Vitens Automates Drinking Water Testing on ML STARlet with KingFisher Presto Integration

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Introduction

The ongoing assessment of drinking water quality is essential in ensuring human safety and health. Drinking water fecal contamination is routinely determined by the presence of gut bacteria such as *E. coli* and Enterococci. The current standard for water testing is culture-based. Vitens laboratory analyzes drinking water samples, also with other more innovative and highly sensitive techniques like Reverse Transcriptase Quantitative PCR (RT-qPCR). This enables faster and more efficient evaluation of fecal pollution, as compared to conventional breeding methods. Especially in situations where quick test results have to be delivered; for example, when leaks in drinking water distribution systems are detected, this is highly desirable. In the Netherlands, the RT-qPCR for *E. coli* is already ISO 16140-validated and widely used by drinking water laboratories.

- **Fast and reliable detection of fecal drinking water contamination**
- **Reduced costs and increased flexibility for analysts, due to replacement of manual operations**
- **Higher capacity of sample numbers to be processed**

For RT-qPCR testing, Vitens chose to detect the 16S rRNA bacteria-specific ribosome sub-unit directly (more then 70 copies/bacterium), rather than the encoding gene itself (i.e. only 2 or 3 copies/cell) for increased sensitivity. However, efficient RNA extraction from samples remains a prerequisite to detect a single colony forming unit (1 CFU) in 100ml of water. Until now, this procedure had to be performed very carefully in a laborious manual way. We demonstrate that this operation can be automated, using the Hamilton STARlet with an integrated KingFisher Presto device, fulfilling these stringent criteria.

Method Description

100ml of drinking water samples are filtered over 0.45µm polycarbonate filters. For RNA extraction, the NucliSENS Magnetic Extraction Reagents (Biomerieux) are used as described by the vendor. The filters are treated with 2ml lysis buffer, supplemented with 10µl lysozyme, by incubation at 37°C for 15 minutes. The filters are then removed from the lysis buffer and subsequently, the lysates are transferred to a 7ml tube and brought to the ML STARlet with KF integration in a 24-tube carrier.

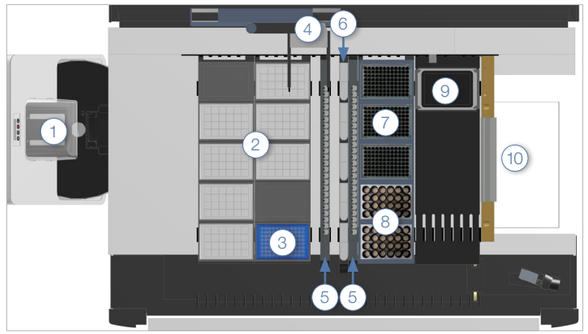
Figure 1: Microlab® STARlet™ with integrated Thermo Fisher Scientific™ KingFisher™ Presto device in the Vitens Laboratory

Next, 50µl of silica magnetic beads are pipetted by the robot to the lysis buffer. After preparation of 24-well plates, filled with dedicated washing and elution buffers by the ML STARlet, resuspension and incubation of the magnetic beads are performed within the KF Presto system as follows: 350µl Wash buffer 1, then 500µl Wash buffer 2 (2 times) and 500µl Wash buffer 3. After washing, elution is performed at 65°C in 100µl of elution buffer within the Hamilton Heater Shaker. Eluates are finally transferred in a 96-well plate by the ML STARlet.

The following two-step RT-qPCR reaction (separate cDNA synthesis and qPCR steps) is performed in a Biorad CFX PCR system. For this, 10µl of each RNA eluate are used in duplicate, and amplification of *E. coli* and Enterococci specific 16S rRNA cDNAs are measured using respective Taqman probes (Texas red for *E. coli*, FAM for Enterococci 16S rRNA specific cDNAs). The abundance of 16S rRNA cDNAs is expressed by C_q values. C_q values of <35 or <37 are considered a positive test result for the presence of *E. coli* or Enterococci respectively and indication of potential fecal contamination. This threshold was estimated by using suspensions of known amounts of viable bacteria. Dilution series for these standard suspensions were performed to reach the detection limit of 1 CFU as estimated by culture (traditional method). The corresponding dilutions were used as inputs in 5 independent replicates for RNA extraction and RT-qPCR.

Figure 2: Visual Workflow

Deck Layout Description



1. Thermo Fisher Scientific KingFisher Presto (24-well head)
2. 2x carrier for deep-well plates
3. 96-well plate position
4. iSWAP plate handler
5. 2x carrier for 24 tubes
6. Reagent carrier with 5x 60ml troughs
7. 3x 1000 µl Tips on carrier
8. 2x 5000 µl Tips on carrier
9. HHS (Hamilton Heater Shaker)
10. Waste position

Figure 3: Deck layout

Kit Description

For RNA extraction

Component	Kit / Vendor
NucliSENS Lysis buffer	NucliSENS Magnetic Extraction Reagents / Biomerieux
Lysozyme (10mg/mL) Sigma	
Magnetic homogeneous silica suspension	
Wash Buffer 1	
Wash Buffer 2	
Wash Buffer 3	
Elution buffer	

For cDNA synthesis

Component	Vendor
cDNA Synthese mix; 10µl/reaction	GC Biotech

For qPCR

Component	Vendor
Bioline SensiMIX II Probe NO-ROX Kit; 25µl/reaction	GC Biotech
Taqman Probes; 0.1 µM/reaction	Biolegio
primer mix; 0.4 µM/reaction	Biolegio

Consumables per 24 samples run

Amount	Component	Part Number / Vendor
7	KingFisher Flex 24 deep-well plate, sterile	95040480 / Thermo Scientific
1	KingFisher deep-well 24 tip comb and plate, sterile	97002620 / Thermo Scientific
24	CO-RE Tips 5000µl	184020 / Hamilton
14	CO-RE Tips 1000µl	235904 / Hamilton
32	CO-RE Tips 300µl	235902 / Hamilton
3	Reagent containers 60ml	56694-01 / Hamilton
1	96 well PCR plate	HSP-9601 / BioRad

Application Software

The method was developed with Microlab® Vector software (VENUS 4), using the VENUS Dynamic Scheduler Software.

