Challenge

Fast and reliable quantification of bacterial load in cooling tower water samples as well as detection of *Legionella spp.* and especially pathogenic *Legionella pneumophila*.

Solution

Efficient automated extraction of bacterial DNA from cooling tower water samples using the InnuPure C16 *touch* and subsequent qPCR analysis by innuDETECT Assays on qTOWER³.

Extraction and Detection of *Legionella* DNA From Cooling Tower Water Samples Using InnuPure C16 *touch* and qTOWER³

Introduction

Bacteria of the genus *Legionella* are the cause of Legionnaires' disease as well as Pontiac fever. A common source of Legionnaires' disease outbreaks are cooling towers. The bacteria are spread via aerosols and infect alveolar macrophages upon inhalation. Legionnaires' disease is most commonly caused by *Legionella pneumophila* (*L. pneumophila*). However, other species of *Legionella* (*Legionella spp.*) can also cause legionellosis.

According to the 42. Bundesimmissionsschutzverordnung (BImSchV), the German Federal Immission Control Ordinance, operators of evaporative cooling systems, cooling towers and wet separators must regularly monitor the parameter *Legionella spp*. to assess the hygienic quality of service water. If specific limit values are exceeded, the additional differentiation into the *L. pneumophila* species is mandatory.

In addition, monitoring the general bacterial load in cooling towers is mandatory as well. Conventional detection methods reliant on bacterial plating and cultivation are challenging due to complex nutritional demands of these bacteria as well as interference by accompanying flora. Cultivation also requires at least a couple of days to generate conclusive results. Detection of general bacterial DNA and/or *Legionella* DNA by means of real-time PCR, however, is a matter of hours.

As little as a 1 mL water sample from a cooling tower is sufficient for extraction of bacterial DNA using InnuPure C16 *touch*. The usage of the innuPREP Bacteria

Lysis Booster, an optimized mix of several lytic enzymes, ensures efficient non-mechanical pre-lysis of a wide range of bacteria. The lysis of the bacteria was included in the automated protocol, making external lysis and subsequent manual addition of the lysate to the reagent plate obsolete.

To analyze the extracted DNA for overall bacterial load as well as for *Legionella* including sub-specification, two distinct innuDETECT Water Pathogen Assays were used in combination with the qTOWER^3 real-time cycler. The probe-based *Legionella*-specific assay can differentiate between *Legionella pneumophila* and *Legionella spp.* in one multiplex real-time PCR. The complete procedure starting with the nucleic acid extraction from cooling water samples to the final result obtained from the qPCR analyses can be done within only a few hours as shown in Figure 1.

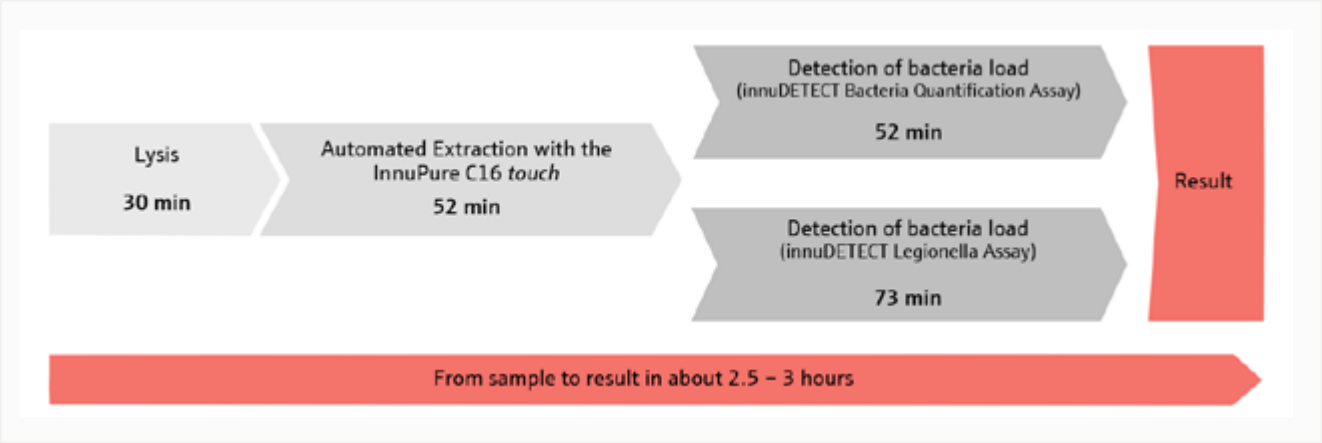

Figure 1: Timeline - overview of the necessary steps to get to the result within a few hours

