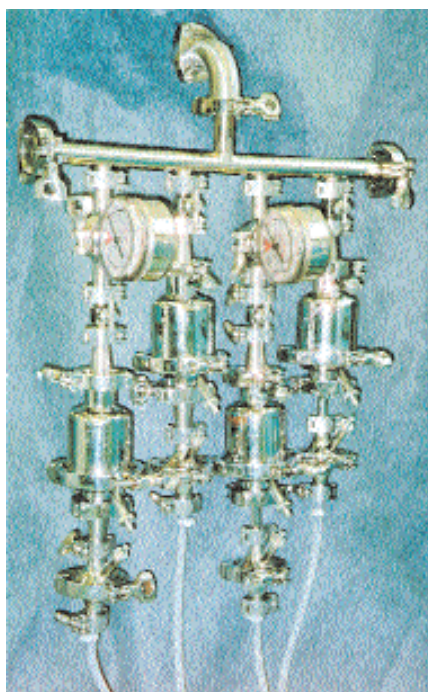


# Validation of Sterile Filtration of Liquid Nitrogen

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Liquid nitrogen (LN<sub>2</sub>) is a widely used freezing and lyophilization agent, but it is also a potential source of contamination. LN<sub>2</sub> can be sterile

filtered, with some difficulty, and FDA has increasingly required validation of that thermally challenging process. This article presents a method of validating the sterile filtration of LN<sub>2</sub>.

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Liquid nitrogen (LN<sub>2</sub>) is widely used in the pharmaceutical, biopharmaceutical, and life sciences industries for lyophilization and quick-freezing of pharmaceutical preparations and storage of cells and microbial cultures. As a refrigerant, LN<sub>2</sub> can act as a vehicle for transmitting contaminant microorganisms. Whether as the original source of contamination or as a conduit, LN<sub>2</sub> has been reported as a potential biohazard (1). Fungal and bacterial contaminants have been found in both the freezers that use LN<sub>2</sub> and the cultures stored in them (2). An outbreak of hepatitis B in patients undergoing cytotoxic treatment has been traced to LN<sub>2</sub>, suggesting that contaminants can move both in and out of cryostorage containers (3). In other instances, storage tanks that use LN<sub>2</sub> were reported to be contaminated by *Bacillus* (4), and storage tanks holding cryopreserved stem cells also were found to be contaminated (5).

Compendial guidelines for aseptic processing offer little direction about how to produce sterile LN<sub>2</sub> and provide only general requirements that a sterilization process be established, followed, and validated (6). To comply with current good manufacturing practices, corporate quality guidelines are based on the General Charter to produce sterile LN<sub>2</sub>.

FDA auditors and corporate quality assurance officials have increasingly indicated that the cleanliness level of processes used in aseptic manufacturing should be consistent with the sensitivity of the product involved so that exposure of product to process fluids does not jeopardize finished-product quality or integrity (7). Some FDA auditors have interpreted this to mean that everything in the vicinity of open product containers, such as those used in lyophilization, or in contact with sealed product containers or cryovials used for quick-freezing pharmaceuticals must be sterile.

Validation of sterile filtration of LN<sub>2</sub> presents yet another dimension of unfamiliarity and uncertainty to many pharmaceutical manufacturers and regulatory officials, and it is an especially difficult task. The sterilizing-grade filter is subject to autoclave sterilization at temperatures >121 °C, followed by sterile filtration of LN<sub>2</sub> at -196 °C—a net temperature differential of at least 317 °C. Bacterial challenge testing follows sterile filtration to ensure that the filter has remained intact and has maintained its bacteria retention capability.

