Determination of Bacteria Retention in the Thermo Scientific Barnstead Smart2Pure Water Purification System

Verena Ruckstuhl, Thermo Fisher Scientific, Langenselbold, Germany and
Julie Foster, Ph.D., Thermo Fisher Scientific, Asheville, NC

Application Note

Key Words
Water purification, lab water, ultrapure water, bacteria, 0.2 micron filter, Thermo Scientific Barnstead, Smart2Pure

Abstract
The bacteria retention of a Thermo Scientific™ Barnstead™ Smart2Pure™ water purification system was evaluated using the membrane filtration method based on the European Pharmacopoeia method described in Chapter 2.6.12.[1]

Introduction
Bacteria are single celled organisms that can be found nearly everywhere in a busy laboratory. Although many of these bacteria are harmless to a person's health, they can create unwelcome variability in an experiment. Because of their abundance and ability to be easily transferred, precautions such as sterile technique were employed. Using bacteria-free water during steps such as sample preparation, system rinsing, or buffer preparation is an easy method of reducing the chance of bacterial contamination.

Water purification systems are a reliable source for bacteria-free water. On average, bacteria such as *Escherichia coli (E. coli)*, which are widely used in the laboratory, have an length of about 2 µm, and an average diameter of 0.5 µm.[2] A 0.2 µm absolute membrane filter at the end of the system is used to remove any particles or bacteria that are larger than the pore size of the filter[3]. Proper maintenance of the water system, including filter replacement as specified in the manual, helps to ensure the water remains bacteria-free.

Ultrapure water from a Barnstead Smart2Pure water system was analyzed for the presence of aerobic bacteria. The Barnstead Smart2Pure 3 system was chosen from the family of Smart2Pure systems, which also includes Smart2Pure UV and UF models. All of these systems have the same feed water requirements, basic flow path and all dispense water through a 0.2 µM final filter. The systems are fed by tap water and utilize two purification steps to create 18.2 megohm lab water. The first step purifies the feed water using a reverse osmosis (RO) membrane to remove the majority of impurities in the water. The second step of purification utilizes an ultrapure cartridge to remove any ions in the water. Additionally, an optional ultraviolet (UV) lamp and/or an ultrafilter (UF) can be added to the system configuration.

Methods
Bacteria retention of the Smart2Pure system using tap water as feed water

The bacterial content of tap water was determined after disinfecting the water outlet with 70% ethanol and rinsing with 1 L water. Afterwards three 1 L sample volumes were collected and tested for bacterial growth using the membrane filtration technique.[1] Three different volume samples were generated from the three 1 L samples: a 1 L, 10 mL, and a 1 mL sample. These three samples were filtered through a 0.2 µm cellulose nitrate (CN) membrane. The membrane was afterwards transferred aseptically to a R2A-Agar and incubated at 35°C for 5 days in a Thermo Scientific™ Heratherm™ compact microbiological incubator (model IMC18). The agar plates with sample volumes of the membrane filtration (1 L and 10 ml) showed after 5 days incubation at 35°C too many colonies to count. Only the 1 ml sample volumes gave results that could be quantitated.

After installing and rinsing of the Smart2Pure 3 system according to the Smart2Pure water purification system operating instructions, the system was ready for sampling. 0.2 L of water from the system was used to rinse the 0.2 µm filter of the Smart2Pure 3 system and then 1 L samples were collected in sterile flasks.

Figure 1. Diagram of a Smart2Pure water system with a 6 L internal reservoir.

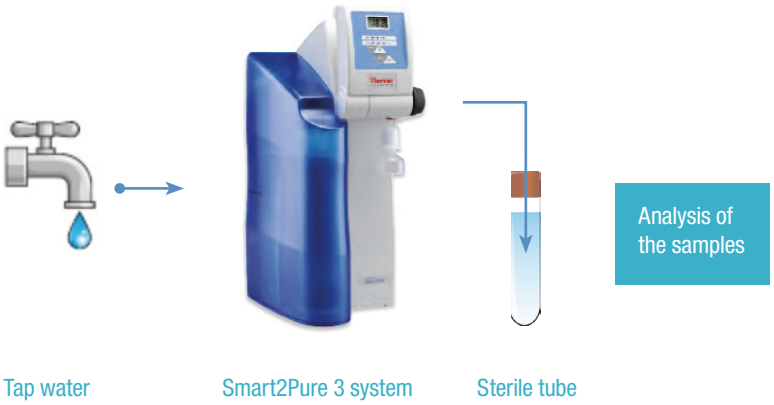


Figure 2. Set up of the filtering of the 1 L sample, using a Thermo Scientific Nalgene analytical test filter funnel, through the 0.2 µm cellulose nitrate (CN) membrane.

