BSR/PDA Standard 05-201x, Consensus Method for Rating 0.1 Mycoplasma Reduction Filters

Draft stage

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Introduction

Mycoplasmas (trivial name for organisms of the class Mollicutes) are well-known microbial contaminants found in biologic processes, particularly cell culture processes. Historical surveys of cell lines have found high rates of mycoplasma contamination in research labs and production facilities [1, 2]. With their ability to establish occult contaminations, mycoplasmas can evade conventional bioburden assays, and even lead to changes in metabolism and phenotype of the cell culture, potentially impacting resultant product quality.

The absence of a rigid peptidoglycan-based bacterial cell wall enables mycoplasmas to pass through sterilizing-grade (0.2 μm) and mycoplasma reduction-grade (0.1 μm) filters, potentially contaminating an entire production process. Filters are rated based on performance and not on an absolute measure of pore size. Because of these invasive capabilities, mycoplasma contamination has garnered special attention by regulatory agencies, resulting in expectations for testing and risk-mitigation [3].

USP <1043> provides categories that are useful for assessing material risks associated with mycoplasma contamination in raw materials [4]. Examples of contamination risks include:

- Process materials, which can provide a suitable environment for mycoplasma to remain present at high levels for at least 6 months [5] ;
- Biological process fluids (typically containing either plant or animal-derived components), prepared with 0.2 μm filtration without a heat inactivation step [6, 7].

The risk of contamination not only depends on the media, but also on where the material is used in the process and whether the process contains subsequent purification (i.e., inactivation or removal) steps. Therefore, pretreatment of raw materials (e.g., heat treatment or irradiation) should be considered, where appropriate.

In a biologics process, 0.1 μm filtration is often used in drug substance manufacturing as a mycoplasma contamination prevention measure. This barrier approach, with risk reduction as the goal, is prevalent in the mammalian cell culture industry. The concept is similar to bioburden reduction filtration used in protein purification processes. For upstream barrier applications, such as cell culture media filtration, process-specific mycoplasma reduction validation is generally not a regulatory expectation. However, based on a risk assessment, an end user may evaluate a process-specific reduction of mycoplasma using the mycoplasma consensus method as described in this standard and an article previously published in the *PDA Journal of Science and Technology* [8].

For manufacturers of raw materials (e.g., serum) that wish to make an *Acholeplasma laidlawii* (*A. laidlawii*) reduction claim based on filtration, validation of the mycoplasma removal filtration process should be performed following the principles outlined in PDA Technical Report 26 [9]. In addition, manufacturers of raw materials should also consider using the mycoplasma method described in this standard to grow the *A. laidlawii* challenge organism for validation purposes.

BSR/PDA Standard 05-201x, Method for Rating 0.1 Mycoplasma Reduction Filters

1 Scope

This test method establishes a standardized method for filter manufacturer rating of nominal 0.1 micron rated filter membrane for retention of mycoplasma using 47 mm discs and using *A. laidlawii* as the test organism. It is also necessary for the filter manufacturer to validate filter devices that may require testing a wide variety of device sizes and configurations. Validation of the actual filter device is not addressed in this standard test method. Any appropriate end-user validation and/or qualification of 0.1 micron rated filter devices incorporating such membrane is also outside of the scope of this standard.

This test is intended to be used by the filter manufacturer to validate a mycoplasma-retentive filter within a manufacturing process and to qualify a filter for a mycoplasma retentive claim. Validation of a drug manufacturing process employing such filter must be done under applicable process-specific conditions.

2 Normative References

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

PDA Technical Report No. 75 Consensus Method for Rating 0.1µm Mycoplasma Reduction Filters (2016) [10].

The Development of a Microbial Challenge Test with *Acholeplasma laidlawii* To Rate Mycoplasma-Retentive Filters by Filter Manufacturers [8].

