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# 0.1-µm Bottle-Top Filtration of Media as a Strategy to Avoid Contamination of Cell Cultures with Mycoplasma

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## Abstract

Preventing contamination with mycoplasma plays a crucial role when working with cell cultures in order to ensure accurate and reproducible results in experimental trials and production of biological targets. To minimize the risk of contamination, it is recommended to implement a number of measures, such as aseptic working, use of mycoplasma-free tested reagents and media, and regular mycoplasma testing. In addition, filtration of media prepared from dry media is an essential element in a prevention strategy. Here, it was shown that the tested vacuum bottle-top filters Sartolab® RF with a pore size of 0.1 µm can effectively remove the mycoplasma strains *A. laidlawii* and *M. hyorhinis*. Cell culture media DMEM, F17, and RPMI + 10% FBS spiked with the respective mycoplasma strains were used to simulate typical conditions during filtration.

# Introduction

The contamination of cell cultures with mycoplasmas poses a risk for the production of safe products and the generation of reliable experimental results and makes preventive measures a necessary prerequisite. Various studies on the prevalence of mycoplasma in cell cultures show the potential risk of contamination with mycoplasma. In their extensive investigations, Barile and Rottem showed that of more than 20,000 cell cultures examined over a period of 30 years, 3,000 (or 15%) were contaminated with mycoplasmas. In another study, Olarerin-George and Hogenesch found mycoplasma in 11% of 9,385 of rodent and primate cell samples. Despite this frequency of cases, only a few species are responsible for 90–95% of all contamination. *M. orale* is the most widespread species, accounting for 20–40%, followed by *M. hyorhinis* (10–40%), *M. arginini* (20–30%), *M. fermentans* (10–20%), *M. hominis* (10–20%), and *A. laidlawii* (5–20%). Cell cultures are most likely to become infected by cross contamination from already infected cell cultures; the organisms can also gain entry from contaminated culture media components, such as fetal bovine serum or, in the case of *A. laidlawii,* from serum-free cell culture media powders. Other sources are the human operator or primary tissue, if it is colonized by mycoplasmas.[1-5]

Infection of a cell culture with mycoplasmas can lead to a number of undesirable effects, such as altered expression of proteins, altered RNA and DNA synthesis, or degradation of host cell DNA, resulting in changes in morphology, cell metabolism, and growth.[6]

Mycoplasmas, a synonymously-used term for the bacterial class of Mollicutes, are among the smallest self-replicating organisms enveloped only by a lipid bilayer. The lack of a cell wall results in mycoplasmas being easily deformed compared to bacteria with a cell wall. Due to their small size of 0.3–0.8 µm and their deformability, it was found that mycoplasmas can pass sterile filtration membranes with a nominal pore size of 0.2 µm. In contrast to 0.2-µm filter media, 0.1-µm depletes the mycoplasma load in liquid cell culture media more effectively, due to the smaller membrane pore size.[6-9]

Preventing the introduction of mycoplasma into cell cultures and the associated reagents and media is crucial for the laboratory user and requires a comprehensive approach rather than relying on individual measures alone. Besides the use of sterile or mycoplasma-free tested materials, aseptic handling, and regular confirmation of the absence of mycoplasma in the cell culture (e.g., by PCR or a cultural test), the filtration of cell culture media prepared from dry media using 0.1-µm filters is a good strategy to minimize the risk of contamination.[10]

To demonstrate the suitability of Sartolab® RF bottle-top vacuum filters equipped with a 0.1-µm PES membrane in the retention of mycoplasmas, two representatives of mycoplasmas were selected as test micro-organisms combined by three different media and phosphate buffer as test matrix. For this purpose, *M. hyorhinis* was chosen as representative of Mycoplasmatales and *A. laidlawii* was chosen as representative of Acholeplasmatales. RPMI + 10% FBS, DMEM, and F17 were selected as frequently used representatives for cell culture media.

