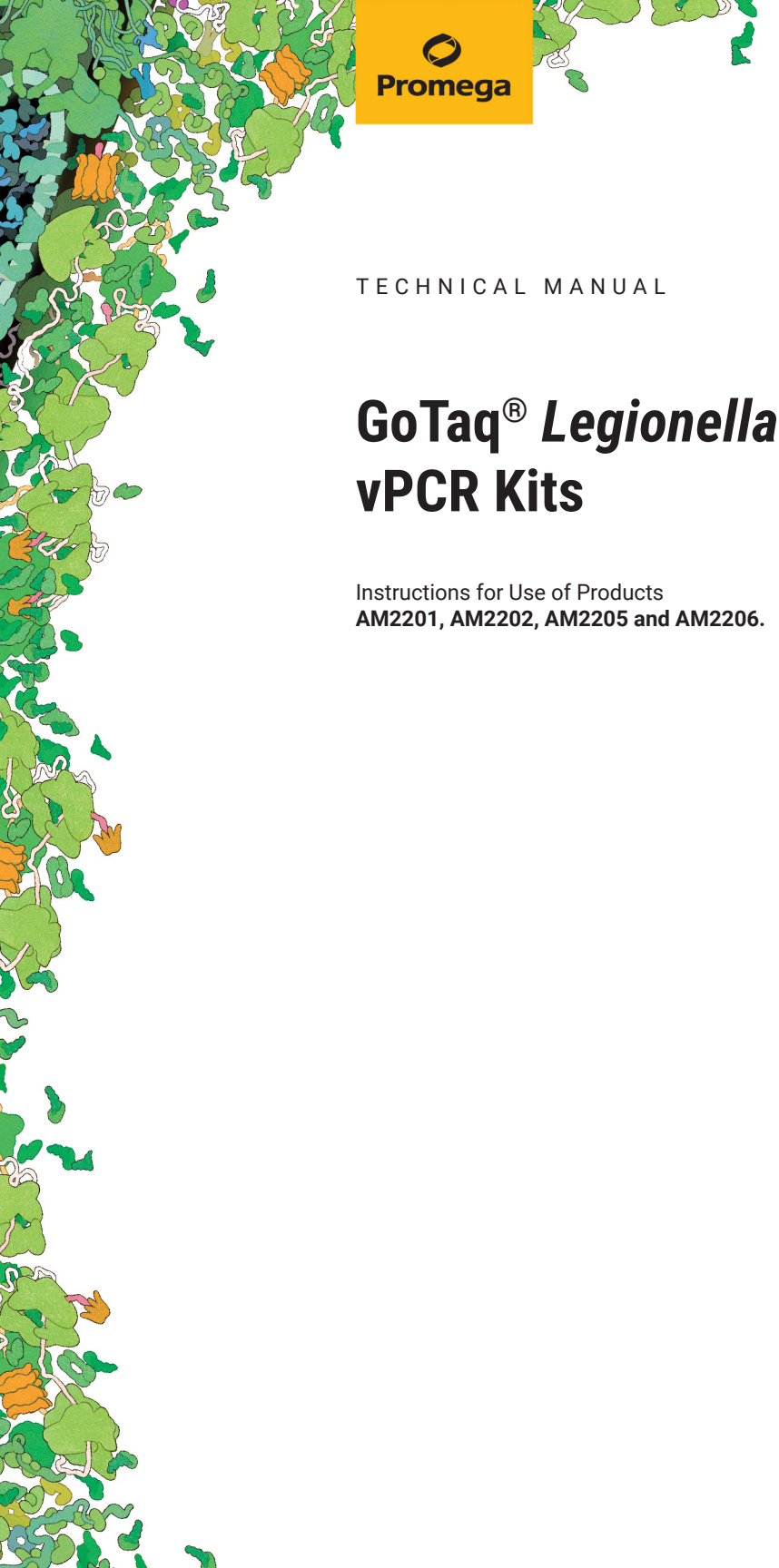

TECHNICAL MANUAL

GoTaq® *Legionella* qPCR and vPCR Kits

Instructions for Use of Products
AM2201, AM2202, AM2205 and AM2206.

11/24
TM725

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GoTaq® *Legionella* qPCR and vPCR Kits

All technical literature is available at: www.promega.com/protocols/
Visit the website to verify that you are using the most current version of this Technical Manual.
Email Promega Technical Services if you have questions on use of this system: techserv@promega.com

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1. Description

Culture-based detection of *Legionella* is commonly used worldwide as part of routine microbial surveillance protocols of water supplies. Despite this widespread acceptance, culture-based approaches are severely limited regarding time to results (7–10 days). These approaches also lack the ability to detect viable but nonculturable (VBNC) *Legionella* bacteria that are often residually present after common disinfection treatments (e.g., chlorination or heat). PCR-based detection directly addresses these issues and is quickly becoming a widely adopted method for molecular-based *Legionella* assays.

The GoTaq® *Legionella pneumophila* qPCR Kit (Cat.# AM2201) and GoTaq® *Legionella spp/pneumophila*/SG1 qPCR Kit (Cat.# AM2202) described in this technical manual provide a rapid, robust and sensitive hydrolysis probe-based multiplex method for quantitative PCR (qPCR) detection of *Legionella* from water samples.