Materials and Methods

Samples and reagents

- innuPREP Bacteria Lysis Booster (845-KA-1000050)
- innuPREP Bacteria DNA Kit-IPC16 (845-IPS-5516096)
- innuDETECT Bacteria Quantification Assay (845-IDF-0031024)
- innuDETECT Legionella Assay (845-IDF-0033024)
- Legionella pneumoniae DNA (strain: DSM 7513) as positive control
- Cooling tower water samples

Instrumentation

- Vortex mixer
- Benchtop centrifuge
- Plate centrifuge
- Thermal shaker
- InnuPure C 16 *touch*
- qTOWER^3

Sample preparation

Three 1 mL samples of cooling tower water were rebuffered in Tris/EDTA buffer and pre-lysed enzymatically by following the instructions of the innuPREP Bacteria Lysis Booster. Subsequently, bacterial DNA was extracted from the samples in a fully automated manner on InnuPure C16 *touch* by using the innuPREP Bacteria DNA Kit-IPC16. Samples were extracted in duplicates (A+B) and subsequently analyzed via real-time PCR. PCR analysis was performed in duplicates as well.
Total bacterial content of the samples was determined by use of the innuDETECT Bacteria Quantification Assay with a temperature time protocol and channel settings as shown in Figure 2. This real-time PCR assay detects the 16S ribosomal RNA gene, a universal bacterial target gene. The assay includes standard DNA with the defined amount of bacterial DNA of 10^7 copies/µL. Amplification of a serial dilution of this DNA allows semi-quantification of the samples by correlating the resulting Ct values.

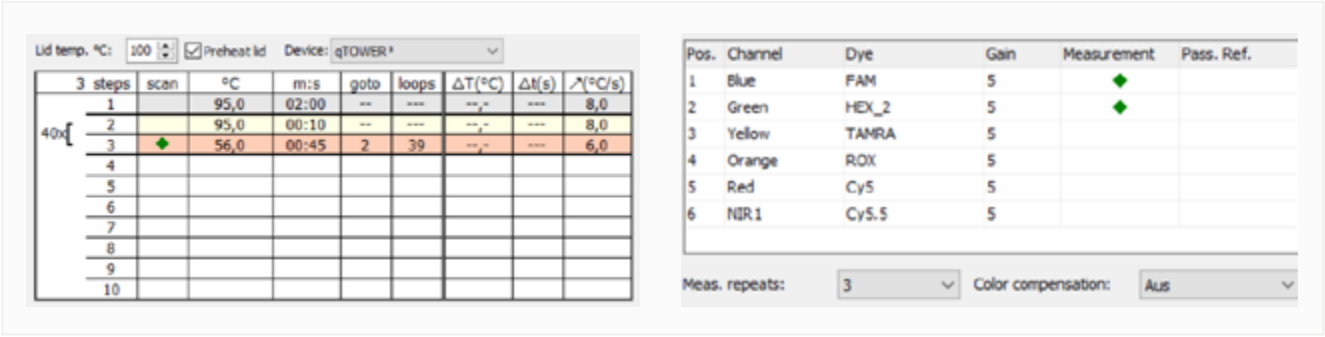

Figure 2: Overview of the settings for bacterial detection in qPCRsoft for the real-time PCR on the qTOWER³. The target DNA is detected in the blue channel, while the internal control is detected in the green channel.

Detection of *Legionella* DNA within the extracted samples was done using the innuDETECT Legionella Assay on qTOWER³. The FAM-labelled probe detects the *Legionella pneumophila*-specific *mip* gene, whereas the Cy5-probe detects a universal target present in all species of the genus *Legionella*. The instrument settings are summarized in Figure 3. An amplification control added to the PCR mixture can be detected in the HEX-channel. To control for the presence of contaminating DNA in the PCR set up, a no template control (NTC) is included in each real-time PCR run. The DNA of *Legionella pneumoniae* (strain: DSM 7513) was used as a positive control.