# Materials and Methods

**Pre-Titration and Preparation of Challenge Cultures**
To pre-determine the count of *A. laidlawii* (ATCC 2306) or *M. hyorhinis* (ATCC 17981), 5 mL seedstock was added to 100 mL of ME liquid, incubated for 24 hours and titrated. The concentration of mycoplasma determined in this way, (defined as "CFU expected"), served as the basis for calculating the minimum volume of challenge suspension. For each challenge run, 5 mL of the same seedstock lot was inoculated into 100 mL of the same ME liquid batch and used to prepare the challenge suspensions, based on the pre-determined count.

**Preparation of Challenge Suspensions**
The minimal challenge of $1 \times 10^7$ CFU/cm$^2$ of effective filtration area was based on the accepted standards for validation of filter retention.[13] The EFA for Sartolab® RF 250 (Sartorius, 180D03-E) is 43 cm$^2$. Therefore, a minimum of $43 \times 10^7$ CFU is required (defined as "CFU required") to challenge the Sartolab® test filters. For each test filter, 100 mL of test matrix, cell culture medium, or phosphate buffer should be spiked with this amount of CFU. For each combination of test fluid and mycoplasma strain, 500 mL of challenge suspension with a target CFU of $2.53 \times 10^9$ CFU/500 mL including a 10% inflation factor was prepared.

To achieve the challenge target, the volume of 24-hr culture added to the test matrix for the challenge suspension was calculated by dividing the "CFU required" by "CFU expected" in 1 mL of 24-hr culture.

Sufficient volume of the 24-hr broth culture was added to 500 mL of the test matrix based on the results of the pre-counts and calculated as shown above.

### Filtration of the Challenge Suspension Through the Test Filter Units

Three Sartolab® 0.1-µm test filter units plus one control Sartolab® 0.22-µm filter (Sartorius, 180E03) unit were attached to the ports on the 6-unit carousel Sartolab® Multistation (Sartorius, SDLC01) but not fully connected to the vacuum train. Challenge suspension was added to the 100-ml mark on the cups (Figure 1). The first two filters were pushed into the full connection position and the vacuum pump started. Runs were timed from when filtrate emerged. The filtrations were run at minus 0.5 bar ± 0.1, approx. 20 °C. After filtration the units were disconnected, and the second pair of units engaged and filtered.

**Figure 1:**
*Experimental Setup for Filtration of Challenge Suspension*

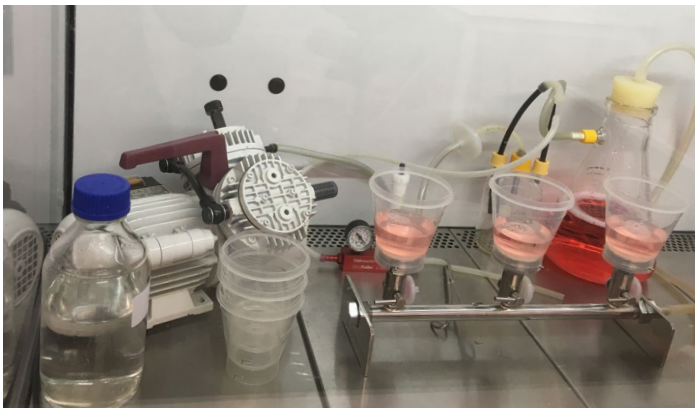
*Note.* Experimental setup for filtration of challenge suspension comprising a vacuum pump, the rotatable vacuum manifold unit Sartolab® Multistation, and the vacuum filtration units Sartolab® RF 250 with an 0.1-µm PES membrane (3X), Sartolab® RF 250 with an 0.22-µm PES membrane (1X). The challenge suspension, consisting of 100 mL of cell culture medium and spiked mycoplasma each, is simultaneously filtered at a vacuum of minus 0.5 ± 0.1 bar. Times required for filtration were taken and the filtrates were then analyzed for the passage of mycoplasmas by means of a further analytical filtration.