The GoTaq® *Legionella pneumophila* qPCR and viability qPCR Kits enable quantification of *L. pneumophila,* while the GoTaq® *Legionella* spp/pneumophila/SG1 qPCR and viability qPCR Kits enable quantification of *L. pneumophila*, *L. pneumophila* Serogroup 1 (SG1) and other *Legionella* species. Each kit contains a quantitative standard DNA, Legionella Quant Standard, that can be used to quantify the *Legionella* DNA present in a sample. In addition, an internal positive control is included in the IPC-Primer-Probe Mixes, to aid in detecting qPCR inhibitors that may be present in collected samples.

The main drawback of existing PCR approaches is the lack of live-dead discrimination in test result analyses. The GoTaq® *Legionella pneumophila* Viability qPCR Kit and GoTaq® *Legionella* spp/*pneumophila*/SG1 Viability qPCR Kit[a] combine the rapidity of PCR with the ability to discriminate between viable and nonviable cells. The kits contain a cell-impermeable reagent (Viability PCR Reagent) that binds exclusively to DNA from membrane-compromised dead, dying or lysed cells. Once bound, the Viability PCR Reagent irreversibly modifies DNA, making it nonamplifiable by PCR. As a result, the qPCR signal results primarily from viable cells. Collected water samples are analyzed in a pairwise fashion, allowing the quantification of total (– Viability PCR Reagent) and viable (+ Viability PCR Reagent) *Legionella* fractions. Assay results are reported as viable genomic units (vGU/ml).

The protocols in this technical manual describe complete workflows, combining filter-concentration of water samples, Viability PCR Reagent treatment, DNA purification and hydrolysis probe-based multiplex qPCR.

GoTaq® *Legionella pneumophila* qPCR Kit (Cat.# AM2201) ISO Conformance Statement:

The automated nucleic acid purification and amplification workflows adhere to the protocol, experimental design, and calculation methodologies outlined in ISO 12869:2019 for the detection and quantification of *Legionella pneumophila* via qPCR.

GoTaq® *Legionella spp/pneumophila*/SG1 qPCR Kit (Cat.# AM2202) ISO Conformance Statement:

The automated nucleic acid purification and amplification workflows employed in this kit adhere to the protocol, experimental design, and calculation methodologies outlined in ISO 12869:2019 for the detection and quantification of *Legionella spp.*, *Legionella pneumophila* and *Legionella pneumophila* serogroup 1 via qPCR.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
GoTaq® *Legionella pneumophila* qPCR Kit	**1 each**	**AM2201**

Contains sufficient reagents for 200 reactions in suspension. Includes:

- 1 × 220µl L. pneumophila IPC-Primer-Probe Mix (20X)
- 2 × 1.1ml Legionella qPCR Master Mix (2X)
- 1 × 100µl Legionella Quant Standard, 4 × 10^6 copies/µl
- 1 × 13ml Nuclease-Free Water

PRODUCT	SIZE	CAT.#
GoTaq® *Legionella spp/pneumophila*/SG1 qPCR Kit	**1 each**	**AM2202**

Contains sufficient reagents for 200 reactions. Includes:

- 1 × 220µl L. pneumophila/spp/SG1 IPC-Primer-Probe Mix (20X)
- 2 × 1.1ml Legionella qPCR Master Mix (2X)
- 1 × 100µl Legionella Quant Standard, 4 × 10^6 copies/µl
- 1 × 13ml Nuclease-Free Water

PRODUCT	SIZE	CAT.#
GoTaq® *Legionella pneumophila* Viability qPCR Kit	**1 each**	**AM2205**

Contains sufficient reagents for 200 reactions in suspension. Includes:

- 1 × 220µl L. pneumophila IPC-Primer-Probe Mix (20X)
- 2 × 5ml Viability PCR Neutralization Buffer (10X)
- 1 × 50µl Viability PCR Reagent, 30mM
- 2 × 1.1ml Legionella qPCR Master Mix (2X)
- 1 × 100µl Legionella Quant Standard, 4 × 10^6 copies/µl
- 1 × 13ml Nuclease-Free Water

PRODUCT	SIZE	CAT.#
GoTaq® *Legionella spp/pneumophila*/SG1 Viability qPCR Kit	**1 each**	**AM2206**

Contains sufficient reagents for 200 reactions. Includes:

- 1 × 220µl L. pneumophila/spp/SG1 IPC-Primer-Probe Mix (20X)
- 2 × 5ml Viability PCR Neutralization Buffer (10X)
- 1 × 50µl Viability PCR Reagent, 30mM
- 2 × 1.1ml Legionella qPCR Master Mix (2X)
- 1 × 100µl Legionella Quant Standard, 4 × 10^6 copies/µl
- 1 × 13ml Nuclease-Free Water

Storage Conditions: Nuclease-Free Water can be stored at room temperature. Store all other components at –30°C to –10°C, protected from light.

Note: Thaw Viability PCR Reagent at room temperature before use. Make sure reagent is completely thawed and vortex before use.

Safety Note: Use proper laboratory personal protective equipment (gloves, lab coat, safety glasses) when handling the Viability PCR Reagent, to reduce risk of direct exposure. See the SDS for more information.