3 Terms and Definitions

- *Acholeplasma laidlawii*: *A. laidlawii* is a mycoplasma in class Mollicutes and order Acholeplasmatales.
- Challenge Concentration: The concentration in Colony Forming Units/mL of the test microorganism in the challenge fluid.
- Challenge Fluid: The carrier fluid in which the test microorganism is suspended and delivered to the test filter.
- Challenge Level: The number of test microorganisms applied to the test filter (per square centimeter) at the completion of the challenge.
- Challenge Volume: The volume of challenge fluid applied to the test filter.
- Colony Forming Units (CFU): A single microorganism or an aggregate of many that forms a single discrete colony on solid agar media after suitable incubation. Colony-forming units are used for bacterial titer determination on solid media.
- Culture Medium: The nutritional medium which supports the growth of the given microorganism.
- Filter Rating: A numerical rating of Filter membrane performance based on the ability of the filter to retain an appropriate model microorganism under given test conditions (generally based on ASTM F838) [11].
- Pa: pascal. The International System of Units derived unit of pressure.
- Log Reduction Value (LRV): Titer Reduction (TR) expressed as a base 10 logarithm.
- Mycoplasma Buffer – Made up of sodium phosphate monobasic, sodium phosphate dibasic, and deionized water as described in Table 2 below.
- Mycoplasma Reduction Filter: A filter that provides a log reduction value (or a titer reduction value) for a specified test mycoplasma according to the PDA Mycoplasma Consensus Method. Typically, these filters are also qualified as sterilizing grade filters.
- Positive control filter membrane (Penetration control): A control filter membrane with a larger pore size rating than the test filter and used to demonstrate the penetrative ability of the test microorganism..
- Psid: Pound-force per square-inch differential: the pressure difference between the upstream (influent) and downstream (effluent) sides of a filter.
- Sterilizing Grade Filter: Described in PDA Technical Report 26 and in FDA guidance as a filter that reproducibly removes all *B. diminuta* test microorganisms from the process stream, producing a sterile effluent [9,12-14].
- Titer reduction (TR): A measure of the degree to which a particular filter removes a microorganism under specified test conditions. Calculated as the ratio of the total number of microorganisms used to challenge the filter divided by the total number of microorganisms that passed through the filter:

$$\boldsymbol{TR} = \frac{Upstream\ microbial\ Titer\ \times Volume\ Applied\ \ = \textbf{Total \# Influent Cells}}{Downstream\ microbial\ Titer\ \times Volume\ Filtered\ \ = \textbf{Total \# Effluent Cells}}$$

4 Acronyms and Abbreviations

CFU - Colony Forming Units

kPa – kilopascal

LRV - Log Reduction Value

MTFB Mycoplasma Task Force Broth

TR – Titer reduction

5 Mycoplasma Filtration

Filter manufacturers use a bacterial challenge test to characterize filter membrane performance during product development and manufacturing lot release. Key elements of the bacterial challenge test include the cultivation and preparation of the bacterial suspension, and the appropriate penetration of the positive control by the bacteria.

The bacterial challenge microorganism should provide a final minimum challenge level of 1.0 x 10^7 CFU/cm² of test filter surface area [11]. For mycoplasma reduction filters, *Acholeplasma laidlawii* obtained from a reputable collection, and qualified strain *(*see section **6.1, Materials, Table 1**) hereinafter referred to as *A. laidlawii*, is used as the challenge microorganism. Like microorganisms of the genus Mycoplasma, *A. laidlawii* has no cell wall and is deformable. This characteristic makes *A. laidlawii* capable of penetrating 0.2 µm filters and some 0.1 µm filters at high challenge levels, providing a means of differentiating 0.1 µm filter retention capabilities. In addition, unlike many mycoplasma, *A. laidlawii* is comparatively easy to grow, robust, non-pathogenic, and is capable of being grown to high titers in a relatively short time. Standardized preparation parameters and media ensures consistent performance across laboratories.

5.1 Summary of mycoplasma challenge testing

In mycoplasma challenge testing, the test microorganism is inoculated directly into the challenge fluid and delivered to the test filter. The test microorganism is suspended in the challenge fluid at a concentration that delivers a minimum challenge level of 1.0 x 10^7 CFU/cm^2 of test filter area. A sample of the influent challenge fluid is titered to determine the actual challenge level. The concentration of the test microorganism in the effluent is also determined, and the number of influent and effluent microorganisms are compared to evaluate the filter membrane performance.