**Table I: Process parameters are met or exceeded in one or more of the challenge phases.**

Process Parameter	Model Process	Phase I LN <sub>2</sub> Phase (performed 3×)	Phase II Conditioning Phase	Phase III Bacterial Challenge Phase
Flow rate	6 L/min	Unspecified	8–14 L/min	1.0 L/min
Pressure	3–5 psi	Unspecified	12–17 psi	19.5–22.5 psig
Sterilization	121 °C, 60 min, SIP	134–146 °C 60 min, SIP (×3)	123 °C, 60 min, autoclave	N/A
Temperature	–196 to +121 °C	–196 to +146 °C	22–123 °C	22 °C
Fluid	LN <sub>2</sub>	LN <sub>2</sub>	Deionized water	Deionized water
Batch size	256 L	5 L (×3)	450–792 L	60 L
Duration	45 min	Unspecified (×3)	60 min	60 min

Although the production of sterile process fluids by sterile filtration using validated submicron membrane filters is not usually problematic, LN<sub>2</sub> presents a special condition. The thermal stresses to which filters are subjected during sterile filtration of LN<sub>2</sub> are far more extreme than with any other gas or liquid. These stresses can destroy the integrity of the membrane filters used to sterilize LN<sub>2</sub>, thereby breaching sterility. This threat is greatly significant to the filtrative sterilization of LN<sub>2</sub> and its validation.

In the study discussed in this article, a method for validating the sterile-filtration process of LN<sub>2</sub> was developed. Sterile filtration was performed by using PTFE cartridge filters (Ultra-dyne TT0.2, Meissner Filtration Products, Camarillo, CA) and by maintaining the nitrogen as a liquid throughout the process. A critical step in complying with CGMPs and satisfying FDA auditors about the sterility of sterile-filtered LN<sub>2</sub> lies in the validation report for this process. In this article, the authors describe the method used to validate the sterile filtration of LN<sub>2</sub> and convey the essence of the process's validation report.

The validation test data are based on the removal of *Brevundimonas diminuta* (ATCC 19146) from a surrogate fluid using 0.2-µm PTFE filters that were conditioned with LN<sub>2</sub>. Meissner Technical Services (MTS), a unit of Meissner Filtration Products, Inc., performed this study. The sterile filtration was performed off site. The test protocol is an adaptation of ASTM F838-83, "Standard Test Method for Determining Bacterial Retention of Membrane Filters Utilized for Liquid Filtration," and follows the guidelines of PDA Technical Report No. 26, "Sterilizing Filtration of Liquids" (8,9).

### Sterile filtration

Three lots of PTFE minicartridges (LTT0.2-2PS, Meissner) were used. The filters had an effective filtration area of 1 ft<sup>2</sup>. Testing involved the following three phases:

**LN<sub>2</sub> phase (Phase I).** Phase I consisted of three cycles of steaming, cooling, and filtering 5 L of LN<sub>2</sub>. During this phase, the filters were exposed to temperature extremes from 134–146 °C for 60 min during the steaming to –196 °C during the LN<sub>2</sub> filtration. The filters were integrity tested using the bubble-point test before and after the conditioning phase in 99% isopropyl alcohol (IPA) using an automated integrity tester. The filters

were then shipped to MTS for Phases II and III.

**Conditioning phase (Phase II).** The second phase involved pumping deionized water through the test and control filters at a rate of 8–14 L/min for 60 min at 12–17 psi to simulate maximum flow rates and the duration of process liquid filtration.

**Bacterial challenge phase (Phase III).** In the third phase, polyethersulfone (PES) analysis disks (STyLUX SM0.2-47, Meissner) were

introduced immediately downstream of the test filters. The filters were challenged with *B. diminuta* in deionized water at 19.5–22.5 psig for 60 min with flow rates of 1.0 L/min. Each filter was then flushed with 1000 mL of deionized water before the analysis filters were plated on tryptic soy agar (TSA) and incubated at 30 °C for one week. Test filters were integrity tested in 60% IPA before and after the challenge. The small size of the *B. diminuta* was confirmed by its passage through a PES 0.45-µm filter (STyLUX LSM0.4-2PS, Meissner) as a positive control. The minicartridges had >11.2/filter log reduction values (LRVs) and had bubble-point integrity test values of 18.5–22.0 psi in 60% IPA, thereby demonstrating their integrity.