3. Suspension Sample Protocol

Use this protocol if test samples have already been collected and/or filter concentrated using externally validated bacterial collection methods (ISO 11731, CDC-9260-J, etc.)

We recommend resuspending *Legionella* bacteria in an isotonic buffer, such as 1X PBS (phosphate-buffered saline) instead of nuclease-free water, to minimize any osmotic shock that could affect detection of *Legionella* with the GoTaq® *Legionella pneumophilia* qPCR and GoTaq® *Legionella spp/pneumophila*/SG1 qPCR Kits.

Viability PCR Reagent concentrations may need to be optimized for the specific water sample and test setup. Modify the volume of reagent added to reactions as needed. If lower concentrations of reagent working stocks are needed, dilute reagent in PBS and dispose of unused Viability PCR Reagent after each experiment.

Viability PCR Reagent is not stable for long-term storage in aqueous solution.

Materials to Be Supplied By the User

- 1.5ml microcentrifuge tubes
- heat block
- microcentrifuge (Section 3.C, manual method only)
- 100% isopropanol (Section 3.C, manual method only)
- **optional:** automated nucleic acid purification platform (e.g., Maxwell® RSC Instrument, Cat.# AS4500 or Maxwell® RSC 48 Instrument, Cat.# AS8500) for Section 3.B
- **optional:** automated Maxwell® RSC PureWater Kit (Cat.# AS2110; Section 3.B) or manual Wizard® PureWater Kit (Cat.# A3130; Section 3.C)

Note: Use either automated (Section 3.B) or manual (Section 3.C) nucleic acid purification workflows to isolate nucleic acid after optional Viability PCR Reagent treatment and neutralizing samples (Section 3.A).

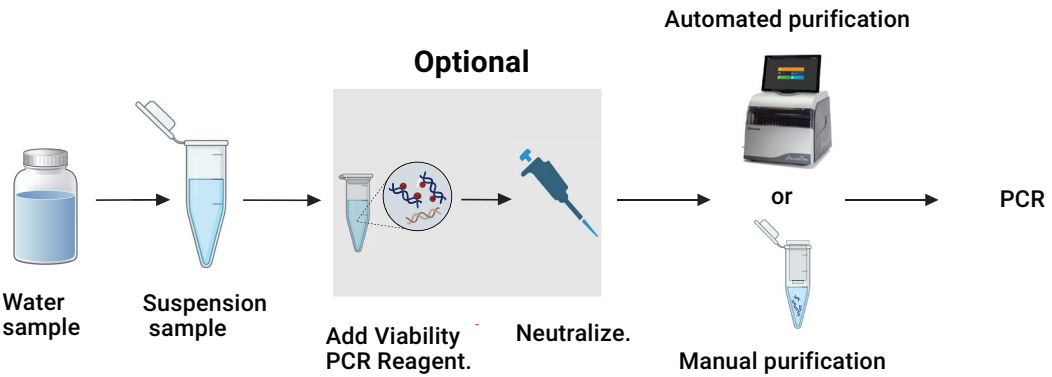

Figure 1. GoTaq® *Legionella* Kit workflow. Created with BioRender.com.

3.A. Optional: Viability PCR Reagent Treatment

Treating samples with Viability PCR Reagent is optional. If Viability PCR Reagent is not used, proceed to either the automated (Section 3.B) or manual (Section 3.C) purification protocol.

1. Add 200µl of each test sample to two separate 1.5ml microcentrifuge tubes.
2. Dilute 30mM Viability PCR Reagent stock to 1mM (1,000µM, 1:30 dilution) in 1X PBS. You will need 1µl of 1mM Viability Reagent per sample.

   Note: Do not dilute Viability PCR Reagent in DMSO; it will inactivate live cells. Viability PCR Reagent is not stable in PBS for extended storage; only dilute the amount of Viability PCR Reagent needed for each day.
3. Add 1µl of Viability PCR Reagent, 1mM, to one of the tubes (5µM final concentration; with Viability PCR Reagent; + VR), leaving the other tube untreated(– VR). Vortex to mix and incubate at 37°C for 30 minutes.
4. Vortex again to mix, then incubate statically at 37°C for an additional 30 minutes.
5. Add 20µl of 10X Viability PCR Neutralization Buffer to each tube. Vortex to mix.
6. Incubate at room temperature for 15 minutes.
7. The samples are now ready for downstream processing (see Section 3.B, Automated Nucleic Acid Purification Protocol or Section 3.C, Manual Nucleic Acid Purification Protocol).

3.B. Automated Nucleic Acid Purification

For details such as Troubleshooting, see the *Maxwell® RSC PureWater Kit Technical Manual*, #TM726. The reagents in this protocol are provided in the Maxwell® RSC PureWater Kit (Cat.# AS2110).

1. Add 200µl of CTAB Buffer and 40µl of Proteinase K to each tube. Vortex to mix.
2. Incubate at 56°C for 10 minutes.
3. Load sample and 300µl of Lysis Buffer into well #1 of a Maxwell® cartridge.
4. Add 80µl of Elution Buffer to the elution tubes.
5. Run the **Maxwell RSC PureWater** protocol.
6. Proceed to Section 5 to perform qPCR on purified nucleic acid. Alternatively, store samples at –30°C to –10°C for later analysis.