### Analytical Filtration of the Test Filter Unit Filtrates

All filtrates were filtered at minus 0.5 ± 0.1 bar through a sterile PES 0.1-µm membrane disc (Sartorius, 15458) in a Biosart® 250 funnel (Sartorius, 16407) placed on a triple Combisart® manifold (Sartorius).

After each filtration was completed, the *A. laidlawii* membrane was placed directly onto AIErt 24 agar and the *M. hyorhinis* membrane was placed onto standard MES (Mycoplasma Experience) in a 60-mm petri dish.

**Figure 2:**
*Experimental Setup for Analyzing Filtrates of Test Filters*



*Note.* Experimental setup for analyzing filtrates of test filters (Sartolab® RF) comprising a vacuum pump, a triple Combisart® vacuum manifold, 0.1-µm PES membrane filter discs, funnels (Biosart® 250), and a receiver flask. The filtrates of the test filters are examined for mycoplasma by performing analytical filtration at a vacuum of minus 0.5 ± 0.1 bar. Finally, the filter discs are placed on AIErt 24 agar plates allowing captured mycoplasma to form countable colonies.

### Verification of Challenge

The challenge suspension for each matrix was diluted in serial ten-fold steps (1 mL + 9 mL) to $10^{-3}$. Duplicate MES plates were inoculated with 20 µl of $10^{-2}$ and $10^{-3}$ dilutions.

### Filter Controls

All challenge runs included a Sartolab® RF filter unit with a 0.22-µm membrane. The test was invalid if organisms were not recovered from the filtrate.

0.2 mL of each challenge suspension was added to 20 mL of mycoplasma broth based and filtered through the analytical membrane to confirm retention of the inocula.

All 20-mL volumes were passed through Sartorius PES 0.1-µm filters (15458) at minus 0.5 ± 0.1 bar and each filter overlaid onto AIErt 24 for *A. laidlawii* suspensions and onto MES for *M. hyorhinis*.

### Incubation and Assessment

All plates were incubated in an atmosphere of 5% $CO_2$ in nitrogen at 36 °C ± 1 °C.

For *A. laidlawii* AIErt 24 plates plus membranes were removed and read at 48 hr. Positive MES plates were removed and read at 48 hr, but negative plates were re-incubated for a further five days. For *M. hyorhinis,* all plates were incubated for seven days and all membranes were stained with Dienes' stain. This enabled any colonies present to be visualized, as mycoplasmas pick up the blue stain.

All apparently negative membranes (no evidence of colored colonies) were scanned at 25-fold magnification before recording as negative. The most appropriate dilution was selected for counting control plates and any filtrates with high numbers of organisms. The averages of the 2-plate count from the control plates were used to calculate the actual challenge titer for each organism.