### Rationale for challenge testing

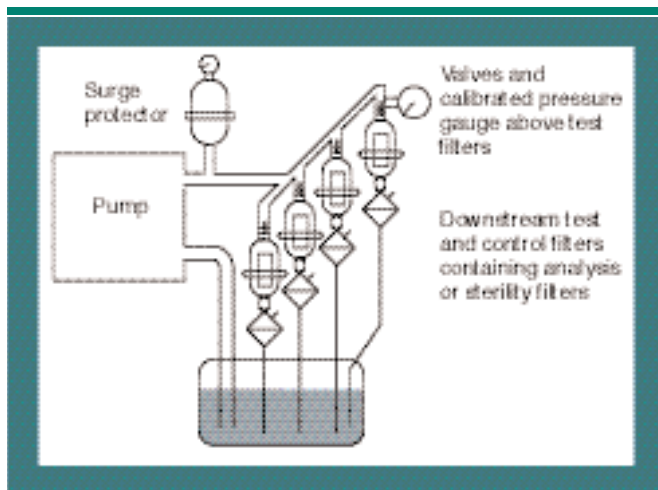
This challenge was designed to simulate filtration of 68 gal. of LN<sub>2</sub> at 1.5 gal./min with a minicartridge for >45 min. A critical parameter was the extreme temperature differential (a total of 342 °C) that the filter cartridges would experience during the steaming in place (SIP) and filtering of the LN<sub>2</sub>. The cartridges were alternately steamed and used three times for small batches of LN<sub>2</sub> filtration.

Although LN<sub>2</sub> has been used for long-term storage of *B. diminuta*, it does not readily lend itself as a challenge fluid because it can freeze the filtration system hoses or boil away. The challenge portion of the validation took place in deionized water, a readily available substitute fluid in which *B. diminuta* survives well.

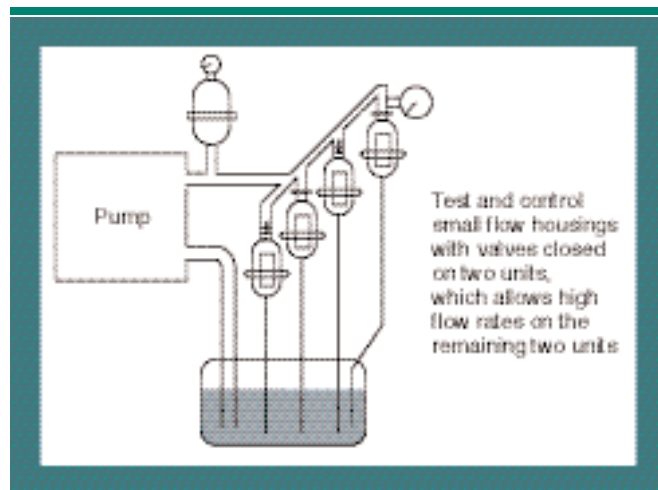
**Critical parameters.** This protocol includes high temperature extremes, high pressures, high flow rates, and high bacterial challenge titers. Table I identifies the critical filtration parameters and the phase of the challenge in which that parameter is met or exceeded.

### Inhibition and viability testing

LN<sub>2</sub> is very cold (–196 °C) and is composed of 80–95% LN<sub>2</sub> and 5–20% nitrogen gas. These properties make it difficult to handle in a laboratory setting, so it was decided that the filters would be challenged in water with *B. diminuta* grown in saline lactose broth (SLB) after the LN<sub>2</sub> conditioning. Challenge titers taken during the course of the challenge ensured the viability of the organism throughout the test.



**Figure 1:** Sterility and challenge orientation.



**Figure 2:** High-flow orientation.

Because the challenge solution, water, is not inhibitory, an inhibition study was not indicated. The system should be flushed with 1.0 L/filter of deionized water to ensure that it is rinsed and that any organisms that manage to pass through the filters have an opportunity to impact the analysis filters. This procedure provides a >10-times flush volume for the space between the test and analysis filter. The initial 4-L volume (1 L × 4 filters) is sufficient to flush the reservoir and pump tubing.

