



BACGene Legionella Multiplex

TEST KIT FOR QUALITATIVE REAL-TIME PCR DETECTION OF *LEGIONELLA* SPP., *LEGIONELLA PNEUMOPHILA* AND *LEGIONELLA PNEUMOPHILA* SUBSP. *PNEUMOPHILA* SG1

5123222601 BACGene Legionella Multiplex

for use on Agilent AriaMx™, Bio-Rad CFX96 Touch™
and CFX96 Touch™ Deep Well PCR platforms

For 96 reactions



**GOLD
STANDARD
DIAGNOSTICS**

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1 INTRODUCTION

The BACGene *Legionella* Multiplex detection kit provides materials for rapid confirmation of *Legionella* colonies, as well as the discrimination of the phylogenetic groups *Legionella* spp., *Legionella pneumophila* and *Legionella pneumophila* subsp. *pneumophila* SG1.

The kit is only intended for the confirmation and identification of presumptive *Legionella* colonies, not for analysis of water samples.

It is intended that this kit is used by trained laboratory personnel.

The BACGene *Legionella* Multiplex kit is validated for use with the Agilent AriaMx™, Bio-Rad CFX96 Touch™ and CFX96 Touch™ Deep Well PCR platforms.

1.1 Test Principle

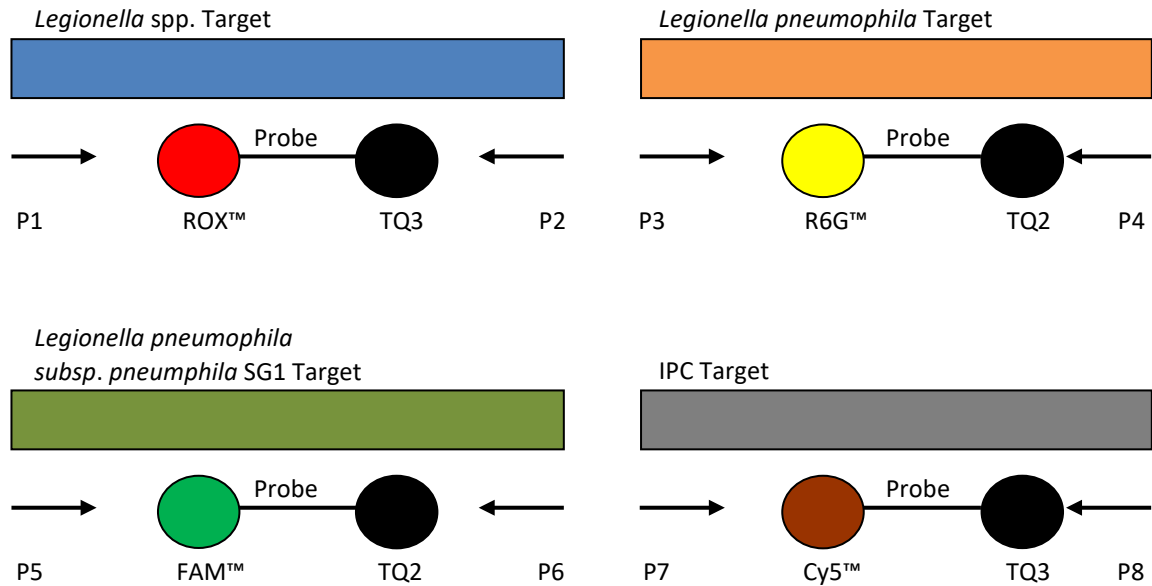
After preparation of water samples according to ISO11731 presumptive *Legionella* colonies are picked, DNA is extracted by a simple thermal lysis step and rapidly analysed by real-time PCR. With this workflow, *Legionella* colonies can be confirmed within maximum 2 h without the necessity of re-streaking presumptive colonies onto BCYE-cys and BCYE agar plates followed by incubation for up to 5 days.

DNA amplification and detection methods take advantage of the nucleotide sequence conservation found in bacterial genomes that ensures the potential for high specificity and sensitivity in detection of pathogenic bacteria.

The BACGene *Legionella* Multiplex kit is designed to differentiate between *Legionella* spp., *Legionella pneumophila* and *Legionella pneumophila* subsp. *pneumophila* SG1 within one PCR reaction. Therefore, further serological tests are not necessary.

The amplified fragments are detected via ROX™, R6G™ or FAM™ fluorescence-labelled hybridization probes quenched by non-fluorescent Tide Quencher™ 2 (TQ2, paired with R6G™ and FAM™) or Tide Quencher™ 3 (TQ3, paired with ROX™).

An internal positive control (IPC) is included in the master mix. IPC DNA is amplified in parallel and detected using a Cy5™ fluorescence-labelled hybridisation probe quenched by non-fluorescent Tide Quencher™ 3 (TQ3). IPC detection indicates the proper functioning of the PCR.



To effectively prevent carry over contamination from and to other PCRs, BACGene *Legionella* Multiplex utilizes Uracil-DNA Glycosylase (UNG).

Deoxyuridine triphosphate (dUTP) substitutes deoxythymidine triphosphate (dTTP) in the PCR-master mix. Thus, newly synthesized amplicons contain deoxyuridine instead of deoxythymidine. The heat labile UNG cleaves hydrolytically at the N-glycosylic bond of uracil and deoxyribose and is subsequently inactivated by the initial denaturation of the PCR.

A PCR preceded by this step exclusively amplifies from native, i.e. thymine containing DNA (e.g. bacterial DNA), reducing the risk of false positive signals due to carry over contamination from previous analyses.

1.2 Components of the Kit

Important Note:

- Store all reagents as indicated below, through to the expiration date printed on the label. Never store components of the kit together with samples or post-PCR products. Shelf life is indicated on the label of the components.
- Do not mix components from different kit lots.