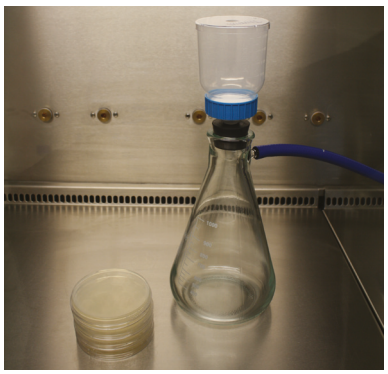
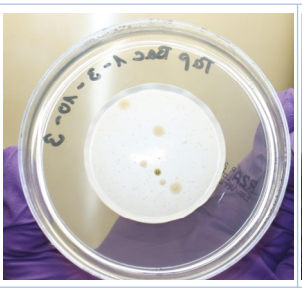
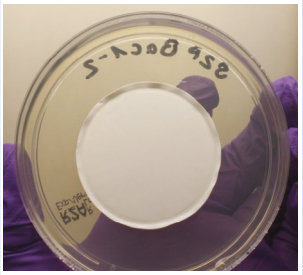
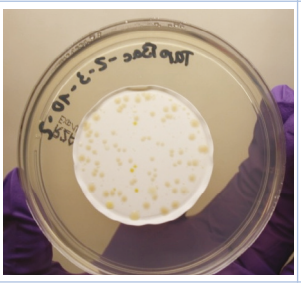


Table 1. Bacteria count from the tap water line (1 L and 10 ml)

	Tap Water Sample (10 ml)	Smart2Pure System Sample (1 L)
1st Run		
2nd Run		

	Tap Water	Smart2Pure System Water
Bacteria Count (CFU/ml)	n.a.	<0.01

n.a. not applicable, the colonies were too numerous to evaluate

The 1 L sample was then filtered through a 0.2 µm cellulose nitrate (CN) membrane, using a Thermo Scientific™ Nalgene™ analytical test filter funnel, as shown in Figure 2. The membrane was afterwards transferred aseptically to a R2A-Agar and incubated at 35°C for 5 days in a Heratherm compact microbiological incubator (model IMC18). The Smart2Pure 3 system was disinfected with the disinfection routine according to operating instructions, and the pretreatment cartridge, ultrapure polisher cartridge and 0.2 µm filter were replaced with new ones to retest the system.

Results

After 5 days, any bacteria colonies found on the R2A-Agar plates were counted and the amount of bacteria per ml in the water were calculated. The data is summarized in Table 1.

Conclusion

The Smart2Pure system, which was directly connected to tap water, was able to remove the bacterial load of the tap water down to < 0.01 CFU/ml.

References

1. European Directorate for the Quality of Medicines & HealthCare (EDQM): European Pharmacopoeia 6.8, *Microbiological Examination of Non-sterile Products: Microbial Enumeration Tests*, 2010

2. Thermo Scientific Waterbook. 2014. Thermo Fisher Scientific. Retrieved 12 June, 2014 from http://www.thermoscientific.com/purewater

3. Operational Instructions for the Thermo Scientific Barnstead Smart2Pure water purification system. Thermo Fisher Scientific, Niederelbert, Germany.