A mycoplasma growth broth (MTFB) is specified here to generate the test cells. However, the validated protocol for generating the frozen stock used to inoculate that growth broth is not specified and is the responsibility of the laboratory performing the work. A validated mycoplasma titer protocol is also the responsibility of the laboratory performing the work.

A bacterial titer is defined as the suspended concentration of bacteria (or, in this case, mycoplasma) in solution. It is generally necessary to perform dilutions when titering to ensure countable plates. For a mycoplasma challenge, the challenge fluid is titered as per each laboratory's validated method.

After the challenge has been completed and the full effluent volume collected, the effluent is titered, using dilutions, or it may simply be filter plated in its entirety without any dilution or titer. If little or no penetration is expected, then filter plating of the entire effluent may be appropriate to evaluate retention. If filter plating is expected to result in uncountable plates, then a titer with dilutions would be appropriate.

A microbial retention challenge test produces two possible outcomes through a test article demonstrated to be integral: 1) no penetration of the filter by the test microorganism under the given test conditions, or 2) some degree of penetration under the given test conditions.

5.2 Test validity criteria

It is expected that 0.1 µm filters will retain high levels of *A. laidlawii* during a challenge test. Therefore, a 0.2 µm filter positive penetration control is necessary to verify that the cells are cultivated appropriately and that the challenge test is valid.

Penetration by the *A. laidlawii* challenge through a 0.2 µm rated filter as a positive penetration control confirms the small size, monodispersion (unclumped cells), and the overall penetrative ability of the test mycoplasma. As a result, the primary criterion for test validity is growth downstream of a 0.2 µm rated filter.

In a challenge test, the positive control filter must be tested in parallel with the test filter(s). This serves to confirm the validity of the test at the time it is performed. Lack of mycoplasma penetration of the 0.2 µm positive control filter invalidates the test. The lack of mycoplasma growth downstream of the 0.2 µm positive control filter may be due to a lack of viability or penetrative ability of the test culture, or a below-specification challenge concentration.

The challenge test is considered valid if the *A. laidlawii* challenge level used was at a minimum of ≥1.0 x 10^7 CFU/cm^2, the cells were monodispersed as outlined in Section 6.2.1, the positive control filter demonstrated penetration, and the filter integrity tests (pre- and post-challenge) passed.

5.3 Summary of test parameters

The test parameters are as follows:

- Test microorganism: *Acholeplasma laidlawii*
- Challenge level: ≥1.0 x 10^7 CFU/cm^2
- Test pressure: 30 psid (207 kPa)
- Challenge volume for a 47 mm disc: 200 mL
- Challenge fluid: Phosphate buffer
- Positive control (penetration control): 0.2 µm sterilizing grade filter
- Mycoplasma Task Force Broth (MTFB) for generating test cells

The culture medium and incubation conditions are as follows:

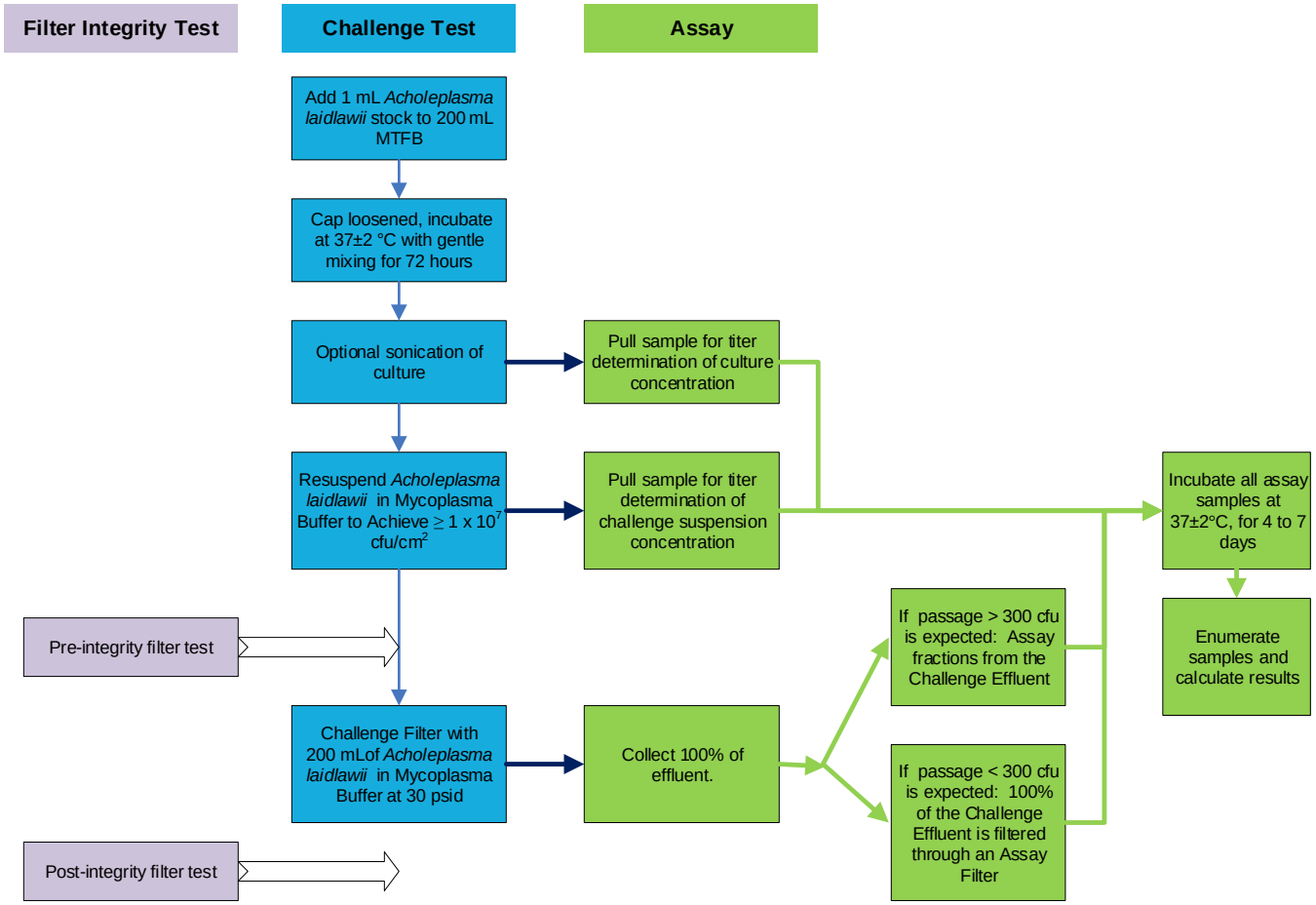
- Temperature of incubation: 37±2 °C
- Duration of incubation: 3 days
- Composition of the MTFB:
  - Mycoplasma Broth Base (beef heart infusion broth)
  - Yeast Extract
  - Horse Serum, heat inactivated

Although the culture medium is not fully chemically defined, cultivation in this medium consistently ensures the production of highly penetrative cells [8]. Both the MTFB and selected solid medium must be shown to have satisfactory nutritive properties to support the growth of *A. laidlawii* under the chosen incubation conditions. However, prequalification of the medium does not completely eliminate—but rather reduces—the risk of test invalidation due to a failure to penetrate even a minimally retentive filter. As previously stated, in an actual test, the 0.2 µm positive control filter must be tested simultaneously (i.e., with the same culture batch on the same day) with the test filter(s) and ultimately serves to validate the test at the time it is performed.

6 Method

The following procedure describes a method for performing a mycoplasma challenge test of 47 mm membrane filter discs using *Acholeplasma laidlawii* as the test microorganism. A mycoplasma challenge test of presumptive 0.1 µm rated filters is validated using 0.2 µm rated positive-penetration control filters. The 0.2 µm rated filters are used to confirm the penetrative ability of the test mycoplasma cells produced by cultivation in the recommended growth media as described in this method. The positive control is intended to be tested under the same conditions with the same challenge suspension pool as the test articles. The 0.2 µm filter challenge conditions should not compromise the ability of the 0.2µm control filter to detect inappropriate sized test organisms.