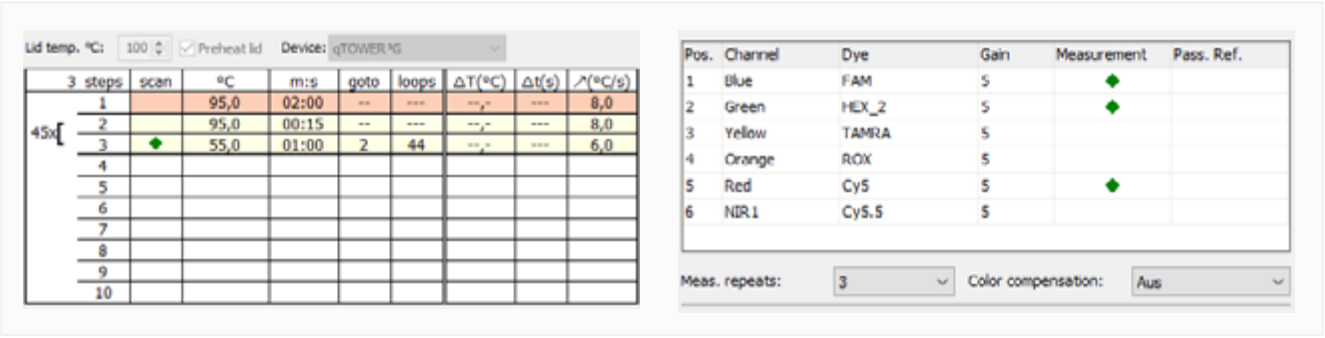

Figure 3: Overview of the settings for *Legionella* detection in qPCRsoft for the real-time PCR on the qTOWER³. *Legionella pneumophila* and *Legionella spp.* can be detected in the blue and red channel, respectively. In the green channel the internal control can be detected.

Results

Determination of total bacterial load

The standard DNA dilutions were amplified and detected with innuDETECT Bacteria Quantification Assay (Figure 4). If the PCR efficiency is 100%, dilutions of 1:10 show a difference in Ct values of 3.3. The average Ct shift for the dilutions of the DNA standard is 3.6, corresponding to a PCR efficiency of 89% (Figure 5). Thus, a good correlation between the Ct values and the bacterial DNA content was achieved (Table 1). The NTC shows a Ct value above 35. It should be noted, that the polymerases used in PCR mixtures are usually generated in bacteria, thus containing trace amounts of bacterial DNA. These can also be detected with this assay, making the NTC necessary to account for this background of bacterial DNA. The determination of the abundance of bacterial DNA in the three samples that were extracted in duplicates (1A+B, 2A+B, 3A+B) was done by inference from their respective Ct values (Table 2) compared to Ct values of the standard DNA. As can be seen in Figure 6, the content of bacterial DNA is very high within the extracted samples, attesting to the very high efficiency of extraction and detection using this system. The amplification of the Internal Control DNA (Figure 7) confirms the correct performance of the PCR as well as the detection system.

Bacterial DNA standard

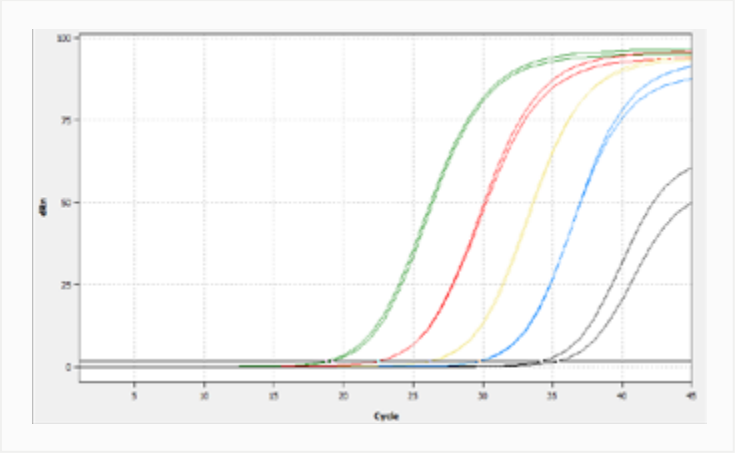

Figure 4: Amplification curves of bacterial DNA standard
Serial dilutions containing 1 x 10^6 (green), 1 x 10^5 (red), 1 x 10^4 (yellow) and 1 x 10^3 (blue) copies of bacterial DNA were recorded in duplicates. The no template controls (NTCs) are shown in black. Signals were detected in the FAM channel on qTOWER^3.

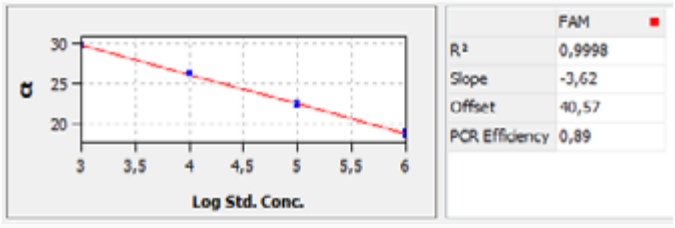

Figure 5: Linear regression of the bacterial DNA standard
The slope of the linear regression when plotting the logarithm of the concentration of the DNA standard against its Ct value, indicates the shift in Ct between the serial dilutions. The efficiency of this PCR is at 89%.