On-demand Nuclease- and Endotoxin-Free Lab Water Using a Thermo Scientific Barnstead Smart2Pure UV/UF Water Purification System

Verena Ruckstuhl, Thermo Fisher Scientific, Langenselbold, Germany,
Kim Knepper and Julie Foster, Ph.D., Thermo Fisher Scientific, Asheville, NC, United States

Application Note

Key Words
Water purification, lab water, ultrapure water, nuclease, endotoxin, pyrogen, RNase, DNase, Thermo Scientific, Barnstead, Smart2Pure, tap to type 1

Abstract
A Thermo Scientific™ Barnstead™ Smart2Pure™ UV/UF water purification system was challenged with RNase, DNase and endotoxins to evaluate the effectiveness of its ability to reduce these impurities below detectable limits and produce nuclease- and endotoxin-free ultrapure water.

Introduction
Nuclease is the general name which includes both ribonuclease (RNase) and deoxyribonuclease (DNase), the enzymes responsible for degrading RNA and DNA respectively. In a controlled application, these enzymes can be very beneficial as they are used in many life science experiments to cleave specific links on RNA and DNA strands. In contrast, nucleases can also be detrimental to experiments if they are present in applications that require the RNA or DNA to be whole. Controlling nuclease contamination can be a challenge, but it is necessary for accurate and reproducible results in these types of experiments. Very durable, nucleases are resistant to heating, are active over a wide pH range, re-nature readily and are easily transferable.[1] They can be plentiful on counter tops, centrifuges, laboratory glassware, buffer and reagent solutions. They can even be found on gloved hands that have touched hair or skin.

It is important to reduce possible contamination of nucleases, and there are multiple ways to achieve this. Using nuclease-free water for buffers and reagents is a good first step. Traditional practices include inactivating RNase in water with the use of the inhibitor Diethyl Pyrocarbonate (DEPC) followed by autoclaving the water to destroy the inhibitor. DEPC hydrolyzes when exposed to trace levels of moisture, so proper storage requires a layer of inert gas in the bottle after each use. DEPC can only be used with glass pipettes as it will dissolve some plastics and is not recommended to be used with common buffers such as Tris. Lastly, if exposed to ammonia, DEPC can decompose to a possible carcinogen, urethane.[2] Alternatively, bottled nuclease-free water is also available, but this adds more consumables to manage, with the additional risk of contaminating the bottle during each use, taking time and resources away from valuable research.

Just as nucleases can be detrimental to many life science experiments, so can endotoxins. Endotoxins are lipopolysaccharides in gram negative bacteria, which are left behind during the course of the bacteria's life cycle. Endotoxins (also referred to as pyrogens), can induce a high fever when injected into mammals. When present in vitro, endotoxins can interfere with the growth of tissue cultures. To utilize water that is endotoxin-free, some labs purchase endotoxin-free bottled water. While this can be convenient, the bottle can also become contaminated and is another consumable that needs to be ordered, shipped, and stored.

Point of use ultrapure water purification systems with ultrafiltration (UF) are designed to effectively reduce nuclease and endotoxin macromolecules to below detection limits. Ultrafilters used in Thermo Scientific water purification systems use polysulfone hollow fibers to provide a powerful and consistent barrier to trap these particles. In the Barnstead Smart2Pure UV/UF system this filter is strategically placed in-line, at the end of the water system's flow path, to help ensure the complete elimination of all nucleases and endotoxins without possible outside contamination. Proper maintenance of the water system, such as regular system cleaning and prompt filter replacement as specified in the operational manual, helps to ensure the ultrapure water remains contaminate free.

Thermo SCIENTIFIC

Systems also incorporating an ultraviolet (UV) light with an ultrafilter create a powerful component to further purify the water. A dual wavelength UV light uses its 185 nm wavelength to reduce total organic carbon (TOC) levels to 1 - 5 ppb, and its 254 nm wavelength to maintain an aseptic environment as the water is circulated throughout the system.[3] A Smart2Pure water system with UV and UF is designed to effectively deliver high quality, ultrapure water on demand with ultralow TOCs, and free of bacteria, nuclease and endotoxin contaminants. This allows for efficient work flow, and productive use of resources and space.