For Lysis:

- 1x **Lysis plate** empty, rippable (high profile)
- 1x **Domed caps***, for use with Lysis plate, set of 12 strips
- 2x **Lysis Buffer X**, 5 mL, safe to store from $-20^{\circ}\text{C} \pm 8^{\circ}\text{C}$ to ambient temperature ($20 - 25^{\circ}\text{C}$)

***Note:** additional domed caps for lysis can be purchased in a package of 250 x 8 cap strips (cat. no. 5613900301).

For PCR:

- 1x **BACGene Legionella Multiplex PCR plate**, with pre-dispensed master mix and PCR support grid. Store at $-20^{\circ}\text{C} \pm 2^{\circ}\text{C}$ and protect from light
- 1x **Optical caps**, for use with PCR plate, 12 strips
- 2x **Legionella positive control DNA**, vial with yellow cap, 50 μL , store at $-20^{\circ}\text{C} \pm 2^{\circ}\text{C}$. Do not freeze/thaw more than 6 times

1.3 Additional Equipment, Consumables and Reagents Required

Equipment

For Lysis:

- 2x **Block heater** (e.g. block heater, digital, VWR)
- 2x **Insert for block heater**, 0.2 mL, 96 wells (e.g. double block, 1x96-well PCR plate, VWR)
- 1x **96 well cooling block**, blue (e.g. cat. no. 5613900501)
- 1x **Lysis support grid**, blue, (e.g. cat. no. 5617702209, pack of 8 grids)
- 1x **Lysis rack** (e.g. cat. no. 5613901401)
- 1x **Strip centrifuge:**
 - Capacity of 2x 8-well strips: (e.g. Carl Roth GmbH, Rotilabo® centrifuge with butterfly rotor)
 - Capacity of 4x 8-well strips: (e.g. Mini Centrifuge IKA Mini Gor VWR, MiniStar silverline)
- OR
- **Plate centrifuge:**
 - Capacity of two times 12x 8-well strips: (e.g. Benchmark Scientific, PlateFuge™ microplate microcentrifuge)
- 1x **Capping/Uncapping tool** for 8-well strips (e.g. MicroAmp® Cap Installing Tool, cat. no. 5613900401)
- 1x **Stepper pipette (with 1 mL tip)**, (e.g. HandyStep® S Brand®)
- 1x **Single channel pipette**, (e.g. Transferpette® S 100 – 1000 µL Brand®)
- 1x **Single channel pipette**, (e.g. Transferpette® S 10 – 100 µL Brand®)
- 1x **Single channel pipette**, (e.g. Transferpette® S, 0.5 – 10 µL Brand®)
- 1x **Pipette filler**, e.g. "Pipetboy acu 2" for serological pipettes

For PCR:

- 1x **PCR support grid**, yellow (e.g. cat. no. 5617702208, pack of 8 grids).
Note: 1x PCR support grid is provided with each kit
- 1x **PCR rack** (e.g. cat. no. 5613901301)
- 1x **Capping/Uncapping tool** for 8-well strips (e.g. MicroAmp® Cap Installing Tool, cat. no. 5613900401)
- 1x **Single channel pipette**, (e.g. Transferpette® S, 0.5 – 10 µL (Brand®))
- AND/OR
- 1x **Multichannel pipette**, (e.g. Transferpette® S-8 Kanal / 0.5 – 10 µL Brand®)
- 1x **Disposable or easily cleanable transfer rack** (e.g. an empty tip box) as mount for the yellow support grid and the PCR plate
- **Real-time PCR Thermocycler:**
 - **Agilent AriaMx™** (up to Software v.1.5), with HEX™, FAM™ and Cy5™ filter set
 - **Bio-Rad CFX96 Touch™** (CFX Manager™ Software v. 3.1 / CFX Maestro™ Software 1.1)
 - **Bio-Rad CFX96 Touch™ Deep Well** (CFX Manager™ Software v. 3.1 / CFX Maestro™ Software 1.1)

- **Windows 7/10 PC** with Microsoft Excel 2010 32 bit (Excel Version 14.7140.xxxx or later)
- **BACGene Evaluation Sheet** version 2

Consumables

For Lysis:

- **DNA-/Nuclease-free pipette tips** with filters, 10 µL
- **Stepper tips**, 1 mL
- **Gloves**, powder free
- **Inoculating loops**, 1 µL (e. g. VWR)

For PCR:

- **DNA-/Nuclease-free pipette tips** with filters 5 µL
- **Nuclease free water** (molecular biology grade)
- **Gloves**, powder free

2 HOW TO USE THIS PRODUCT

Important Notes:

- Store all reagents as indicated in section 1.2.
- Do not use the reagents beyond the expiration dates printed on the labels.
- Never store kit components in the vicinity of samples or post-PCR products.
- The BACGene *Legionella* Multiplex kit is used for confirmation and identification of presumptive *Legionella* colonies and is not validated for direct analysis of water samples.
- In case of positive *Legionella* colonies the concentration of amplicons after the PCR run is very high. Therefore, strictly follow all steps mentioned in section 2.3.

2.1 Safety Precautions

- All samples should be handled with caution as they are potentially infectious.
- In order to safeguard the health of laboratory personnel, it is essential that the whole of this method be carried out only by skilled personnel using good laboratory practices.
- A BSL-2 laminar flow biosafety cabinet is recommended for activities with potential for producing aerosols of pathogens.
- Relevant national Health and Safety Regulations relating to this organism must be adhered to.
- Care must be taken in the disposal of all infectious materials.
- Do not eat, drink or apply cosmetics in the work area in which the test is performed.
- Do not pipette by mouth.
- Avoid contact of kit components with injured skin.