Table 1: Ct values of a serial dilution of the bacterial DNA standard detected in the FAM channel

Dilution	Ct value	Copies
1 :10	19.69 20.04	1.0 x 10^6
1:100	23.51 23.49	1.0 x 10^5
1:1,000	27.17 27.27	1.0 x 10^4
1:10,000	30.63 30.74	1.0 x 10^3
NTC	36.32 35.18	-

Detection of total bacterial DNA

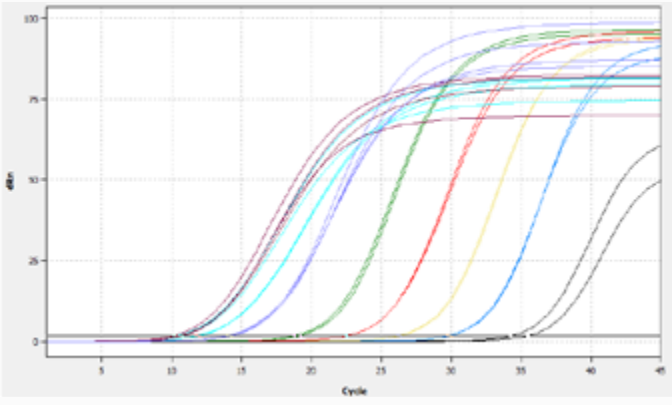

Figure 6: Bacterial DNA in unknown samples
Detection of bacterial DNA via real-time PCR on qTOWER^3. Standard curves as shown in Figure 4. Duplicate curves of 3 duplicate samples are shown in cyan (sample 1A+1B), brown (sample 2A+2B), and lilac (sample 3A+3B), the NTCs are shown in black. Ct values and corresponding copy numbers of bacterial DNA in the samples are listed in Table 2.

Table 2: Calculation of total bacterial DNA in cooling tower water samples

Sample name	Ct value	Copies
Sample 1A	10.29 10.48	2.2 x 10^8
Sample 1B	11.69 11.80	9.3 x 10^7
Sample 2A	10.01 10.60	2.3 x 10^8
Sample 2B	10.40 10.31	2.3 x 10^8
Sample 3A	13.83 14.15	2.2 x 10^7
Sample 3B	14.08 14.13	2.1 x 10^7

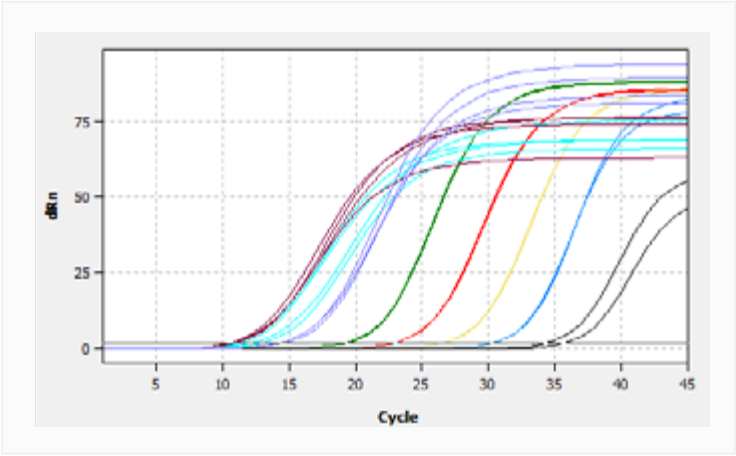

Figure 7: Internal control DNA
Amplification control via the Internal Control (IC) DNA, added to the PCR. The HEX-labeled IC-specific probe can be detected in the green or yellow color module of the qTOWER³. Here, the detection in the green channel is shown.

Legionella detection

The amplification of the gene conserved across the *Legionella* genus (Figure 8) shows that sample 1 is only weakly positive, with one extraction duplicate having a high Ct value above 37, and the second duplicate not showing a Ct. Sample 2 is negative for this gene. Sample 3 shows a positive signal in both extraction duplicates and therefore contains *Legionella* species. The strong signal of the positive control confirms that the real-time PCR set up and run was performed correctly. Whereas the NTC with no Ct values shows that there was no contamination within the PCR reagents.
To ascertain if sample 1 or sample 3 contain *Legionella pneumophila* DNA, the amplification of *mip* (Figure 9) was analyzed. Neither of the samples shows any amplification of this *L. pneumophila*-specific gene (Table 4). The positive control and negative control (NTC) confirm that the PCR results are valid, and reagents were not contaminated.