3.C. Manual Nucleic Acid Purification

For details such as Troubleshooting, see the *Wizard® PureWater Kit Technical Manual*, #TM729. The reagents and tubes in this protocol are provided in the Wizard® PureWater Kit (Cat.# A3130).

1. Preheat 100µl of Elution Buffer to 60°C.
2. Add 200µl of CTAB and 20µl of Proteinase K to each tube. Invert to mix
3. Incubate at 56°C for 10 minutes
4. Add 300µl of Lysis Buffer to 200µl of each sample. Mix by inversion.
5. Add 500µl of 100% isopropanol to each sample. Mix well by inversion.

3.C. Manual Nucleic Acid Purification (continued)

6. Add 700µl of sample to a ReliaPrep™ Binding Column. Centrifuge sample into Collection Tube at 10,000rpm for 1 minute. Discard flowthrough.
7. Repeat Step 6 with the remaining sample using the same ReliaPrep™ Binding Column. Discard flowthrough.
8. Add 300µl of Column Wash A to each Binding Column. Centrifuge sample into collection tube at 10,000rpm for 1 minute. Discard flowthrough.
9. Add 300µl of Column Wash B to each Binding Column. Centrifuge sample into collection tube at 10,000rpm for 1 minute. Discard flowthrough.
10. Repeat Step 9. Centrifuge for 30 seconds to remove any residual wash solution.
11. Transfer the ReliaPrep™ Binding Column to a new 1.5ml Elution Tube and add 40µl of preheated (60°C) Elution Buffer to the column. Allow the Elution Buffer to soak into the Binding Column filter for approximately 1 minute.
12. Centrifuge at 10,000rpm for 1 minute to elute.
13. Repeat the elution with 40µl of preheated Elution Buffer, for a total of 80µl. Centrifuge at 10,000rpm for 1 minute to elute.
14. Proceed to Section 5 to perform qPCR on purified nucleic acid. Alternatively, store samples at –30°C to –10°C for later analysis.

4. Filter-Concentrated Sample Protocol

Use this protocol to filter concentrate test samples followed by optional on-filter Viability PCR Reagent treatment and nucleic acid purification.

See the following Materials list for suggested filter concentration equipment and reagents.

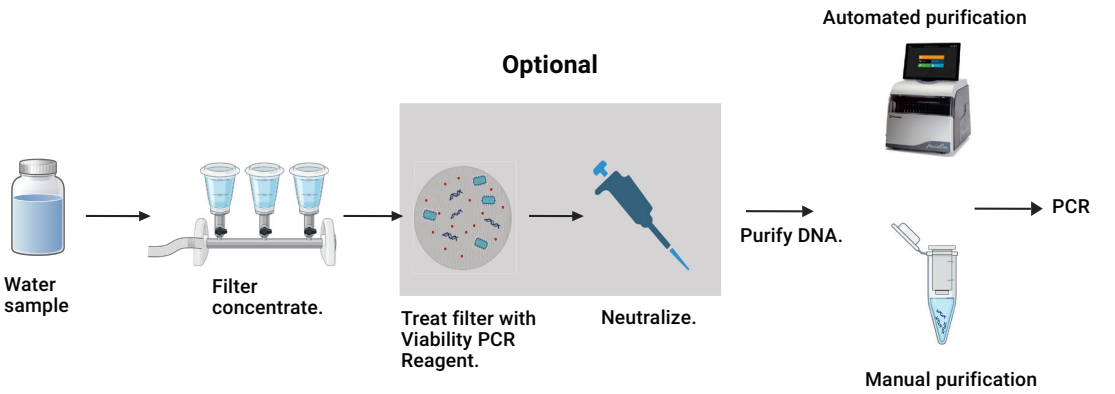

Figure 2. Protocol diagram for GoTaq® *Legionella* Kits used with filter-concentrated water samples. Created with BioRender.com.

Materials to Be Supplied By the User

- vacuum manifold (e.g., Cytiva Cat.# 4889)
- standard adaptor (e.g., Cytiva Cat.# 4892)
- 25mm filter funnel (e.g., Cole Parmer Cat.# EW-35200-55)
- rubber stoppers (VWR Cat.# 59581-367)
- 0.2µm polycarbonate membrane filters 25mm diameter (e.g., Millipore Cat.# GTTP02500)
- Welch® vacuum pump (Model 2522B-01 for North America, Cat.# A6720; Model 2522C-02 for Europe, Cat.# A6722) or other vacuum source
- 1X PBS (phosphate-buffered saline)
- 100% isopropanol
- scalpel
- 1.5ml microcentrifuge tubes
- 2.0ml microcentrifuge tubes (e.g., Eppendorf Cat.# 0030108426; for manual purification protocol only)
- sterile petri dishes
- heat block
- vortex mixer
- microcentrifuge
- **optional:** automated nucleic acid purification: Maxwell® RSC PureWater Kit (Cat.# AS2110)
- **optional:** manual nucleic acid purification: Wizard® PureWater Kit (Cat.# A3130)