2.2 Working Guidelines

- Comply with Good Laboratory Practice (refer to EN ISO 7218 standard).
- Refer to EN ISO 22174:2005 for the general requirements for the in vitro amplification of nucleic acid sequences.
- Refer to associated guidelines for lab equipment maintenance e.g. ISO/DIS20836 for thermal performance testing of thermal cyclers.
- Perform cleaning protocol (outlined in next section).
- Clearly separate the workspaces for picking colony material, for performing DNA extractions and particularly for PCR setups.
- Use different equipment (e.g. pipettes) for sampling a colony, performing lysis and PCR
- Use DNA-, nuclease-free and sterile labware.
- Wear gloves.
- Change gloves between DNA extraction and PCR setup.

2.3 Cleaning Protocol

Assuring workspaces are clean, before, during and after working with BACGene is key to ensure the validity of the results obtained.

Efficiently cleaning needs to ensure the disinfection and decontamination of the working area, to avoid:

- Bacterial contamination – prevented by regular disinfection,
- DNA contamination – prevented by regular decontamination.

Please refer to the ISO 7218:2007 for regular disinfection procedures, to contribute towards the safety of the laboratory personnel by preventing risks of infection and to ensure the validity of food microbiology examinations by ensuring that the general techniques used for conducting these examinations are standardised, helping achieve homogeneous results in different laboratories.

For DNA decontamination of surfaces, see below examples of suitable cleaning agents and respective suppliers. We do not take any guarantees if the end user does not strictly follow the instructions for use or does not use recommended procedures by the cleaning agents' suppliers.

Cleaning Agent	Supplier
ROTI®Nukleinsäurefrei	Carl Roth GmbH
DNA Away	ThermoFisher Scientific™
DNA-ExitusPlus™	VWR

2.4 Waste Disposal

Dispose of any waste which is potentially contaminated with pathogenic bacteria according to your internal and local regulations.

2.5 Before You Begin

- Place cooling block for lysis at 4 °C overnight.
- Set heating block to 95 ± 2 °C.
- Place the lysis plate into the lysis rack and aliquot 100 µL of lysis buffer X into each well. One tube of lysis buffer X is sufficient for 48 reactions.
- Seal the wells tightly with the domed caps and the capping/uncapping tool dedicated to the lysis area.
- Lysis buffer X can be stored at –20 ± 8 °C or ambient temperature until expiry date printed on the label.

2.6 Lysis and PCR Protocol

Lysis

1. Depending on the size of the colony to be tested, different tools have to be used for picking:

- Colony with diameter of 1 mm – 10 mm: use sterile 10 µL pipette tip
- Colony with diameter of < 1 mm: use sterile 1 µL loop

Pick a presumptive *Legionella* colony with a pipette using a 10 µL pipette tip by dipping the end of the tip into a full grown colony or, in case of colonies < 1 mm pick the whole colony using the loop.

Note: Avoid touching the agar or scraping entire colonies above 1 mm diameter to avoid DNA overload of the PCR.

2. Add harvested colony to the appropriate well of lysis plate/strip placed in the blue lysis support grid on top of the lysis rack.

Note: Do not add any material into wells A1 and B1 to ensure the same layout across all PCR runs (A1: positive control, B1: negative control).

3. Pipette up and down 3 – 5 times in order to remove the colony material from the pipette tip or carefully stir colony into the lysis buffer X when using the loop.

4. Seal the strips using the capping/uncapping tool.

5. Proceed to the next strip when all colonies to be characterized have been picked.

6. Place lysis plate/strip in blue lysis support grid onto the 95 °C (± 2 °C) heating block for 10 min.

7. Transfer lysis plate/strip in blue lysis support grid onto the 96 well cooling block (stored at 4 °C ± 2 °C) for 5 min.

8. In order to remove condensate remaining in the lids:

- Transfer the lysis plate/strips to a 96-well plate centrifuge or a centrifuge with adapter for 0.2 mL in 8-strip format
- Spin down samples for 30 sec between 400 – 2 000 x g
- If condensate remains in the lids, repeat this step

9. Continue with PCR setup.

PCR Setup

Before you begin

- Make sure you have switched on the computer, the PCR instrument and ensure the sample layout for the PCR plate is suitably documented and programmed.
- Only remove the number of PCR strips, containing pre-dispensed master mix, necessary for testing the amount of samples to be analysed and store the remainder at $-20\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$.

Important: In case of cutting an 8-strip into smaller parts it is crucial to let the plastic warm up at room temperature for around 1 minute prior to cutting. Do not place the strip into a cooling block.

Note: Frequent freezing and thawing might cause inactivation of the reagents. Do not freeze/thaw more than 3 times.

- Spin down the strips with master mix for 30 sec between $400 - 2\,000 \times g$.
 - Visually check that all master mix is in the bottom of the wells.
10. Place PCR strips, together with the yellow PCR support grid, into an appropriate support rack. Avoid exposing the PCR plate to light for long periods of time.
 11. The lysis and PCR plate should be arranged in the same orientation in order to facilitate handling with the multichannel pipette and in order to avoid cross contamination of wells (place the lysis plate i.e. behind the PCR plate).
 12. Open lysis plate as described in section 2.6.
 13. Open the corresponding PCR strip by fixing the upper and lower end with one hand and removing the foil starting with the loose end from top to bottom in 180 degree angle (see Figure 1A).

Note: Open each strip individually, add samples and close. Only then proceed to the next strip.

14. Transfer 5 μL of each lysate by using an 8-well multichannel pipette to the corresponding PCR strip.

Note: Make sure that the lysate is taken from the upper half of the lysis volume, avoid touching a potential pellet of debris. Ensure the lysate is pipetted directly on top of the master mix.

15. Add 5 μL positive control DNA (vial with yellow cap) to well A1.

Note: Do not freeze/thaw positive control more than 6 times.