A Barnstead Smart2Pure UV/UF water system was challenged with RNase A and DNase I, nucleases commonly used to qualify ultrapure water systems for nuclease reduction. The Smart2Pure UV/UF system was also challenged with *E. coli* O55:B5 endotoxin. This system was chosen from the family of Barnstead Smart2Pure systems, which also includes the Smart2Pure 3 UV/UF and Smart2Pure 6 UV/UF models. All of these systems have the same feed water requirements, basic water flow pattern, ultrafilter filtration, UV lamp and dispense water through a 0.2 µM final filter. The Smart2Pure UV/UF system was connected to a storage reservoir with a high concentration solution containing RNase, DNase, and endotoxins to help determine if the increased bio-load would impact the system's ability to reduce these impurities.

Methods

Nuclease and endotoxin performance testing in Smart2Pure UV/UF system

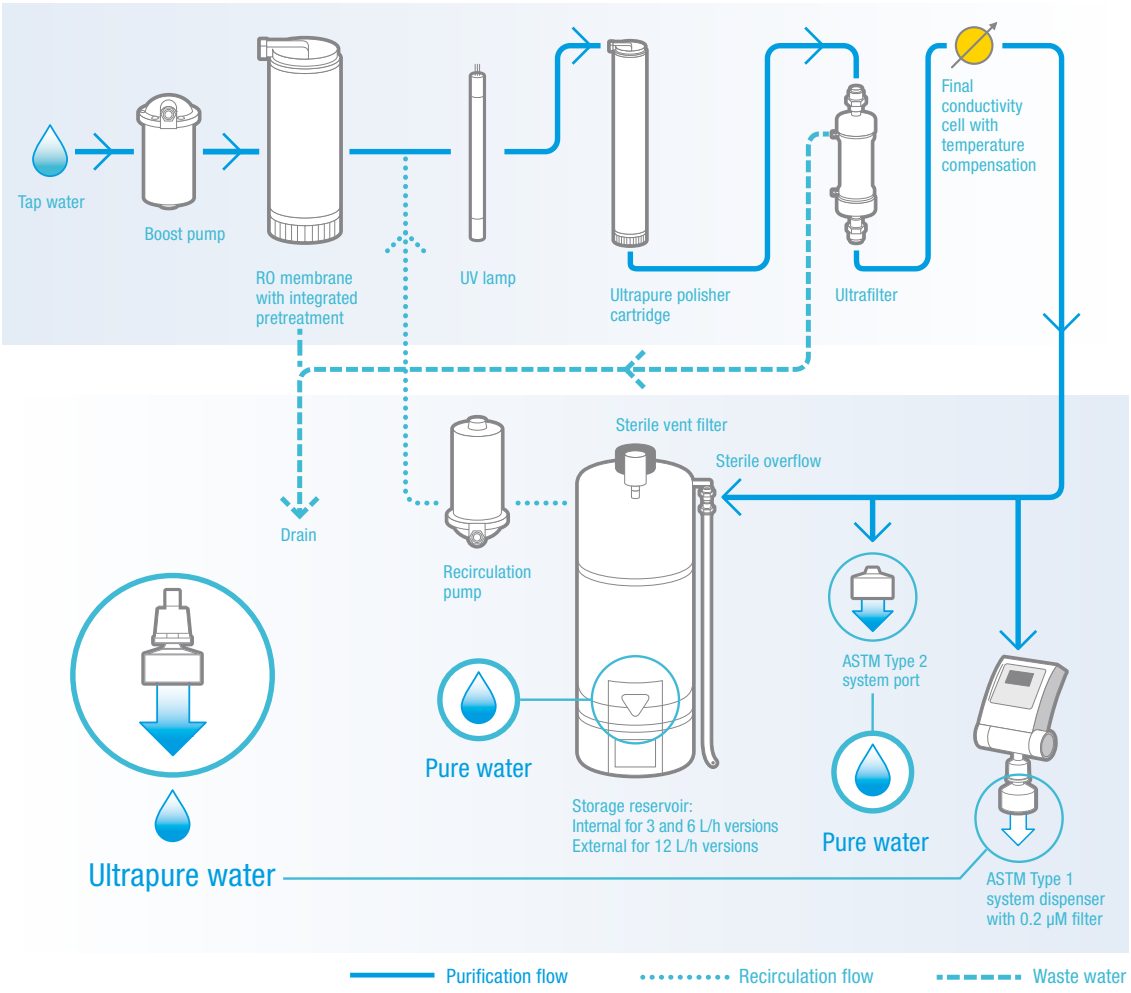
The Smart2Pure 12 UV/UF system is an ultrapure water system, which requires tap water as feed water. An external reservoir is required for the system to operate and contains the Type 2 water that is generated from the reverse osmosis (RO) membrane and deionization (DI) cartridge. For the experimental set up described below, a 60 L Thermo Scientific storage reservoir was utilized to introduce known challenge solutions into the Smart2Pure UV/UF system, and was set up per its operation manual.[4] The system was set up as demonstrated in Figure 1.