4.A. Filter Collection and Concentration

1. Collect 200ml of each water sample to be tested. If performing optional On-Filter Viability PCR Reagent Treatment (Section 4.B) collect 2 × 200ml water samples.
2. Filter concentrate the sample(s) onto 0.2µm polycarbonate filter(s).
3. **Optional:** If performing Viability PCR Reagent treatment, label one filter "untreated" (total *Legionella*) sample and other filter "treated" (intact *Legionella*).
4. Aseptically transfer the filter(s) to separate sterile petri dishes. Slice the filters into halves or quarters using a sterilized scalpel.
5. Aseptically transfer the filter fragments to separate 1.5ml (if using the automated Maxwell® protocol) or 2ml (if using the manual Wizard® protocol) tubes.
6. Add 700µl of 1X PBS to each tube.

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4.B. Optional: On-Filter Viability PCR Reagent Treatment

Treating samples with Viability PCR Reagent is optional. If Viability PCR Reagent is not used, proceed to automated (Section 4.C) or manual (Section 4.D) purification protocol.

1. Dilute the amount of 30mM Viability PCR Reagent stock required for your experiment to 3mM (3,000µM, 1:10 dilution) in 1X PBS. .

   Notes: Do not dilute Viability PCR Reagent in DMSO; it will inactivate live cells. Viability PCR Reagent is not stable in PBS for extended storage; only dilute the amount needed for each day.

2. Add 4.6µl of 3,000µM Viability PCR Reagent to the treated sample. Pipet gently to mix, then close the tube cap and invert three times.
3. Incubate both treated and untreated samples at 37°C for 30 minutes.
4. Pipet gently to mix, then close the tube cap and invert three times.
5. Incubate both treated and untreated samples at 37°C for an additional 30 minutes.
6. Add 70µl of 10X Neutralization Buffer to both treated and untreated samples. Invert three times to mix.
7. Incubate at room temperature for 15 minutes.
8. The samples are now ready for downstream processing (see Section 4.C, Automated Nucleic Acid Purification or Section 4.D, Manual Nucleic Acid Purification).

   Note: These nucleic acid purification protocols (Sections 4.C and 4.D) are suggested options. Other validated manual or automated nucleic acid purification workflows and platforms are acceptable.

4.C. Automated Nucleic Acid Purification

For details such as Troubleshooting, see the *Maxwell® RSC PureWater Kit Technical Manual*, #TM726. The reagents in this protocol are provided in the Maxwell® RSC PureWater Kit (Cat.# AS2110).

1. Centrifuge tubes with filters at 10,000rpm for 1 minute in a microcentrifuge.
2. Carefully remove liquid from filters, taking care to pipet from the center of tube bottom to avoid removing any pelleted bacteria.
3. Add 700µl of CTAB. Vortex for 30 seconds.
4. Incubate at 95°C for 5 minutes.
5. Cool at room temperature for 2 minutes, then vortex for 1 minute.
6. Add 40µl of Proteinase K and 20µl of RNase A. Vortex to mix.
7. Incubate at 70°C for 10 minutes.
8. Add 700µl of liquid sample and 300µl of Lysis Buffer to well #1 of the Maxwell® cartridge.

   Note: Leave filters in the tube; do not add filters to the Maxwell® cartridge.

9. Add 80µl of Elution Buffer to each collection tube.

10. Run the **Maxwell RSC PureWater Kit** protocol.
11. Proceed to Section 5 to perform qPCR on purified nucleic acid. Alternatively, store samples at –30°C to –10°C for later analysis.

4.D. Manual Nucleic Acid Purification

For details, including Troubleshooting, see the *Wizard® PureWater Kit Technical Manual* #TM729. The reagents for this protocol are provided in the Wizard® PureWater Kit (Cat.# A3130).

1. Centrifuge tubes with filters at 10,000rpm for 1 minute in a microcentrifuge.
2. Carefully remove liquid from filters, taking care to pipet from the center of tube bottom to avoid removing any pelleted bacteria.
3. Add 700µl of CTAB Buffer. Vortex for 30 seconds.
4. Incubate at 95°C for 5 minutes.
5. Cool at room temperature for 2 minutes, then vortex for 1 minute.
6. Add 20µl of Proteinase K and 10µl of RNase A. Vortex to mix.
7. Incubate at 70°C for 10 minutes.
8. To 700µl of each sample, add 300µl of Lysis Buffer. Mix by inversion.
9. Add 700µl of 100% isopropanol to each sample. Mix well by inversion.
10. Preheat 100µl of Elution Buffer to 60°C.
11. Add 700µl of liquid sample to a ReliaPrep™ Binding Column.
    Note: Leave the filters in the 2.0ml tube. Do not transfer filters to the ReliaPrep™ Binding Column.
12. Centrifuge sample into the Collection Tube at 10,000rpm for 1 minute. Repeat this step two more times to load the entire sample onto the ReliaPrep™ Binding Column. Discard flowthrough.
13. Add 300µl of Column Wash A to each ReliaPrep™ Binding Column. Centrifuge at 10,000rpm for 1 minute. Discard flowthrough.
14. Add 300µl of Column Wash B to each ReliaPrep™ Binding Column. Centrifuge at 10,000rpm for 1 minute. Discard flowthrough.
15. Repeat Step 14. Centrifuge for 30 seconds to remove any residual Column Wash B solution.
16. Transfer the ReliaPrep™ Binding Column to a new 1.5ml elution tube and add 40µl of preheated (60°C) Elution Buffer to the column. Allow the Elution Buffer soak into the Binding Column filter for approximately 1 minute.
17. Centrifuge at 10,000rpm for 1 minute to elute. Repeat elution with another 40µl of preheated Elution Buffer, for a total of 80µl of Elution Buffer.
18. Proceed to Section 5 to perform qPCR on the purified nucleic acid. Alternatively, store samples at –30°C to –10°C for later analysis.