16. Close the PCR strip with an optical cap strip with clean, gloved hands (see Figure 1C).
17. Re-close the lysis strip with fresh domed caps (not supplied with the kit).
18. Repeat steps 12 – 17 with the remaining strips.
19. Transfer the PCR plate to the real-time instrument and start the run (refer to section 3 for Agilent AriaMx™ + section 4 for Bio-Rad CFX96 Touch™).
20. Store the lysis plate at $-20 \pm 2\text{ }^{\circ}\text{C}$ in case a re-run of the PCR is required.

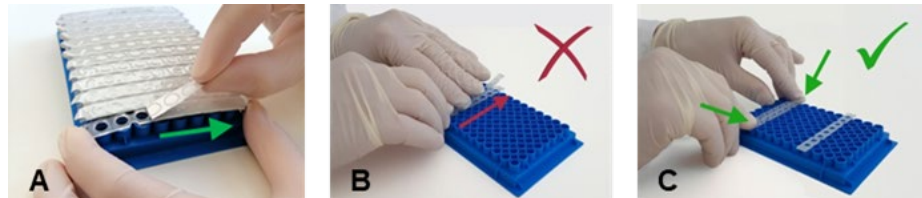


Figure 1: A. Removing foil from PCR strips. B. Incorrect way to close lids in one direction. C. Correct way to close lids by first softly fixing the ends of the cap strip to the reaction tubes, then applying even pressure to tightly close the PCR strips.

Plate Setup

The following plate document shows the recommended distribution of reactions:

	1	2	3	4	5	6	7	8	9	10	11	12
A	C+	7	15	23	31	39	47	55	63	71	79	87
B	C-	8	16	24	32	40	48	56	64	72	80	88
C	1	9	17	25	33	41	49	57	65	73	81	89
D	2	10	18	26	34	42	50	58	66	74	82	90
E	3	11	19	27	35	43	51	59	67	75	83	91
F	4	12	20	28	36	44	52	60	68	76	84	92
G	5	13	21	29	37	45	53	61	69	77	85	93
H	6	14	22	30	38	46	54	62	70	78	86	94

Plate layout for 94 samples;

C+ = positive control

C- = negative control

3 AGILENT ARIAMX™: INSTRUCTIONS FOR USE WITH BACGENE LEGIONELLA MULTIPLEX KIT

The BACGene Legionella Multiplex kit is validated for the use on the Agilent AriaMx with software version up to 1.5.

The PCR platform should be prepared prior to commencing the practical work. For a more detailed description of the instrument programming please refer to the user manual of the Agilent AriaMx™.

3.1 PCR Setup

The following instructions outline how to setup a PCR run on the Agilent AriaMx™ using the touchscreen on the instrument.

Note: The Aria software is able to interface with a Laboratory Information System “LIMS”. Please contact your local sales support for more information.

3.1.1 Import Template to Platform

1. Turn on Agilent AriaMx™.
2. Insert USB containing the “BACGene_Legio_AriaMx_Template.amxt” file into the slot at the front of the platform.
3. Select “New experiment”.
4. Select “Open Template”.
5. Choose “Import”. Select “BACGene_EV_Legionella_Multiplex.amxt” from USBDisk. The template is now imported onto the platform.

Note: This procedure only needs to be carried out the first time of usage.

3.1.2 Running PCR Using Template (for Agilent AriaMx™)

1. Turn on Agilent AriaMx™.
2. Select “New experiment”.
3. Select “Open Template”.
4. Open “BACGene_EV_Legionella_Multiplex.amxt”.
5. Select “Sync Plate”.
 - a. Please check that following parameters are included in the template:
 - Plate Setup Tab:
 - Fluorophores:
 - ROX™, HEX™, FAM™ and Cy5™ are selected for all 96 wells
 - Target Names: If more than one system is analysed on one plate, please use the following target names for respective wells of the BACGene Legionella Multiplex kit:



BACGene Legionella Multiplex	Target Name	Dye Name
<i>Legionella spp.</i>	2022a	ROX™
<i>Legionella pneumophila</i>	2022b	HEX™
<i>Legionella pneumophila subsp. pneumophila</i> SG1	2022c	FAM™
IPC Legionella	2022i	Cy5™

- Sample type:
 - A1 should be defined as “Calibrator”
 - B1 should be defined as “Buffer”
 - All other wells should be defined as “Unknown”

Thermal Profile:

UNG Digestion	1 HOLD	42 CYCLES	
UNG	enzyme activation	denaturation	annealing & extension
2 min at 50°C	15 min at 95°C	15 sec at 95°C	60 sec at 60°C
No data collection	no data collection	no data collection	data collection



Note: The run should always be initiated using the provided template!

6. Select “Run Experiment” in the “Thermal Profile” tab to start the PCR run. Save the experiment under desired name.

3.2 Evaluation (for Agilent AriaMx™)

After run completion, data must be evaluated using the BACGene Evaluation sheet. The data exports from the Agilent AriaMx™ are described below.

3.2.1 Export of Data (for Agilent AriaMx™)

1. When running the Agilent AriaMx™ without a connected PC, follow these instructions:
 - a. Insert USB into slot on the front of the platform.
 - b. Go to “Saved files” and select the run file.
 - c. Copy and paste this to the USB drive (listed on left side).
 - d. Transfer file to desired location.
2. Open Agilent AriaMx™ software (up to v1.5) on your PC for data evaluation and open the run file.
3. To name samples:
 - a. Create a text (.txt) file with the following format: WELL NAME, SAMPLE ID e.g.
A1, C+
B1, C-
C1, 12345
...
 - b. In the run file, right click on any well and select “Import Well Name...”
 - c. Select the .txt file to import and click “Open”.
4. Ensure all wells are selected in the “Plate Setup” screen.
5. As a final check before export go to “Graphical Displays” tab and ensure that the “Background Based Threshold” is set from Cycle Range “5 thru 9” and “Sigma Multiplier 10” (this option can be found under “Amplification Plots”).
6. To prepare a default configuration for result export, follow these instructions.

Note: This step only needs to be carried out the first time you use the software.