### Method for validation

**Conditioning: SIP and LN<sub>2</sub>.** Three lots of PTFE filters (Ultradyn lots 3927D, 5028C, and 6900A, Meissner) were conditioned, which involved several steps. The first step was to wet the filters with 99% IPA and perform the bubble-point test using an automated integrity tester. The excess IPA was removed by flowing nitrogen gas at 20 psig through the filters for ~10 min. Next, the filters were steamed in place for 60 min in the forward direction between 138 and 145 °C, and then dried overnight with ~5 psig nitrogen gas. The filters were used for 5 L of LN<sub>2</sub> and allowed to warm. The SIP step was repeated with temperatures from 142 to 145 °C for 60 min, then the filters dried overnight. Each filter processed 5 L of LN<sub>2</sub>, and then were allowed to return to room temperature. The filters were steamed a third time for 60 min at 134–144.5 °C and dried overnight. A 5-L batch of LN<sub>2</sub> was passed through each filter a third time, and the filters were allowed to warm and dry before being wetted with 99% IPA. Then the filters were integrity tested again by the same bubble-point procedure. All filters were integral before and after conditioning. The conditioned filters were returned to MTS where they were integrity tested using a manual bubble-point test in 60% IPA. All the filters passed the test.

**Equipment preparation.** Three stainless steel in-line small-flow housings (HS2D-2P-0SH1S, Meissner) loaded with the pre-conditioned test filters were autoclaved for 60 min at 123 °C. A hydrophilic PES 0.4-μm membrane filter (STyLUX LSM0.4-2PS, Meissner) was bubble-point tested for the positive control and autoclaved together with eight 47-mm stainless steel filter holders loaded with 0.2-μm PES sterility and analysis filters (SM0.2-047, Meissner). Assorted hoses, four valves, two auto-

clavable gauges, a manifold, and a pressure reservoir were autoclaved at the same time. A large pump (model 925 VIRG, Watson-Marlow Bredel Pumps, Wilmington, MA) was sanitized by recirculating 60% IPA for 60 min. These components were allowed to cool to ambient temperature before assembly in a laminar-flow test area.

**Challenge-inoculum preparation.** *B. diminuta* was inoculated into 5 mL of tryptic soy broth (TSB) for 2–3 h at 37 °C until turbid and then vortexed and transferred into 500 mL of SLB for use in the challenge. The SLB was incubated at 30 °C overnight with stirring. This process resulted in a turbid stock solution of ~10<sup>9</sup> cfu/mL of stressed *B. diminuta* suitable for use in challenges.

**Calculation of the challenge titer.** The stock culture was titered concurrently with the challenge. To ensure that a suitable turbidity level had been reached, an aliquot was measured spectrophotometrically at 625 nm and compared with a 0.5 McFarland standard.

*B. diminuta* stock solution in 1-mL aliquots was diluted 10-fold in 0.1% peptone water to 10<sup>-6</sup>. Then, dilutions from 10<sup>-4</sup> to 10<sup>-6</sup> were plated in triplicate on TSA using sterile 0.01-mL loops. These plates were incubated for two days at 30 ± 2 °C and then read. The number of plates with 30–300 colonies was counted, multiplied by the dilution factors, and averaged to obtain the stock titer, which is expressed in colony forming units per milliliter (cfu/mL). The number of cfu added to the challenge solution was calculated by multiplying the stock titer by the number of milliliters used.

Similarly, aliquots of the working challenge solution that were taken at the beginning and end of the challenge were titered to ensure the nontoxicity of the challenge fluid to the *B. diminuta*. One milliliter of the 10<sup>-6</sup> dilution was vacuum filtered through a gridded 0.45-μm cellulose nitrate disk (Nalge Nunc International Inc., Rochester, NY) and plated onto TSA. These beginning and ending counts, multiplied by the dilution factor, are averaged to give the challenge concentration expressed in cfu/mL. The challenge concentration constantly changes as bacteria are removed by the filters and added to the reservoir.

The challenge cfu total is the number of bacteria added to



**Table II: Pre- and postbacterial challenge bubble points (psi) in 60% IPA from Table III.**

TT0.2 Lot #	3927D	5028C	6900A	Average
Presterilization (after three SIP and LN <sub>2</sub> cycles)	19.0	19.5	21.0	19.8
Prechallenge	18.5	18.5	20.5	19.2
Postchallenge	19.5	20.0	22.0	20.5

the challenge solution. The challenge level, expressed as cfu/cm<sup>2</sup>, is the number of bacteria that land on each square centimeter of each test or control filter. The challenge per filter is calculated by the total challenge cfu divided by the number of test filters. The challenge per filter is divided by 1115, which is the effective filtration area (EFA). The result is the challenge per cm<sup>2</sup> or cfu/cm<sup>2</sup> as shown by

$$\text{challenge per cm}^2 = \frac{\text{total cfu added}}{\text{number of filter tested}} \div 1115 \text{ cm}^2 \text{ EFA}$$