Figure 1. Summary Workflow



6.1 Material and equipment

The equipment needed to perform the challenge test is listed in **Table 1**.

Table 1 Material and equipment required

Filters
Test Article: 47 mm sterilized test filter discs (presumptive 0.1 µm rating, possibly unrated)
Positive penetration control filter disc(s): 47 mm sterilized filter discs, 0.2 µm sterilizing grade Note: It is the responsibility of the laboratory conducting this test to select the challenge conditions, polymer, and brand of 0.2 µm sterilizing grade filter and to perform studies demonstrating suitability for this test. Assay Recovery Filters: Sterile filter discs validated for use in *A. laidlawii* assay Note: It is the responsibility of the laboratory conducting this test to select the polymer and brand of recovery filter and to perform studies demonstrating suitability for this test.

Equipment	Test Microorganism, Reagents, and Media
Sterile borosilicate glass test tube with closure Stir plate, magnetic, Stir bars, sterilized Petroff-Hausser counting chamber (optional) Stopwatch Sterile pipettes/pipettors Pressure vessel with fittings Incubator 37±2 °C, with agitation Vortex mixer Filter holders to hold 47 flat disc membranes Autoclave Filter forceps Tubing Pressure gauges 0 to 100 psig (0 to 689 kPa) Biological safety cabinet (BSC) or laminar flow hood (LFH) (Optional) Ultrasonic bath, capable of producing a culture that penetrates a 0.2 µm control filter	*Acholeplasma laidlawii* (Sabin 1941) Edward and Freundt 1970 (A. laidlawii) [15] History: ATCC 23206 <- R. Wittler <- D.G.ff. Edward, PG8 <- Nat. Inst. Med. Res. London, UK (A) <- P.P. Laidlaw & W.J. Elford Collections, for example, include: ATCC® No. 23206 ™, CIP 75.27, NCTC 10116, DSM 23060, NBRC 14400 (formerly IFO 14400), or equivalent. Other designations: A, PG8; PG8 The test microorganism should be identified as being of the required species by comparison to type cultures. Sterile deionized (DI) water Sodium Phosphate, monobasic Sodium Phosphate, dibasic Agar, purified grade Mycoplasma Broth Base Dilution blanks, for serial 10-fold dilutions, mycoplasma buffer Yeast Extract Horse Serum, heat inactivated

6.1.1 Preparation of media and buffer

The information provided in **Table 2** below describes steps for preparing the frozen stock broth, culture medium, mycoplasma buffer and recovery agar for the challenge test.

Table 2 Preparation of Media & Buffer

Mycoplasma Buffer:	Composition: Sodium Phosphate Monobasic, CAS 7558-80-7 3.36 g Sodium Phosphate Dibasic, CAS 7558-79-4 10.22 g Sterile deionized water 1 L Preparation: 1. Dissolve 3.36 g of Sodium Phosphate Monobasic and 10.22 g of Sodium Phosphate Dibasic in 1 L of deionized water. 2. Adjust the pH of the solution to a final pH of 7.1 ± 0.1 3. Sterilize by filtration or autoclave for15 min @ 121 °C. 4. After sterilization, buffer can be stored at room temperature until used. Prepare fresh buffer each time.

Recovery Agar:	1. Select an appropriate nutritional culture agar previously validated for *A. laidlawii* culture and titer.
Culture Media: Mycoplasma Task Force Broth (MTFB)	Composition: Mycoplasma Broth Base 20 g Yeast Extract 25 g Deionized water 900 mL Horse Serum 100 mL Preparation: 1. Dissolve 20 g of mycoplasma broth base and 25 grams of yeast extract in 900 mL of deionized water. 2. Autoclave the culture medium using a validated sterilization cycle. 3. Once the broth has cooled, aseptically add 100 mL of heat inactivated horse serum. 4. Store refrigerated (4°C to 8°C). Time to be determined by the laboratory.

6.2 Preparation of challenge microorganism

Use *Acholeplasma laidlawii* from a culture collection (see Table 1) stored at -20 °C or lower as the stock culture for challenge testing. Organism should not be used more than 15 passages from reference strain obtained from the culture collection. The stock is used to inoculate MTFB to obtain an *A. laidlawii* working culture [15,16].