Clean techniques were used throughout to reduce the chance of nuclease or endotoxin contamination. Three samples were routinely collected so that one sample was sent for nuclease analysis, one for endotoxin analysis, and one was archived to protect against shipping errors. The samples were stored at -20°C until they were analyzed.

Negative Controls:

A 60 L reservoir was filled with 15 L Type 2 water generated from the Smart2Pure 12 UV/UF system and 10 L was rinsed through the Smart2Pure UV/UF system without the 0.2 µM final filter, followed by 1 L rinse with the 0.2 µM final filter. After a 0.2 L rinse from the 60 L reservoir spigot, three 10 ml samples were taken directly from the reservoir to determine the nuclease and endotoxin

Figure 1. Flow diagram of the Smart2Pure 12 UV/UF system with an external storage reservoir.



levels of the water feeding the Smart2Pure UV/UF system (see "Smart2Pure Reservoir Water" in Table 1). After 0.2 L of water was dispensed from the Smart2Pure system, three 10 ml samples were collected to establish a non-challenged baseline ("Pre-Challenge Water" samples listed in Table 1).

Nuclease Challenge Protocol

A challenge solution with 1 µg/mL RNase and 100 U/L DNase was prepared by adding 500 µL of 10 mg/mL RNase A stock solution and 110 µL of 4.5 U/µL DNase to 5 L of UltraPure™ DNase/RNase-Free distilled bottled water from Life Technologies. The 60 L storage reservoir was drained of remaining water, and the challenge solution was introduced to the reservoir that was connected to the Smart2Pure UV/UF system. Water was dispensed continuously from the Smart2Pure UV/UF system dispenser and three 10 mL samples were taken at specific volume intervals: 2.5 L, 5 L, 10 L, 20 L, 30 L, 40 L, and 50 L. Type 2 water was used to replenish water in the 60 L challenge reservoir to complete the sampling. After collecting all samples, the Smart2Pure UV/UF system was sanitized and consumables changed per the system's operational manual[5] and the entire procedure

	RNase Concentration (ng/mL)		DNase Concentration (pg/µL)	Endotoxin Concentration (EU/mL)	
Smart2Pure Reservoir Water	Run 1: > 0.003	Run 2: < 0.003	<0.002	Run 1: 0.0243	Run 2: 3.98
Pre-challenge water	<0.003		<0.002	<0.001	
2.5 L post challenge	<0.003		<0.002	<0.001	
5 L post challenge	<0.003		<0.002	<0.001	
10 L post challenge	<0.003		<0.002	<0.001	
20 L post challenge	<0.003		<0.002	<0.001	
30 L post challenge	<0.003		<0.002	<0.001	
40 L post challenge	<0.003		<0.002	<0.001	
50 L post challenge	<0.003		<0.002	<0.001	

Table 1. RNase, DNase, and endotoxin detection in ultrapure water produced by a Barnstead™ Smart2Pure™ UV/UF water purification system. Data for both runs were identical unless noted otherwise.