**Sterility check.** Sterility of the filtration lines was verified by running 1000 mL of sterile water through each test filter assembly (see Figure 1). This water also served as the flush to remove the 60% IPA used in the integrity test. The test-filter outlets were directly connected to 47-mm holders loaded with 0.2- $\mu$ m PES sterility filters. These sterility test filter holders were removed and taken to the biosafety hood where the membranes were removed, plated on TSA, and incubated at 30  $\pm$  2  $^{\circ}$ C for two days. All sterility test filters were sterile. This step represents the negative control of the test.

**Bacterial challenge testing.** Three lots of 1-ft<sup>2</sup> PTFE filter cartridges (TT0.2, Meissner) were tested along with a 1-ft<sup>2</sup> PES positive control cartridge (SM0.4, Meissner). The filters were conditioned two at a time by pumping deionized water through them at high flow rates for 1 h each (see Figure 2).

After completing the high flow rate phase of the challenge, analysis filters were added downstream of each test (3) and control filter (1). The pump speed was adjusted to 1 L/min to accommodate the small surface area of the analysis filters. Stock *B. diminuta* in 125-mL aliquots was added to 4 L of deionized water in the reservoir at 15-min intervals for >60 min until 500 mL of stock had been added. This was followed by a 1.0 L/filter deionized-water flush. All the challenge solution and the flush were then passed through the in-line analysis filters directly downstream of the test filters.

**Filtrate analysis.** The filtrates from the test and control filters were 100% analyzed in-line through the 0.2 PES analysis disks. The deionized-water flush ensured that all bacteria could be flushed through the system onto the analysis disks. After the flush, the analysis filters were removed from the assembly, the holders were opened in the biosafety hood, and the membranes were plated onto TSA and incubated at 30  $\pm$  2  $^{\circ}$ C for seven days. All negatives were checked microscopically at 7 $\times$  magnification after seven days.

Any growth found on the analysis filters was examined for colonial morphology, and Gram stain, oxidase, catalase, and Kligler's iron agar tests were performed to confirm the identity

of *B. diminuta*. Only the positive control analysis filter showed *B. diminuta* growth.

**Pressure and flow rates.** The model for the LN<sub>2</sub> filtration process uses a 6 L/min flow rate with an LTT0.2-2PS 1-ft<sup>2</sup> cartridge at 3–5 psi. The validation study was performed with full-size filter units at higher pressures than that which the actual filtration process is expected to reach. Pressures were 12–17 psi during 1 h with deionized-water flow rates of 8–14 L/min. During the 60-min bacterial challenge phase, filter differential pressures increased to 21–22.5 psig and rose to 30 psig during the flush. Flow rates were determined by measuring the volume of deionized water filtered during the various phases of the challenge. Flow during the bacterial challenge was 1 L/min—a reduced flow rate as a result of the flow restriction caused by the downstream analysis disk filters, which have only 1% of the surface area of the cartridge test filters.

**Bubble-point tests.** Filter integrity was confirmed by performing bubble-point tests at all critical test stages. An automated integrity test instrument was used to determine the bubble points with 99% IPA before and after conditioning the filters with three SIP and liquid-nitrogen cycles. MTS used 60% IPA for the manual bubble-point tests before and after sterilization and after the challenge because it is easy to use with PTFE filters. The hydrophilic 0.4- $\mu$ m PES control filter was bubble-point tested in deionized water before sterilization and after the challenge. It was integrity tested in 60% IPA after sterilization so that its test conditions were identical to those of the test on PTFE filters.

The bubble point is defined as the pressure at which bulk flow of test gas through the largest pores of a wetted membrane filter occurs. Product bubble points were not determined. The manufacturer's minimum validated bubble point for TT0.2 PTFE filters is 16 psi in 60% IPA.

Table II shows that the pre- and postchallenge bubble points of the tested filters are very similar; the postchallenge values are slightly higher as a result of the plugging of the membrane by the challenge bacteria.