1. Inoculate the MTFB medium 72 hours prior to use.
2. Add 1 mL of thawed *A. laidlawii* stock per 200 mL of MTFB.
3. Incubate the broth culture, cap loosened and with gentle agitation at 37±2 °C for 72 hours.
4. (Optional) After incubation and prior to use, sonication in an ultrasonic bath may be performed.

6.2.1 Preparation of the challenge suspension

1. Prepare the *A. laidlawii* (ATCC® No. 23206™ or equivalent) challenge suspension to achieve ≥ 80% monodispersion using the above working culture.
2. Assess monodispersion by loading a bacterial counting chamber (such as a Petroff-Hauser counting chamber) as described by the manufacturer. View the cells at 1000X. Use of a green filter on the light source can facilitate this imaging.
3. Randomly select a minimum of 5 boxes on the grid. Count the number of individual vs. the number of clumped (doubles or more) microorganisms observed. At least 20 or more cells or cell clusters must be counted before determining a percent monodispersion.
4. Calculate the percent monodispersion using the following equation:

% Monodispersion = 100 x **Number of single cells counted/total number of bodies* counted**

$$\%\ Monodispersion\ = 100x\frac{Number\ of\ Single\ Cells\ Counted}{Total\ Number\ of\ Bodies\ Counted}$$

*Where "bodies" refers to single cells and cell clusters. One multi-celled cluster observed is counted as one body when determining total count.

5. Add sterile mycoplasma buffer to a pressure vessel sufficient for the application of 200 mL of challenge suspension per test filter and control filter.
6. Calculate the volume of *A. laidlawii* working culture needed for the challenge suspension using the following equations (based on a single filter):
    a. Determine the required challenge concentration and multiply as necessary depending on the number of filters to test:

$$\textbf{Challenge Concentration}\ (\textbf{CFU/mL}) = \frac{Target\ Challenge\ Level\ (CFU/cm^2) \times\ Filter\ Area\ (cm^2)}{Total\ Challenge\ Volume\ (\ mL)}$$

    b. Determine the volume of *A. laidlawii* working culture required:

$$\textbf{Volume of Working Culture}(\textbf{mL}) = \frac{Challenge\ Concentration(CFU/mL) \times\ Total\ Required\ Volume(mL)}{Working\ \ Culture\ Concentration\ (CFU/mL)}$$

    c. After adding the required amount of the culture to the mycoplasma buffer from step b, place the vessel on a magnetic stirrer; add a sterilized magnetic stir bar, and gently stir to uniformly maintain the culture in suspension throughout the duration of the challenge test. Maintain the pressure vessel at ambient room temperature.
7. Suspend sufficient mycoplasma cells in mycoplasma buffer to yield a final minimum *A. laidlawii* challenge level of 1.0 x 10^7 CFU/cm2 of test filter area.

6.2.2 Challenge test apparatus

Figure 2 presents one example of a two-filter (one-test and one positive control) challenge test apparatus for a constant pressure normal flow filtration test. All components are designed to withstand at least 100 psig (689 kPa) and at least 135 °C.

In designing a test system, consideration should be given to ensure negligible pressure drop due to filter housing fittings on the downstream side of the filter. Additionally, confirm that the manifold pressure (G-2) reflects the target inlet pressure of 30 psi at the filter flow rates expected for the testing. Otherwise, a pressure gauge is needed immediately upstream of the filter housing and, if housing outlet pressure drop is not negligible, immediate downstream or one could use a differential pressure gauge connected immediately upstream and immediately downstream of the filter housing. Additionally, if more than one filter at a time is tested, it must be shown that the pressure differential is maintained at 30 psid for each filter. **Table 3** reflects the challenge test parameters for the apparatus.