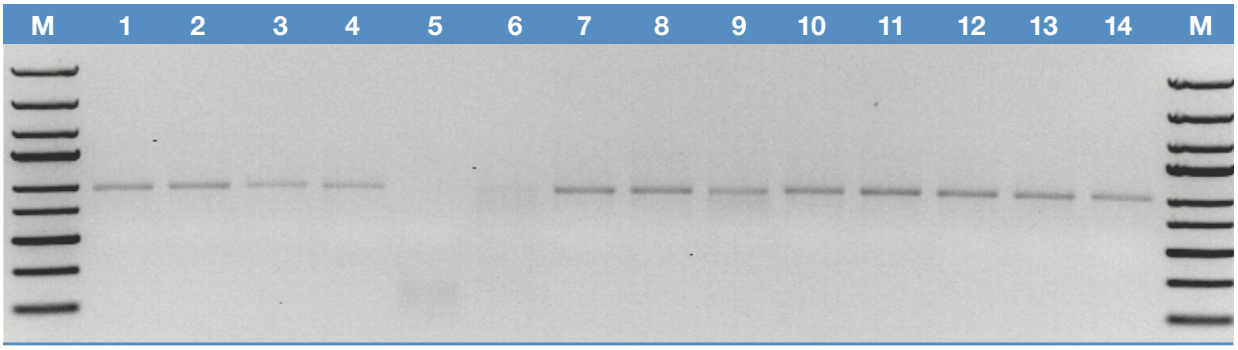


Figure 2: Ribonucleases Assay: Electrophoresis gel with 1% agarose buffer and ethidium bromide stain. M: GeneRuler™ Express DNA Ladder; Lanes 1,2: Negative control; Lanes 3, 4: 30 ng/15 µL RNA transcript control; Lanes 5, 6: Smart2Pure 60 L reservoir water; Lanes 7, 8: Pre-challenge water; Lanes 9, 10: 2.5 L post challenge; Lanes 11, 12: 5 L post challenge; Lanes 13, 14: 10 L post challenge. Sample is considered have RNase activity if degradation of RNA is observed.

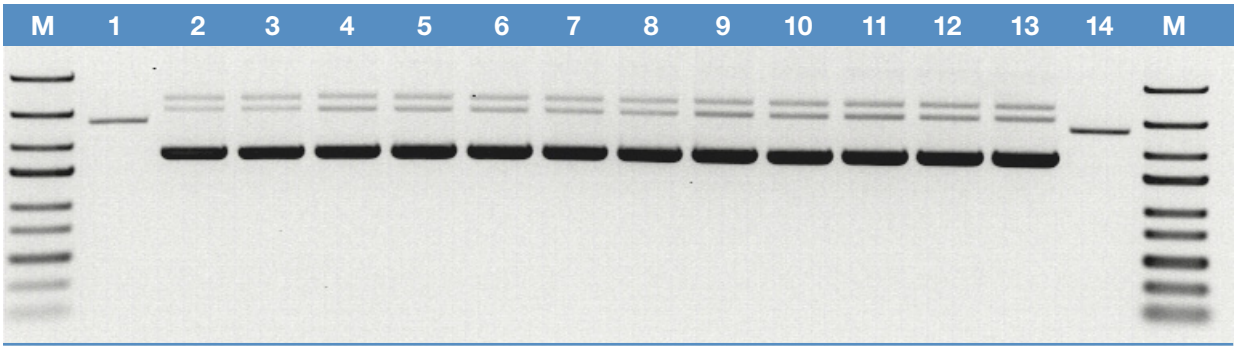


Figure 3: Double-stranded Endodeoxyribonucleases Assay: Electrophoresis gel with 1% agarose buffer and ethidium bromide stain. M: GeneRuler Express DNA Ladder; Lanes 1, 14: 40 ng/20 µL linear form control; Lanes 2, 3: Negative control (not incubated); Lanes 4, 5 Negative control, (incubated); Lanes 6, 7: Smart2Pure Reservoir water; Lanes 8, 9: Pre-challenge water; Lanes 10, 11: 2.5 L post challenge; Lanes 12, 13: 5 L post challenge. Sample is considered to have DNase activity if conversion of supercoiled plasmid DNA to nicked or linearized is observed.

was repeated to create the run 2 data set. Samples were shipped to Thermo Fisher Scientific Baltics UAB, Lithuania for analysis.