5. qPCR Protocol

Note: To avoid contamination of samples with external sources of *Legionella* DNA template, perform all steps with filtered pipette tips.

Materials to Be Supplied By User

- 1.5ml tubes
- 0.5ml low-bind tubes (e.g., Eppendorf Cat.# 022431005)
- vortex mixer
- qPCR plates or strip tubes with caps
- qPCR thermocycler with FAM™, HEX™/VIC®, Quasar® 670 (Cy®5) and Texas Red®-XN (ROX™) channels

5.A. Assemble Legionella qPCR Standard Curve

1. Thaw the Legionella Quant Standard, 4×10^6 copies/µl, avoiding long exposures to ambient temperature.
2. Dilute the Legionella Quant Standard, 4×10^6 copies/µl, 100-fold by adding 3µl of DNA to 297µl of Nuclease-Free Water, for a final concentration of 4×10^4 copies/µl.
3. Perform subsequent serial tenfold dilutions in low-binding 0.5ml tubes. For example, combine 10µl of DNA with 90µl of Nuclease-Free Water to obtain the following standard curve dilutions (4×10^4–4 copies/µl; see Table 1 and Figure 3). Vortex each dilution for 3–5 seconds prior to removing an aliquot for the next dilution. Change pipette tips between dilutions.

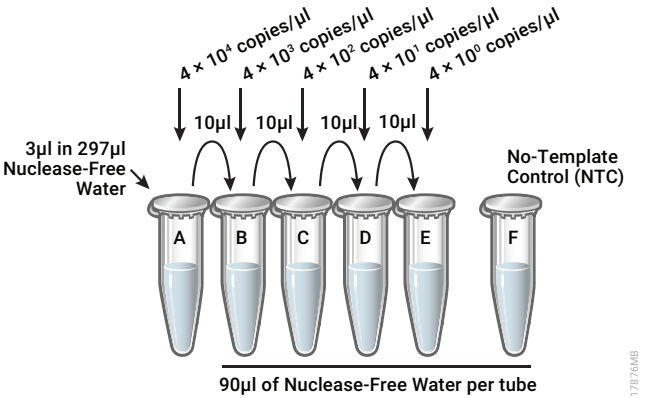

Figure 3. Dilution scheme for Legionella Quant Standard, 4×10^6. Add 3µl of *Legionella* Quant Standard to 297µl of Nuclease-Free Water (tube A), then prepare serial dilutions as shown in the diagram.

Table 1. Standard Curve Dilutions for Legionella Quant Standard, 4×10^6. Tubes A–E correspond to the tube labels in Figure 3.

Tube	Legionella Quant Standard (copies/µl)	Copies/Well (5µl of sample/20µl reaction)
A	4×10^4	2×10^5
B	4×10^3	2×10^4
C	4×10^2	2×10^3
D	4×10^1	2×10^2
E	4×10^0	2×10^1

5.B. Assemble qPCR Master Mix

Calculations below are based on three technical replicates. If increased statistical power is desired, increase the number of technical replicates accordingly.

Total Reaction Wells Needed (n) = (# samples × 3) + (15-sample standard curve) + (3 no-template control) + 3

Table 2. Reaction Mixture Worksheet for 20µl Reaction Volume.

Component	Volume per Reaction (X)	Number of Reactions (n)	Final Volume (X × n)
Legionella qPCR Master Mix (2X)	10µl		
Legionella IPC/Primer/Probe Master Mix (20X)	1µl		
Nuclease-Free Water	4µl		

1. Vortex the Legionella qPCR Master Mix briefly before use to ensure homogeneity. Centrifuge briefly to collect contents at bottom of tube.
2. Determine the number of reaction wells needed. This should include reactions for the combined quantitative DNA standard curve and negative controls. Add three reactions to this number to compensate for pipetting error. While this approach consumes a small additional amount of each reagent, it ensures that enough qPCR amplification mix will be available for all samples. It also ensures that each reaction contains the same qPCR amplification mix.
3. Assemble the reaction mix by combining the Legionella qPCR Master Mix (2X), L. pneumophila IPC-Primer-Probe Mix (20X) or L. pneumophila/spp/SG1 IPC-Primer-Probe Mix (20X) and Nuclease-Free Water calculated in Step 2 and Table 2.
4. Pipet 15µl/well of reaction mix from Step 3 into 96-well qPCR plates.
5. Add 5µl of extracted nucleic acid, Legionella Quant Standard or Nuclease-Free Water for no-template control. The final reaction volume should be 20µl.