Verification

Handling of standard samples proved that this system is able to extract RNA from 1 CFU/100ml *E. coli*, which can also be detected by RT-qPCR.

Technology

The Thermo Scientific KingFisher Presto device, as part of the Hamilton automated liquid handling system, enables high-throughput sample processing. Utilizing proven KingFisher magnetic particle-based technology, it provides high-quality yields of target nucleic acids, suitable for various downstream applications.

Throughput and Capacity

Currently, it takes 75 minutes for the isolation of 24 samples. Optimization of the workflow is planned to reach the goal of processing 24 samples within 60 minutes. Depending upon the washing volumes required by individual kits, capacities could be scaled-up by switching to a 96-well format.

Results

Table 1: RT-qPCR results achieved after RNA extraction using Hamilton STARlet with KingFisher Presto integration from control sample dilutions of **A)** Enterococci and **B)** *E. coli* suspensions. C_q Values of 16S rRNA cDNA amplification curves are indicated for 5 repeated extractions and RT-qPCR reactions performed in duplicates. Average C_q values below 35 for *E. coli* or below 37 for Enterococci were considered as positive (pos) in final calls. For comparison, CFU values are provided as determined by culture in 7 replicates (14x and 50x dilutions), while 100x and 250x dilutions are estimated numbers. N/A means that there is no C_q value available because no amplification had occurred. This means that the the result is negative.

Table 1 A

dilution of culture solution	Enterococci culture CFU/100 ml	# test	average C_q value	final call
positive control 14x diluted in PBS	Between 8 and 24, average 15,6	1	28,8	pos
		2	29,7	pos
		3	30,6	pos
		4	29,0	pos
		5	29,0	pos
positive control 50x diluted in PBS	Between 1 and 7, average 4,6	1	31,4	pos
		2	29,9	pos
		3	32,2	pos
		4	29,2	pos
		5	32,3	pos
positive control 100x diluted in PBS	Estimated CFU average is 2,3	1	33,4	pos
		2	31,8	pos
		3	34,6	pos
		4	35,1	pos
		5	32,4	pos
positive control 250x diluted in PBS	Estimated CFU average is 1	1	N/A	neg
		2	N/A	neg
		3	35,2	pos
		4	33,7	pos
		5	32,6	pos
Undiluted positive control	average 106		25,5	pos
Negative Test Control	none		N/A	neg

Table 1 B

dilution of culture solution	*E. Coli* culture CFU/100 ml	# test	average C_q value	final call
positive control 14x diluted in PBS	Between 0 and 6, average 4	1	31,8	pos
		2	31,5	pos
		3	31,1	pos
		4	31,6	pos
		5	31,1	pos
positive control 50x diluted in PBS	Between 0 and 5, average 1,8	1	32,4	pos
		2	32,2	pos
		3	33,6	pos
		4	31,5	pos
		5	32,0	pos
positive control 100x diluted in PBS	Estimated CFU average is 1	1	32,4	pos
		2	32,8	pos
		3	32,0	pos
		4	N/A	neg
		5	32,4	pos
positive control 250x diluted in PBS	Estimated CFU average is 0,5	1	N/A	neg
		2	34,1	pos
		3	N/A	neg
		4	33,7	pos
		5	33,1	pos
Undiluted positive control	average 60		28,0	pos
Negative Test Control	none		N/A	neg

Summary

Sensitivity tests have shown that by using manual handling, it is possible to extract and detect RNA from 1 Colony Forming Unit (CFU) *E. coli* and Enterococci.
Using the ML STARlet with KF Presto integration makes it possible to extract low levels of *E. coli* and Enterococci RNA from drinking water samples at average amounts of 2.3 and 1.8 CFUs respectively. The RT-qPCR results are positive at these levels. Because of dilution biases, the physical presence of bacteria at lower concentrations is not guaranteed in all cases. Therefore, some test replicates at these levels return positive, while others are negative. However, the ML STARlet with KF Presto integration meets the requirements concerning sensitivity, by its ability to isolate 1 CFU of *E. coli* or Enterococci, which can be detected by RT-qPCR. Additionally, rapid and reproducible RNA extraction is enabled with established protocols for 24 samples per run.

System Requirements

Description	Units	Part Number
ML STARlet with Autoload	1	173021-M
UV Kit STARlet	1	188129APE
Customized Housing Extension	1	188123APE
Modular Arm for 4/ 8/ 12 Ch. / MPH	1	173051
iSWAP Plate Handler	1	190220
Pipetting Channels 1000 µl	4	173080
Pipetting Channels 5000 µl	2	184090
Landscape Shaker Carrier Base	1	187001
Set of 3x Carrier for 24 Tubes	3	185249
Reagent Carrier 5x 50ml	1	185435
Tip carrier, Landscape	1	182085
Carrier for deepwell format, Landscape	2	182090
Hamilton Heater Shaker, flat bottom adapter	1	199034
VENUS four software	1	911264
Dynamic Liquid Classification	1	911183
VENUS Dynamic Scheduler	1	911095
KingFisher Presto 24 Well Head Instrument	1	10102428

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