Conserved *Legionella* gene

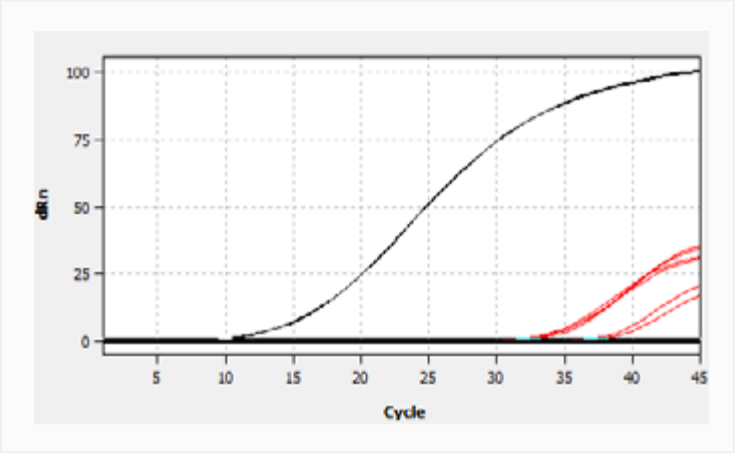

Figure 8: Amplification of the conserved gene
Detection of *Legionella pneumophila* as well as other *Legionella* species (*Legionella spp.*) in the Cy5 channel.
The positive control is shown in black; samples are shown in red. Each sample is analyzed in duplicate.

Table 3: Detection of an universal *Legionella* gene in the Cy5 channel

Sample name	Ct value	*Legionella spp.*
Sample 1A	No Ct	(weakly) positive
Sample 1B	37.62	
Sample 2A	No Ct	negative
Sample 2B	No Ct	
Sample 3A	32.70	positive
Sample 3B	32.29	
Positive Control	10.42	positive
NTC	No Ct	negative

L. pneumophila-specific *mip* gene

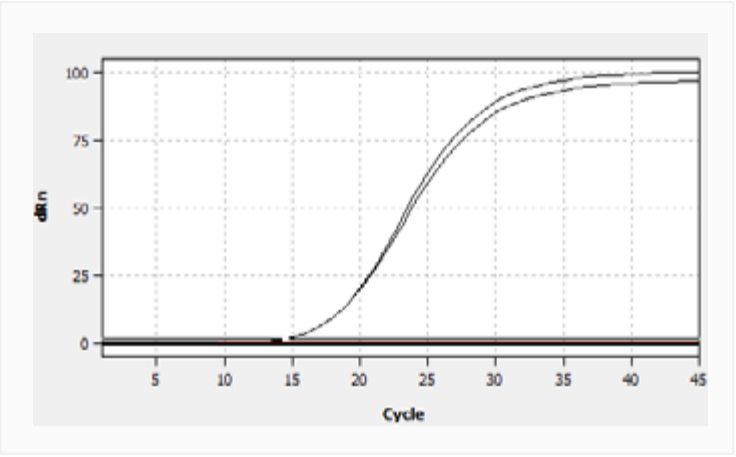

Figure 9: Amplification of *Legionella pneumophila*-specific *mip* gene
Detection of *mip* gene amplification in the FAM channel.
The positive control is shown in black; samples are shown in red.

Table 4: Detection of *Legionella pneumophila*-specific *mip* gene in the FAM channel

Sample name	Ct value	*Legionella pneumophila.*
Sample 1 A+B	No Ct	negative
Sample 2 A+B	No Ct	negative
Sample 3 A+B	No Ct	negative
Positive Control	15.15	positive
NTC	No Ct	negative

Conclusion

The results show that bacterial DNA in general and *Legionella* DNA specifically, can be efficiently and automatically extracted from cooling tower water samples using the InnuPure C16 *touch*. Overall bacterial load can be accurately determined with the innuDETECT Bacteria Quantification real-time PCR Assay. The innuDETECT Legionella Assay performed on the qTOWER[3] allows for differentiation of *pneumophilic* from non-*pneumophilic Legionella* species within the extracted samples.
This exemplary application shows that real-time PCR is a reliable and time-saving alternative method to conventional cultivation-based methods. It facilitates detection of *Legionella spp*. including subspecification of *Legionella pneumophila* as well as quantitative analysis of general bacterial load.

Literature

- https://www.gesetze-im-internet.de/bimschv_42/
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- Paschke, Anne et al. "Legionella transmission through cooling towers: towards better control and research of a neglected pathogen." The Lancet. Respiratory medicine vol. 7,5 (2019): 378-380. doi:10.1016/S2213-2600(19)30041-4

Reference

[1] TechNote_qTOWER3_0009_en.docx

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