- a. Go to “Export Data” tab.
- b. To set up a default export:
 - From the “Definition” drop down menu select “Add new”.
 - Name this “BACGene Legio export”.
 - In “File Type” select “Text”.
 - In items list, select “Amplification plots” and “Tabular Results”.
 - Press save icon beside the “Definition” drop down menu to save this export configuration.

7. To export results, follow these instructions (for Agilent AriaMx™):

- a. Go to “Export Data” tab.
- b. Select “BACGene Legio export” from the Definition drop-down menu.
- c. Edit Tabular results by clicking on the blue pen and select all columns.

Note: In the current software version, these columns will need to be selected EVERY time you export the results.

- d. Select “Export Data”. Save to chosen location.

Note: it is necessary to save both export files in the same folder.

- e. Proceed to “BACGene Evaluation Sheet” for data interpretation.

4 BIO-RAD CFX96 TOUCH™ INSTRUCTIONS FOR USE WITH BACGENE LEGIONELLA MULTIPLEX KIT

The BACGene Legionella Multiplex kit is validated for use with the Bio-Rad CFX96 Touch™ and Bio-Rad CFX96 Touch™ Deep Well with software version 3.1 / CFX Maestro™ software version 1.1.

The PCR platform should be prepared prior to commencing the practical work. For a more detailed description of the instrument programming please refer to the user manual of the Bio-Rad CFX96 Touch™ or CFX96 Touch™ Deep Well.

4.1 PCR Setup

Note: The CFX Manager software can be configured for use with a Laboratory Information System “LIMS”. Furthermore, the software enables the users to create their own protocol template. Please contact your local sales support for more information.

4.1.1 Running PCR using template

1. Turn on Bio-Rad CFX96 Touch™ (Deep Well).
2. Open the Bio-Rad CFX Manager™ 3.1 / CFX Maestro™ 1.1.
3. Choose “Run setup”.
4. Select instrument CFX96.
5. Select run type “User-defined”.
6. In the protocol tab choose “Select existing...”.
7. Navigate to and select the protocol template “BACGene_UNG_protocol” for the Bio-Rad CFX96 Touch™ and “BACGene_UNG_DW_protocol” for Bio-Rad CFX96 Touch™ Deep Well.
8. Select “Next”.
9. In the plate tab choose “Select existing...”.

Navigate to and select the plate template “BACGene_EV_Legionella_Multiplex_plate”.

Optional for naming the samples:

- Select “Edit Selected...”.
- Select “Spreadsheet View/Importer” from the panel or from “Editing tools” menu.
- Include the sample names into the list.
- Select “OK”.
- Select “OK” again to leave the plate view.
- Select “Next” to get to the “Start run” tab.

The run should always be started using the provided protocol and plate templates!

Please check that the following parameters are included in the protocol (for Bio-Rad CFX96 Touch™ and Deep Well):

- Sample volume: 25 µL
- Thermal cycler times and temperatures

UNG Digestion	1 HOLD	41 REPETITIONS	
UNG	enzyme activation	denaturation	annealing & extension
2 min at 50°C	15 min at 95°C	15 sec at 95°C	60 sec at 60°C
No data collection	no data collection	no data collection	data collection

Please check that the following parameters are included in the plate setup:

- Plate Type: BR White
- Scan Mode: All Channels
- Fluorophores: ROX™, VIC™, FAM™ and Cy5™ are selected for all 96 wells
- Target Names: If more than one system is analysed on one plate, please use the following target names for respective wells of the BACGene Legionella Multiplex kit:



BACGene Legionella Multiplex	Target Name	Dye Name
<i>Legionella</i> spp.	2022a	ROX™
<i>Legionella pneumophila</i>	2022b	VIC™
<i>Legionella pneumophila</i> subsp. <i>pneumophila</i> SG1	2022c	FAM™
IPC <i>Legionella</i>	2022i	Cy5™

- Sample type:
 - A1 should be defined as “Positive control”
 - B1 should be defined as “Negative control”
 - All other wells should be defined as “Unknown”

4.2 Evaluation (for Bio-Rad CFX96 Touch™)

After run completion, data should be evaluated using the “BACGene Evaluation Sheet”. The data exports from the Bio-Rad CFX96 Touch™ or Bio-Rad CFX96 Touch™ Deep Well are described below.

4.2.1 Export of Data (for Bio-Rad CFX96 Touch™)

1. When running the Bio-Rad CFX96 Touch™ (Deep Well) without a connected PC, follow these instructions:
 - a. Insert USB into slot on the front of the platform.
 - b. Go to “Saved files” folder and choose “Real Time Data”.
 - c. Select desired file and choose “File Options” to export to the USB.
 - d. Transfer file to desired location.
2. Open Bio-Rad CFX Manager™ software version 3.1 / CFX Maestro™ software 1.1 and select the saved run. Save the resulting “optical file” (*.pcrd) in the desired folder.
3. Once the optical file is opened, go to “Settings” tab and ensure the following options are selected:
 - a. Cq Determination Mode = Single Threshold.
 - b. Baseline Setting = Baseline Subtracted Curve Fit.
 - c. Ensure that “Apply Fluorescence Drift Correction” is selected under “Baseline Settings”.
 - d. Make sure that the plate type “BR White” is selected. Therefore select “View/Edit plate” under “Plate Setup” and choose the right plate type under “settings”.
4. To export results, follow these instructions:
 - a. Go to “Export” tab.
 - b. Select “All Data Sheets” and export as text files (*.txt). Choose location for files to be saved.
 - c. Select “Custom Export” in text format with “Tab” separator (*.txt) with at least the following columns (in the same order as stated below) and save in chosen location:

Note: save all export files in the same folder.

- Well
 - Fluorophore
 - Target Name
 - Content
 - Sample Name
 - Cq
 - End RFU
5. Proceed to “BACGene Evaluation Sheet” for data interpretation.