## Results

Results are reported as the LRV per filter. The equation used is

$$\text{LRV per filter} = \log_{10} \frac{\text{total cfu per filter in challenge}}{\text{total cfu in filtrate}}$$

When the filtrate is sterile, a value of 1 is substituted in the denominator, and the results are expressed as greater than (>) that result.

Table III lists the pre- and postchallenge bubble points for three lots of tested filters as well as the *B. diminuta* challenge level and the LRV for each filter. Growth of *B. diminuta* after passing through the 0.4- $\mu$ m PES membrane confirmed that the organism was uninhibited, not aggregated, and a sufficiently small size to provide a valid challenge test (see Table III).

In addition, a fourth, similar, lower-bubble-point 0.2- $\mu$ m PTFE filter cartridge that had been subjected to three SIP and liquid-nitrogen cycles was challenged with *B. diminuta*, accord-

**Table III: *B. diminuta* removal with a PTFE test filter. All bubble points (BPs) are in 60% IPA except for the control presterilization and post-test, which are deionized-water values.**

TT0.2 Lot #	Presterilization BP (psi)	Pretest BP (psi)	Post-test BP (psi)	Challenge Level cfu/cm <sup>2</sup>	cfu in Filtrate	LRV per Filter
3927D	19.0	18.5	19.5	1.4 × 10 <sup>8</sup>	0	>11.20
5028C	19.5	18.5	20.0	1.4 × 10 <sup>8</sup>	0	>11.20
6900A	21.0	20.5	22.0	1.4 × 10 <sup>8</sup>	0	>11.20
<b>Control SMO.4</b>						
6319Q	35.0	14.0	40.0	1.1 × 10 <sup>8</sup>	305	8.30

**Table IV: *B. diminuta* removal with PTFE 0.2- $\mu$ m cartridge. PTFE bubble points (BPs) are in 60% IPA, and PES control bubble points are in deionized water.**

PTFE 0.2 Lot #	Presterilization BP (psi)	Pretest BP (psi)	Post-test BP (psi)	Challenge Level cfu/cm <sup>2</sup>	cfu in Filtrate	LRV per Filter
5949B	16.0	16.0	16.0	1.2 × 10 <sup>8</sup>	0	>11.11
<b>Control PES 0.4</b>						
5138T	46.0	46.0	47.0	1.5 × 10 <sup>8</sup>	472	6.51

ing to ASTM Standard F838-83. This filter, along with a positive control membrane, was challenged at 30 psi for 39 s. It was steamed a total of 187 min between 130–140 °C (see Table IV).

## Conclusion

In this study, sterile filtration of LN<sub>2</sub> by using PTFE cartridge filters was performed, and the process was validated by MTS. *B. diminuta* removal was evaluated by using three lots of 0.2- $\mu$ m PTFE minicartridges after they had been conditioned with LN<sub>2</sub> under extreme temperature conditions. Filters were steamed at  $\leq$ 146 °C for 1 h and frozen at –196 °C while filtering LN<sub>2</sub> three times before being challenged with *B. diminuta* suspended in deionized water at concentrations of 10<sup>8</sup> cfu/cm<sup>2</sup> for 1 h.

An LRV of >11.2, indicating sterility, was obtained for filters with 1-ft<sup>2</sup> effective filtration area and pretest bubble points of 18.5–20.5 psi in 60% IPA. An additional low-bubble point 0.2- $\mu$ m PTFE minicartridge filter that had been similarly steamed and frozen produced a sterile filtrate after an ASTM Standard F838-83 challenge with a pretest bubble point of 16 psi in 60% IPA.

These results validated the efficacy of sterile filtration of LN<sub>2</sub> under the conditions previously described. Validation of the process is readily achievable by pharmaceutical and biopharmaceutical companies using the methods described in this article.

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## FYI

### ISPE Annual Meeting

The International Society for Pharmaceutical Engineering (ISPE) will hold its annual meeting 3–7 November 2002 in Lake Buena Vista, Florida.

The event program comprises classroom seminars, interactive workshops, discussion forums, tabletop exhibits, a student poster competition, and a plenary session. Attendees also may participate in local recreation such as a golf tournament and tour of the Kennedy Space Center.

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