The RNase analysis was performed by incubation of 80 ng of 2 kb RNA transcript for 4 hours at 37°C with 8.2 µL of the water sample in RNase assay buffer with Mg^{2+}, in a 20 µL total reaction mixture. After incubation, the integrity of RNA was analyzed on a 1% agarose gel and stained with ethidium bromide. Samples were considered to have RNase activity if degradation of RNA was observed. RNase contamination is not detectable with a detection limit of 1×10^{-7} Unit per reaction (0.003 ng/mL). Figure 2 shows a gel with some of the samples, and the data for all the RNase challenge samples are in Table 1.

DNase testing was conducted by incubation of 1.2 µg of supercoiled pUC19 DNA/SmaI with 15.6 µL of the water sample in DNase assay buffer with Mg^{2+} for 17 hours at 37°C, in a 24 µL total reaction mixture. After incubation, the DNA was analyzed on 1% agarose gel and stained with ethidium bromide. Samples were considered to have DNase activity if conversion of supercoiled plasmid DNA to nicked or linearized was observed. DNase is not detectable with a detection limit of 1×10^{-6} Unit per reaction (0.002 pg/µL). Figure 3 shows a gel with some of the samples, and the data for all of the DNase challenge samples are in Table 1.

Endotoxin Challenge of the Smart2Pure UV/UF system

A challenge solution was prepared adding 5 vials of 1,250,000 EU/vial *E. coli* O55:B5 Endotoxin to 5 L of UltraPure™ DNase/RNase-Free distilled bottled water. The challenge solution was introduced to the 60 L reservoir that was connected to the Smart2Pure UV/UF system. Water was dispensed and sampled as above in the RNase/DNase challenge. The endotoxin analysis was conducted by Nelson Laboratory, Salt Lake City, UT. Samples were analyzed using the Bacterial Endotoxins Test: Kinetic Chromogenic Method or Limulus Amebocyte Lysate (LAL) test to detect and quantify bacterial endotoxin. Endotoxin was not detectable with a detection limit of 0.001 EU/mL. The data for the endotoxin challenge is in Table 1.

Results

Table 1 lists the results from runs 1 and 2 for the RNase, DNase and endotoxin analysis and is a summary of the data partially shown in Figures 2 and 3. For results that were identical in both runs, only one result is reported. DNase levels were below detection limits in the Smart2Pure 60 L storage reservoir water, but for run 1 there were detectable amounts of RNase found, as listed in the table as “Smart2Pure Reservoir Water”. The pre-challenge water samples in the table refer to the samples collected from the Smart2Pure UV/UF system dispenser before the challenge solution was introduced. Here again, the levels were below the detection limit. After the challenge solution was introduced into the 60 L reservoir for the Smart2Pure UV/UF system feed water, samples were taken at timed intervals. All post-challenge samples were determined to be below the RNase/DNase detection limits.

The endotoxin analysis, on the other hand, indicated endotoxins were already present in the 60 L reservoir feed water to the Smart2Pure system even before the system was challenged. The pre-challenge water sample taken directly from the Smart2Pure system was below the level of detection, so any endotoxins naturally present in the feed water was reduced by the system. All endotoxin levels in samples taken at specific volume intervals were found to be below the level of detection.

Conclusion

In-line ultrafiltration combined with UV oxidation provided an easy and efficient method of reducing nucleases and endotoxins from water below detectable limits, even when the Barnstead Smart2Pure UV/UF water purification system was challenged with 5mg RNase A, 500 U DNase I, and 6,250,000 EU *E.coli* O55:B5 endotoxin.

References

1. The Basics: RNase Control. 2011. Retrieved 25 September, 2014 from http://www.lifetechnologies.com/us/en/home/references/ambion-tech-support/nuclease-enzymes/general-articles/the-basics-rnase-control.html

2. Diethyl Pyrocarbonate Product Information. 1999. Sigma Aldrich. Retrieved 25 September, 2014 from http://www.sigmaaldrich.com/catalog/search?interface=All&term=depc&N=0&mode=match+partialmax&focus=documents&lang=en&region=US

3. Thermo Scientific Waterbook. 2014. Thermo Fisher Scientific. Retrieved 12 June, 2014 from http://www.thermoscientific.com/purewater

4. Operational Instructions for the Thermo Scientific Barnstead Smart2Pure water purification system. Thermo Fisher Scientific, Niederelbert, Germany.