5 DATA INTERPRETATION

Data is interpreted using the “BACGene Evaluation Sheet” provided and imported as described therein. Interpretation of final sample results is summarized in the following table:

<i>Legionella</i> spp.	<i>Legionella pneumophila</i>	<i>Legionella pneumophila</i> subsp. <i>pneumophila</i> SG1	Internal Positive Control	Final results
+	+	+	Not relevant	Positive for <i>Legionella pneumophila</i> subsp. <i>pneumophila</i> SG1
+	+	-	Valid	Positive for <i>Legionella pneumophila</i>
+	+	-	Invalid	Positive for <i>Legionella pneumophila</i> & questionable* for <i>Legionella pneumophila</i> subsp. <i>pneumophila</i> SG1
+	-	-	Valid	Positive for <i>Legionella</i> spp.
+	-	-	Invalid	Positive for <i>Legionella</i> spp. & questionable for <i>Legionella pneumophila</i> and <i>Legionella pneumophila</i> subsp. <i>pneumophila</i> SG1
-	-	-	Valid	Negative for <i>Legionella</i> spp.
-	-	-	Invalid	Questionable for <i>Legionella</i> spp.
+	-*	+	Not relevant	Positive for <i>Legionella</i> spp. and <i>Legionella pneumophila</i> subsp. <i>pneumophila</i> SG1 & questionable* for <i>Legionella pneumophila</i>
-*	-*	+	Not relevant	Positive for <i>Legionella pneumophila</i> subsp. <i>pneumophila</i> SG1 & questionable* for <i>Legionella</i> spp. and <i>Legionella pneumophila</i>
-*	+	+	Not relevant	Positive for <i>Legionella pneumophila</i> and <i>Legionella pneumophila</i> subsp. <i>pneumophila</i> SG1 & questionable* for <i>Legionella</i> spp.
-*	+	-	Not relevant	Positive for <i>Legionella pneumophila</i> & questionable* for <i>Legionella</i> spp. and <i>Legionella pneumophila</i> subsp. <i>pneumophila</i> SG1

* Inconclusive result, refer to troubleshooting (section 6: “Observation: A “Q” result is observed for sample in the BACGene Evaluation sheet”)

6 TROUBLESHOOTING

6.1 Problems Upon Delivery/During Storage and Reception

Observation:

Master mix plate is not frozen upon arrival

Possible Cause	Solution
Plates defrosted during shipping or were not stored at -20°C upon arrival.	Contact your local sales support or your local distributor.

6.2 Incorrect Data Export and Import to BACGene Evaluation Sheet

Observation:

No results displayed in BACGene Evaluation Sheet after successful import of results

Possible Cause	Solution
The content has not been enabled and Macros are not running properly.	Ensure the content is enabled by following instructions in links provided in the "BACGene Evaluation Sheet" or on the official Microsoft Office website (http://office.microsoft.com/). Then repeat the evaluation.
C+ or C- have not been defined.	Ensure that both C+ and C- have been defined. This can be done by defining "Task" in the drop-down menu for the specific wells in the "Results-tab view" worksheet.
The correct columns for Tabular results (Agilent AriaMx™) or Custom Export (Bio-Rad CFX96 Touch™ (Deep Well)) were not selected when exporting results from the PCR run file.	Ensure the correct columns are selected for the export as described and repeat the export and evaluation of results.

Observation:

Data import into the BACGene Evaluation Sheet was not successful

Agilent AriaMx™:

Possible Cause	Solution
ROX™, HEX™, FAM™ or Cy5™ dye was deselected during examination of the run file.	Ensure all ROX™, HEX™, FAM™ or Cy5™ channels are selected and repeat result export. Also ensure all wells are selected.
Tabular results and/or amplification plots were not exported correctly.	Ensure result export was in the correct format as described in “Instruction” tab of the “BACGene Evaluation Sheet” and in section 3.2.1 above. Then repeat the evaluation.

Bio-Rad CFX96 Touch™ (Deep Well):

Possible Cause	Solution
The correct columns were not selected in custom export.	Ensure result export was in the correct format as described in “Instruction” tab of the “BACGene Evaluation Sheet” and in section 4.2.1 above. Then repeat the evaluation.
Custom export text file was exported with “Comma” separator instead of ‘Tab’ separator.	Ensure that the Custom Export process is carried out correctly as described in the “Instruction” tab of the “BACGene Evaluation Sheet” and in section 4.2.1 above. Then repeat the evaluation.

Observation:

Second export file was not automatically detected during import into evaluation sheet

Possible Cause	Solution
All required export files were not exported.	Ensure all required export files are successfully exported. For further information please refer to the Instructions tab in the “BACGene evaluation sheet”.
The names of the export files were changed.	When prompted, manually choose files or repeat export.
Both files were not saved in the same folder.	When prompted, manually choose appropriate file or ensure all export files are saved in the same folder.

Observation:

Fluorescent drift observed in curves from a Bio-Rad CFX96 Touch™ (Deep Well) PCR run

Possible Cause	Solution
The “Apply Fluorescent Drift Correction” option was not selected during the run evaluation.	Ensure this option is selected and repeat the export of results. Then continue with the evaluation.

6.3 Inconclusive Results Post Evaluation

Observation:

C- negative control is positive (in one of the Legionella system)

Possible Cause	Solution
Cross contamination occurred.	Follow decontamination procedure. Repeat lysis of colonies and repeat PCR using fresh aliquots of all reagents. If C- continues to show a positive signal, contact your local sales support.

Observation:

C- not in range (in the IPC system) – evaluation is not possible

Evaluation of the IPC is dependent on the C- control reaction. If this reaction is not in range, the IPC cannot be evaluated in the sample reactions.