5.B. Assemble qPCR Master Mix (continued)

6. Seal the plate, vortex to mix and centrifuge at approximately 300rpm for 1 minute to ensure all liquid is collected at the bottom of the plate wells. Protect from light and elevated temperatures before cycling. The samples are now ready for thermal cycling.

Example Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	Sample 1 + VR	Sample 1 + VR	Sample 1 + VR							2×10^5	2×10^5	2×10^5
B	Sample 1 – VR	Sample 1 – VR	Sample 1 – VR							2×10^4	2×10^4	2×10^4
C	Sample 2 + VR	Sample 2 + VR	Sample 2 + VR							2×10^3	2×10^3	2×10^3
D	Sample 2 – VR	Sample 2 – VR	Sample 2 – VR							2×10^2	2×10^2	2×10^2
E	Sample 3 + VR	Sample 3 + VR	Sample 3 + VR							2×10^1	2×10^1	2×10^1
F	Sample 3 – VR	Sample 3 – VR	Sample 3 – VR							Legionella Quant Standard standard curve		
G	Sample 4 + VR	Sample 4 + VR	Sample 4 + VR							NTC	NTC	NTC
H	Sample 4 – VR	Sample 4 – VR	Sample 4 – VR									

Figure 4. qPCR plate diagram. If not performing optional Viability PCR Reagent treatment, omit "+VR" wells.

5.C. qPCR Thermal Cycling

1. Run qPCR as shown below on reaction plate assembled in Section 5.B.

Step	Temperature (°C)	Time	Number of Cycles
GoTaq® Hot Start Polymerase activation	95	2 minutes	1
Denaturation	95	15 seconds	40
Annealing/extension	61	60 seconds	

2. Collect data from following fluorescence channels at the end of the 61°C annealing/extension step:

GoTaq® *Legionella* Kit	Channel
GoTaq® *Legionella pneumophila* Kits (Cat.# AM2201, AM2205)	FAM, HEX/VIC
GoTaq® *Legionella* spp/pneumophila/SG1 Kits (Cat.# AM2202 AM2206)	FAM, HEX/VIC, ROX, Cy5

Note: Internal assay optimization has determined that reference dye normalization is not necessary for optimal assay performance (see Section 7).

3. Proceed to Section 6 for data analysis.

6. Data Analysis

6.A. qPCR Channel List

	Legionella pneumophila Kits	*Legionella spp/pneumophila*/SG1 Kits
Legionella pneumophila	FAM	FAM
Legionella pneumophila SG1	N/A	ROX
Legionella spp.	N/A	Cy5
IPC	HEX/VIC	HEX/VIC

6.B. Evaluate qPCR Assay Standard Curve (FAM, Cy5, ROX)

Common qPCR analysis software packages apply a linear regression to the standard dilution series data and calculate the best fit of the standard curve using y = mx + b, where x = log concentration, y = C_q/C_t, and m = slope. r^2 measures goodness of fit to the regressed line and m efficiency, where m = −3.3 indicates 100% PCR efficiency (e.g., amplification product is doubled at each cycle). The y-intercept (b in the equation) is the y value C_q at x = 0. For example, b corresponds to the C_q value for a sample with a concentration of one copy/reaction ($Log_{10}(1) = 0$).

In general, the standard curve for each PCR target has an average slope (m) in the range of −2.84 to −4.1 (corresponding to a qPCR efficiency of 75% < E < 125%) and an r^2 value > 0.970. We recommend monitoring y-intercept values for any significant changes from run to run.

6.C. Analyze IPC Signal (HEX)

Amplification performance for the IPC can be used to evaluate overall performance of the *Legionella* qPCR amplification and/or detect qPCR inhibitors in the DNA sample.

Depending on the qPCR instrument and analysis software used, the IPC C_q should be in the range of 20–30.

If the IPC C_q is shifted >1 C_q compared to NTC well, PCR inhibitors are present in the experimental sample. Repeat purification if necessary.

If IPC fails to amplify, no conclusions can be made about the absence of *Legionella* in a sample.

6.D. Analyze qPCR Signal for Sample Replicates

If a sample yields no detectable amplification for *Legionella* DNA but exhibits IPC amplification (C_q = 20–30), the sample can be categorized as *Legionella* negative.

If Viability PCR Reagent was used, analyze qPCR signal from paired + Viability Reagent and – Viability Reagent samples.

– Viability Reagent Signal	+ Viability Reagent Signal	Conclusion
Negative	Negative	No *Legionella* Present
Positive	Negative	Nonviable *Legionella* and/or DNA present
Positive	Positive	Viable *Legionella* present in sample

6.E. *Legionella* Quantification

Limit of detection (LOD) is defined as the lowest amount of analyte in a sample that can be detected with 95% probability. The LOD for the qPCR assay is 10 copies of nucleic acid per reaction.

Limit of quantitation (LOQ) is defined as the lowest amount of analyte in a sample that can be quantitatively determined with less than 25% coefficient of variance. The LOQ for the qPCR assay is 20 copies per reaction.

The LOD of the entire workflow (filtration, purification and qPCR) depends on sample volume, filter recovery efficiency and purification strategy.