Table 3 Challenge test parameters

Challenge Test Mode:	Normal flow filtration with constant pressure
*Test Pressure:	30 psid (207 kPa)
Challenge volume for a 47 mm disc:	200 mL

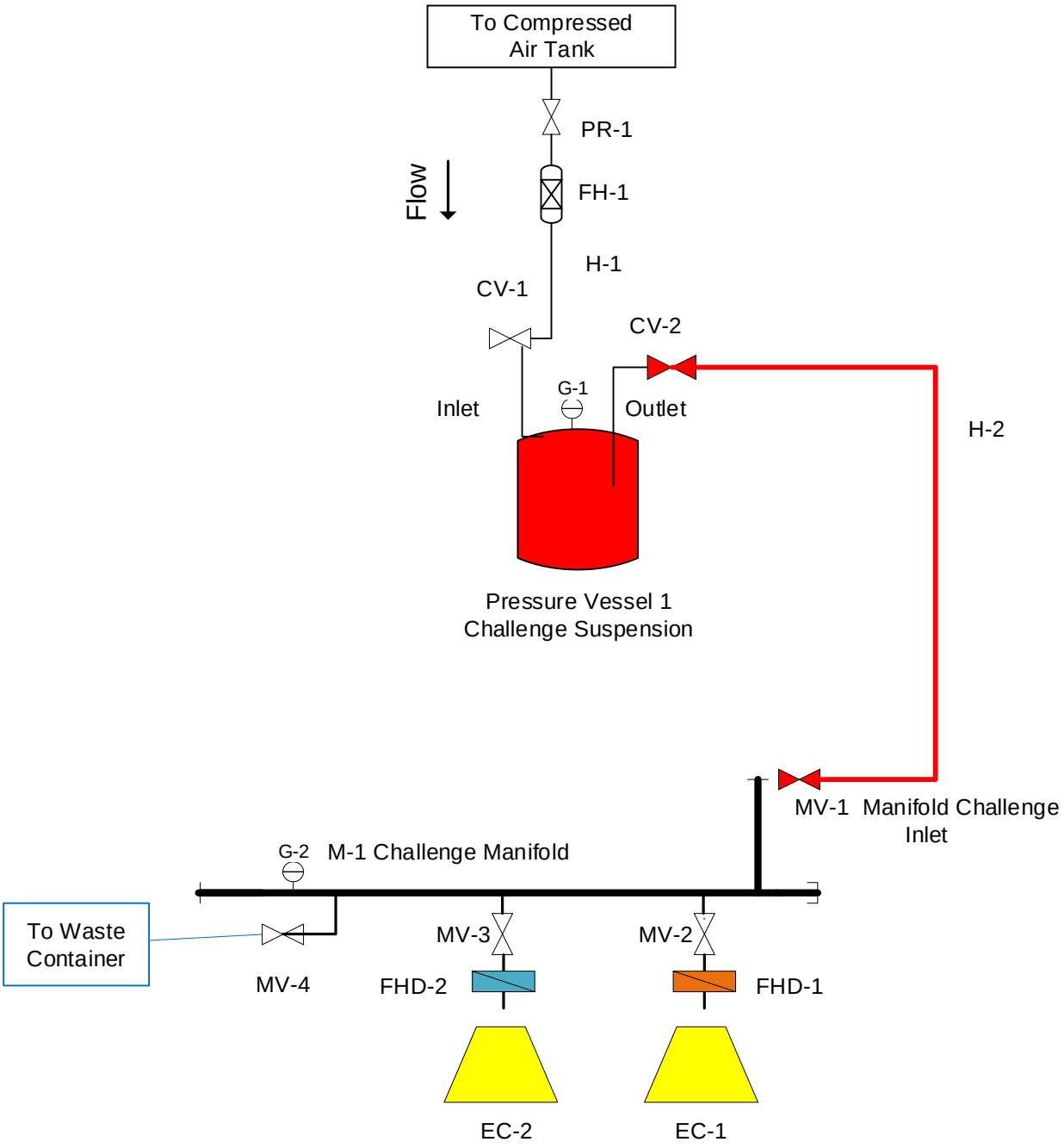
*Maintain a constant test pressure by means of a pressurized vessel, and monitor the differential pressure using pressure gauges upstream of the test filter housings.

To perform the challenge test, ensure all valves are closed, then pressurize the Challenge Suspension vessel to 30 psi. Next, open valves to fill the challenge manifold with challenge suspension. Open the valve above each

filter to allow 200 mL of challenge suspension to filter while collecting the effluent in an effluent collection vessel.