Possible Cause	Solution
Automated threshold setting failed.	The threshold is automatically set by the software (Agilent AriaMx™ software or Bio-Rad CFX96 Touch™) and should not be manually manipulated. Please refer to section 3.2.1 (AriaMx™) or 4.2.1 (Bio-Rad CFX96 Touch™ (Deep Well)) to ensure correct export settings are used.
Master mix plate was freeze-thawed more than 6 times	The master mix plate should not be frozen and thawed more than 6 times as it may cause inactivation of the reagents.
95°C lysis step was not performed correctly.	Ensure the heating block reaches the correct temperature (95°C ± 2°C) by measuring with a thermometer. Ensure this step is performed for the correct amount of time (10 min).

Observation:

Cq value of the C+ is late or negative (in the Legionella system) – evaluation is not possible

Possible Cause	Solution
Pipetting error where no positive control or too little positive control material was added.	Repeat PCR ensuring 5 µL of the positive control material is pipetted into appropriate well (A1).
The positive control was not stored correctly.	If there is suspicion of incorrect storage, use a fresh tube of positive control material. When starting a new kit, always use the positive control material from that kit.
The positive control material has undergone too many freeze-thaw cycles.	Use a fresh tube of positive control material. Do not freeze/thaw more than 6 times. If C+ continues to show a negative signal, contact your local sales support.

Agilent AriaMx™:

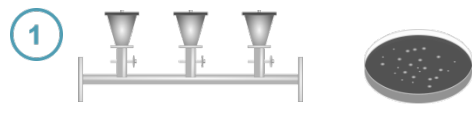
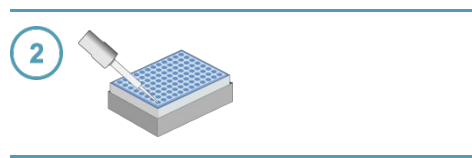
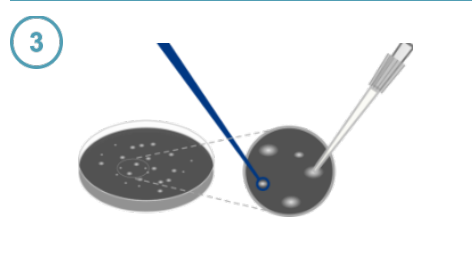
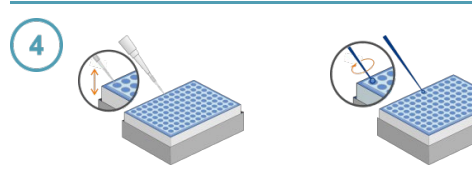
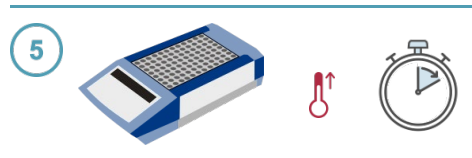
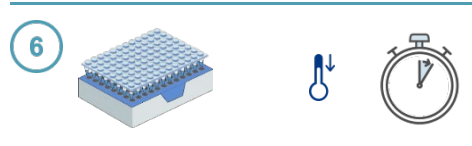
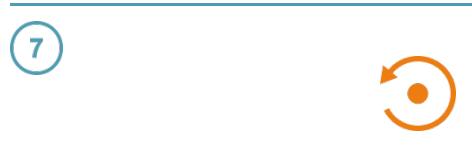
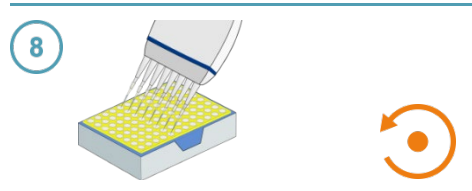
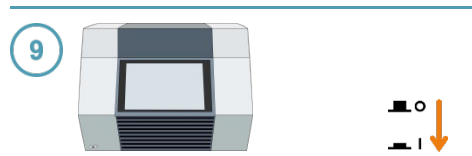
Possible Cause	Solution
Automated threshold setting failed. In the automatic analysis mode, the AriaMx™ software sometimes does not set the “Background Based Threshold” (Cycle Range 5 thru 9 and Sigma Multiplier 10) in the early exponential amplification phase.	Check amplification plots and thresholds for ROX™, HEX™, FAM™ and Cy5™. Set the threshold for the <i>Legionella</i> system (ROX™, HEX™ and FAM™) manually in the early exponential phase of the amplification plots and ensure that the C+ amplification plot is included (if desired).

Observation:

A “Q” result is observed for sample in the BACGene Evaluation sheet

Possible Cause	Solution
Missing PCR tubes.	Check layout of tubes in PCR platform to ensure it matches the correct layout.
The master mix was not in the bottom of the PCR tube, but in the lid.	Always visually check the level of the master mix before use. If the master mix is not at the bottom of the tubes, spin down.
95°C lysis step was not performed correctly.	Confirm that the heating block reaches the correct temperature (95°C ± 2°C) by measuring with a thermometer. Repeat lysis from colony material and repeat the PCR.
Sample lysate was not taken from upper half of tube when transferring to PCR.	Repeat lysis from colony material and repeat PCR. Ensure to take sample from upper half of tube.
Inhibition of PCR or inconclusive results (refer to “Data interpretation” table, section 5).	Repeat colony lysis and repeat PCR. If PCR remains inhibited, dilute the lysate sample with DNA-/Nuclease free water (one part sample plus four parts water and one part sample plus nine parts water).
IPC amplification not possible because of very strong <i>Legionella</i> signals (Cq-values of at least one <i>Legionella</i> system < 20).	Dilute the lysate sample with DNA-/Nuclease free water depending on the Cq-values of the <i>Legionella</i> system with the earliest Cq: Cq <10: dilute 1:1000 (1 part lysate, 999 parts water) Cq 10-15: dilute 1:100 (1 part lysate, 99 parts water) Cq 15-20: dilute 1:10 (1 part lysate, 9 parts water)

