplasma qPCR Detection Kit	25245	50 tests
e-Myco™ VALiD Mycoplasma PCR Detection Kit	25239	48 tests
e-Myco™ plus Mycoplasma PCR Detection Kit	25237	48 tests
e-Myco™ Mycoplasma PCR Detection Kit (ver.2.0)	25235	48 tests
MycoClean™ plus Mycoplasma Prevention Spray	21084	200 mL
G-spin™ Total DNA Extraction Mini Kit	17045	50 col.
	17046	200 col.
AutoXT Total gDNA Kit	17188-48	48 tests
	17188-96	96 tests
Miracle-AutoXT Automated Nucleic Acid Extraction System	IMC-NC15PLUS	SYSTEM
FaSTARamp96 Real-time PCR System	IMC-FAST-A96	SYSTEM