Determine the challenge suspension influent concentration and the post-challenge effluent (filtrate) pool concentration using a previously validated *A. laidlawii* titer determination method. Where necessary, perform a serial dilution in mycoplasma buffer. The results can be reported as an LRV or a TR (Section 6.3).

Figure 2 Example of a Challenge Test Apparatus



PR-1	Pressure Regulator
FH-1	Air Filter Housing Cartridge, 0.2 µm, sterilizing grade
G-1	Pressure Gauge on Challenge Suspension Pressure Vessel
H-1	Hose 1: Deliver compressed air to Pressure Vessel 1: Challenge Suspension Vessel
H-2	Hose 2: Deliver Challenge Suspension to Challenge Manifold
CV-1	Inlet Valve to Challenge Suspension Vessel
CV-2	Outlet Valve from Challenge Suspension Vessel
G-2	Pressure Gauge on Challenge Manifold
M-1	Challenge Manifold
MV-1	Challenge Manifold Challenge Suspension Inlet Valve
MV-2, MV-3	Challenge Manifold Valves to Disc Filter Housings
MV-4	Challenge Manifold Valve to direct waste to vented vessel
FHD-1	Filter Housing for 0.2 µm control filter
FHD-2	Filter Housing for Disk Membrane (Test Sample)
EC-1	Effluent Collection Vessel
EC-2	Effluent Collection Vessel

6.3 Reporting results

Determine the challenge suspension influent concentration and the post-challenge effluent (filtrate) pool concentration using a previously validated *A. laidlawii* titer determination method. Where necessary, perform a serial dilution in mycoplasma buffer; the results can be reported as an LRV or a TR.

Titer reduction is the ratio of the total number of mycoplasma used to challenge the filter (influent) divided by the total number of mycoplasma that passed through the filter (effluent) using the following equation:

$$\boldsymbol{TR} = \frac{Upstream\ Microbial\ \ Titer\ (CFU/mL) \times Volume\ Applied}{Downstream\ Microbial\ Titer\ (CFU/mL) \times Volume\ Filtered}$$

The Log Reduction Value (LRV) is the titer reduction expressed as a base 10 logarithm.

$$\boldsymbol{LRV} = Log10\left(\frac{Upstream\ Microbial\ \ Titer\ (CFU/mL) \times Volume\ Applied}{Downstream\ Microbial\ Titer\ (CFU/mL) \times Volume\ Filtered}\right)$$

The filter can be considered fully retentive (under the given conditions) when all of the filter effluent is analysed and no A. laidlaiwii is detected, where the positive control filter exhibits growth and where the challenge level is ≥ 1.0 x 10^7 CFU/cm2.

Non-fully retentive filters can be considered reductive (under the given conditions) when a titer reduction (or log reduction value) can be determined as per the equations above.

6.4 Test acceptance criteria

The test is valid if it meets the following criteria:

A. **Monodispersion:** Samples of the mycoplasma challenge suspension must be ≥80% monodispersed as determined microscopically. If monodispersion cannot be achieved, then do not proceed with the test.

B. **Challenge Level:** A minimum challenge level of ≥1.0 x 10^7 CFU/cm^2 must be met. If the challenge does not meet the minimum challenge concentration, then the test is invalid.

C. **Positive Control:** *A. laidlawii* must be detected in the effluent (filtrate) of the 0.2 µm positive control filter. If *A. laidlawii* is not detected in the effluent, then the challenge test is invalid.

D. **Filter Integrity:** All test article filters should pass a pre-established pre-challenge integrity test. The positive control must also pass the post-challenge integrity test. For developmental filters, integrity test values are recorded for future determination of allowable limits.

   a. If the filter fails integrity, rewet the filter according to the manufacturer's recommendations and repeat the test. If the filter integrity test fails again, then proceed to Step b.

   b. (Optional unless there is a failure in Step a.)

      Flush the filter using a lower surface tension solution, such as alcohol in water, as recommended by the filter manufacturer, and perform the integrity test using the lower surface tension solution. If the filter integrity test fails again, then the challenge test is invalid.

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