7 PROTOCOL AT A GLANCE

	Follow ISO11731:2017 up to isolation plates BCYE, BCYE+AB, GVPC and MWY. Choose presumptive positive colonies for PCR confirmation.
	Dispense 100 µL of lysis buffer X into lysis strips/plate
	Depending on size of the colony to be tested either Dip a 10 µL pipette tip into the colony (for colony diameter 1 mm – 10 mm). Take the whole colony with a 1 µL loop (for colony diameter < 1 mm). <i>Try to avoid overloading PCR by following these recommendations closely.</i>
	Add harvested bacteria into appropriate well of lysis strips/plate, leaving well A1 + B1 free for negative and positive controls Pipette up and down 3 – 5x for pipette tip or stir loop in lysis buffer X to re-suspend colony. Seal the strips using the capping /uncapping tool.
	Place lysis strips/plate into 95 °C (+/- 2 °C) heating block for 10 min.
	Transfer lysis strips/plate into pre-chilled 96 well cooling block (4 °C +/- 2) for 5 min.
	Spin down lysis strips/plate using a 96-well plate centrifuge or a centrifuge with adapter for 0.2 mL in 8-stripe format.
	Place PCR strip into an appropriate rack and transfer 5 µL of sample and controls into the PCR strips. Spin down PCR strips.
	Transfer PCR strip to real-time instrument and start the run. Review results according to user guide instructions.

8 PRODUCT WARRANTIES, SATISFACTION GUARANTEE

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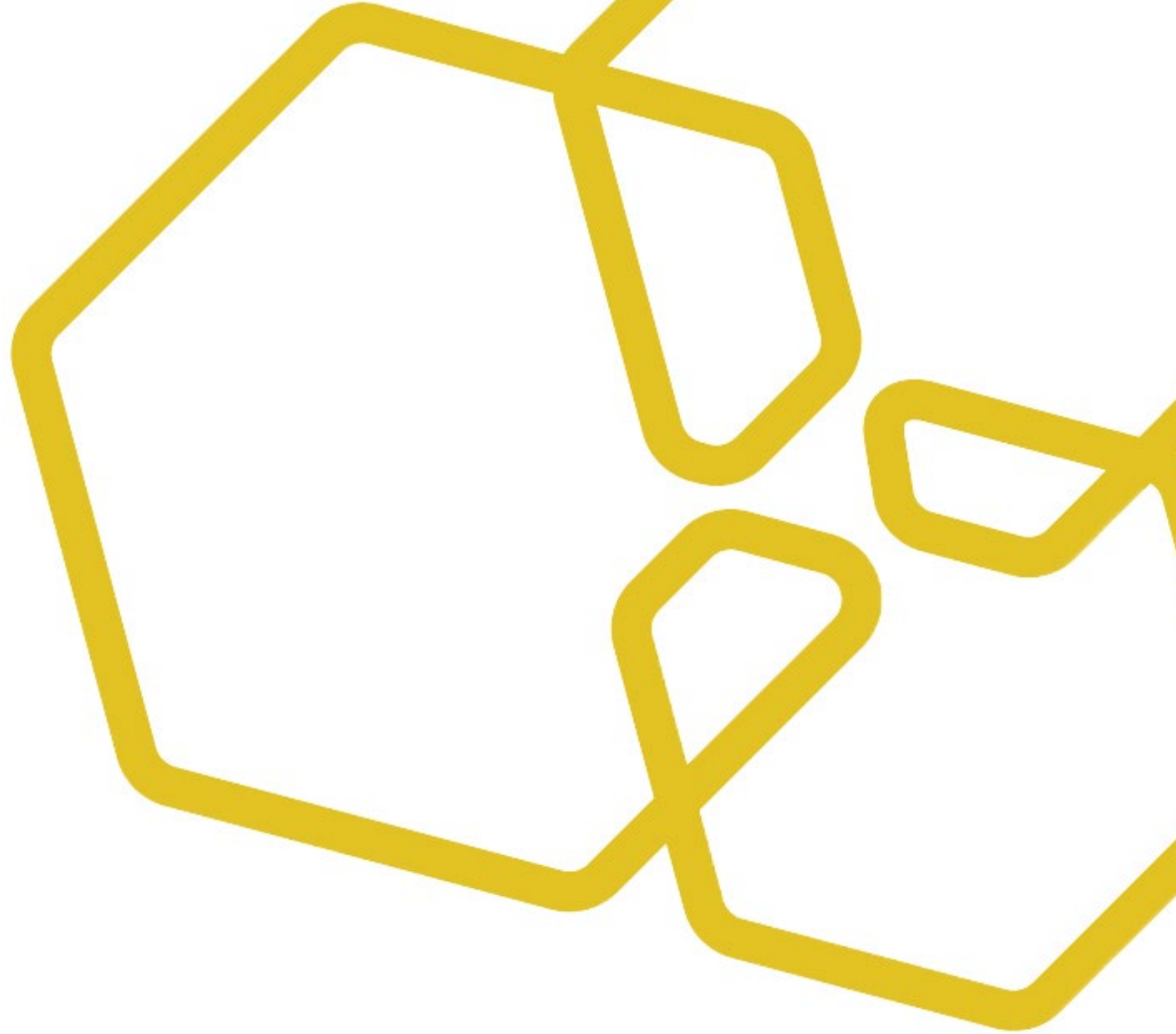
9 IMPORTANT NOTES

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10 VERSION CONTROL

Version	Date	Changes
V1.6	15.12.2022	• Adaptation to Gold Standard Diagnostics layout for BACGene kits • Change of contact address • Add section "Version Control" • Change of file name • Adjustment of structuring in some sections • Remove/correct some catalogue numbers • Change the section "Cleaning Protocol" • Add some notes in section "How to use this Product" • No product or assay-specific changes



TECHNICAL SUPPORT SERVICE

For technical assistance and more information, please contact Gold Standard Diagnostics Budapest's customer service or your local distributor.

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