If Viability PCR Reagent treatment was performed, viable genomic units/ml (VGU/ml) can be used as a more informative surrogate compared to the common CFU/ml notation. If Viability PCR Reagent treatment was not performed, results can be reported as genomic units/ml (GU/ml).

Note: Due to the multicopy nature of the 16sRNA gene fragment used to detect *Legionella spp*., the VGU/ml or GU/ml value calculated for the *Legionella* channel (Cy5) should be **divided by four** to achieve a more accurate estimated value of viable *Legionella* in the sample.

1. Calculate genomic units (GU) in qPCR.
   a. Generalized equation of qPCR standard curve:

      C_q = slope × log(GU) + y intercept

   b. Using experimentally derived C_q value, solve for GU:

      GU = $10^{(C_q - \text{y intercept})/\text{slope}}$

   Note: Many qPCR software packages will perform this calculation automatically.

2. The following formula can be applied to calculate the *Legionella* concentration of an original sample:

Concentration factors for suspension protocol (Section 3):

- Maxwell® RSC PureWater Kit: 2.5
- Wizard® PureWater Kit: 2.5
- If sample was concentrated before using the kit, multiply the upstream concentration factor by 2.5 to get the total concentration factor for entire workflow.

Concentration factor for on-filter protocol (Wizard® and Maxwell® Kits, Section 4):

- 2,500X

$$\text{Concentration of } \textit{Legionella} \text{ genome in water sample (copies/ml)} = \frac{\text{Copies in qPCR (copies)}}{\text{Volume of nucleic acid extract used in qPCR (ml)} \times \text{Concentration factor*}}$$

If 5µl of nucleic acid extract is used in qPCR, the value in milliliters is 0.005.

$$\text{*Concentration factor} = \frac{\text{Water sample volume used (ml)}}{\text{Volume of nucleic acid extracted (ml)}}$$

Note: The viable cell fraction is reported in viable genomic units/ml (vGU/ml).

7. qPCR Instruments and Reference Dye Requirements

Certain qPCR instruments (Applied Biosystems 7500 and 7500 FAST Real-Time PCR System; Applied Biosystems 7300 and 7900HT Real-Time PCR System; QuantStudio® Real Time PCR Systems; Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR Systems; Stratagene Mx3000P® and Mx3005P® Real-Time PCR Systems; Stratagene Mx4000® Multiplex Quantitative PCR System) often require the use of a reference dye standard, per manufacturer's instructions. We recommend not using the reference dye normalization option, as our assay optimization has demonstrated reference dye normalization to not be necessary for optimal assay performance.

8. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: **www.promega.com**. Email: **techserv@promega.com.**

Symptom	Causes and Comments
Flat amplification curves and no C_q values detected for any targets, including the IPC, for a subset of samples in the 96-well plate	Verify that reaction mix was added to the failed amplification reactions.
	Examine the liquid volume in each well of the reaction plate to verify that evaporation did not occur during cycling. Ensure that all wells were adequately sealed to prevent evaporation during thermal cycling.
	Verify that the thermal cycler was programmed correctly (see Section 5.C).
Nonlinear (r^2 values: <0.97) standard curve and/or standard curve slope outside of optimal range (efficiency: 86–115%)	Be sure that all reagents were thawed completely and mixed well before use.
	Repeat standard curve calculations with four highest concentrations of the standard curve (200–200,000 copies/well).
	Calibrate pipettes to minimize variability (especially important if using multichannel pipettes). Amplify each DNA standard in duplicate or triplicate to minimize the effects of variation on the standard curve.
	Briefly vortex assembled, sealed qPCR reaction plate, followed by low speed spin before inserting plate into qPCR instrument.
	Dilute Legionella Quant Standard in low-bind tubes to minimize assay variability.
	An error was made while diluting the Legionella Quant Standard. Verify all calculations and repeat the dilution. Avoid pipetting volumes less than 1µl.
	Incorrect copy number values were entered into qPCR analysis software. Verify that all DNA standard values were entered correctly.
	Discrepancy in the well location of standards between the plate and sample map. Verify that the standard wells were identified correctly in the sample map.

Symptom	Causes and Comments
Noisy amplification plots, especially with lower DNA concentration	Ensure that IPC target channel is enabled (HEX/VIC) in standard and sample wells. This ensures that proper spectral calibration is performed by the qPCR analysis software.
	For recent versions of ABI qPCR analysis software (Design and Analysis, etc.), selecting **relative threshold** in the 'primary analysis setting' window can reduce noise in qPCR analysis.

9. Related Products

Product	Size	Cat.#
Maxwell® RSC Instrument	1 each	AS4500
Maxwell® RSC 48 Instrument	1 each	AS8500
Maxwell® RSC PureWater Kit	48 preps	AS2110
Wizard® PureWater Kit	48 preps	A3130
Welch® Vacuum Pump (Model 2522B-01 for North America)	1 each	A6720
Welch® Vacuum Pump (Model 2522C-02 for Europe)	1 each	A6722

Product	Size	Cat.#
Viability PCR Reagent System	100 reactions	A8881
Viability PCR Reagent System, High Concentration	100 reactions	A8883
Nuclease-Free Water	500ml	P1197

[a]Patent Pending.