## Results and Discussion

### Filtration Times for Cell Culture Media and Buffer

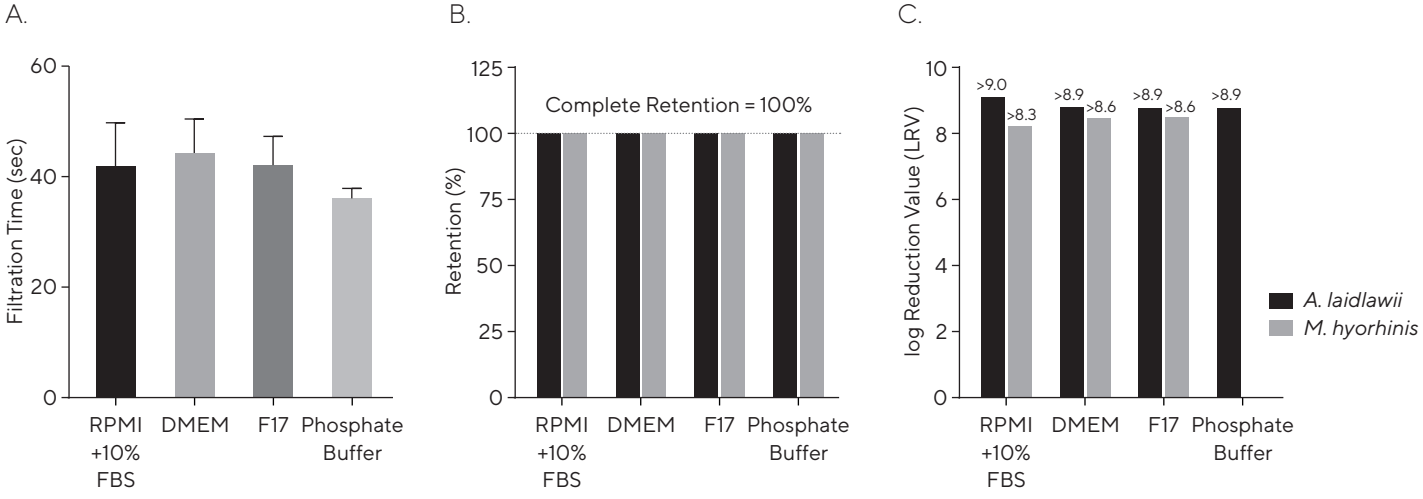
The filtration time required for vacuum filtration at minus 0.5 ± 0.1 bar of 100 mL each of the cell culture media RPMI + 10% FBS, DMEM, F17, and phosphate buffer using Sartolab® RF 250 was determined (Figure 3A). The fastest filtration time was found for phosphate buffer with an average of 38 s. Whereas the 100-mL cell culture media were filtered with an average of 43 s (RPMI + 10% FBS), 46 s (DMEM), and 44 s (F17). The slightly increased filtration time of the cell culture media of approx. 17% compared to phosphate buffer can be explained by the more complex composition of cell culture media and the associated higher viscosity of fluids and/or clogging of smaller pores.

### Sartolab 0.1-µm Bottle Top Filters Completely Retain *A. laidlawii* and *M. hyorhinis*

The retention of mycoplasmas was determined by spiking samples of 100-mL cell culture medium or phosphate buffer with mycoplasmas challenge suspensions of *A. laidlawii* or *M. hyorhinis,* filtering at minus 0.5 ± 0.1 bar through the Sartolab® test filters, and then analyzing the resulting filtrates for mycoplasmas penetrating the filter membrane.

The number of mycoplasmas added to the samples before filtration, the so-called challenge level, was thereby determined to be $2.2 \times 10^7$ CFU/cm² (RPMI + 10% FBS), $1.7 \times 10^7$ CFU/cm² (DMEM), $1.7 \times 10^7$ CFU/cm² (F17), and $2.0 \times 10^7$ CFU/cm² (phosphate buffer) for *A. laidlawii* and $0.5 \times 10^7$ CFU/cm² (RPMI + 10% FBS), $0.8 \times 10^7$ CFU/cm² (DMEM), and $1.0 \times 10^7$ CFU/cm² (F17) for *M. hyorhinis*. Differences in the challenge levels within the preparations of a test organism are explained by the cultivation of the challenge suspension on different test days.

**Figure 3**




*Note.* In each case, 100 mL of the cell culture media RPMI + 10% FBS, DMEM, F17, and phosphate buffer spiked with *A. laidlawii* or *M. hyorhinis* was filtered at 0.5 ± 0.1 bar through a 0.1-µm PES Sartolab® RF 250 with a filter area of 43 cm² EFA. The respective filtration times (cell culture media: n = 12; phosphate buffer: n = 6) (A), retention (n = 3) (B) and the associated LRV were determined (n = 3) (C). No experiments were performed with phosphate buffer and *M. hyorhinis*.

Following filtration, all 0.1-µm rated Sartolab® RF test filters gave complete retention when challenged with *A. laidlawii* or *M. hyorhinis,* regardless of which cell culture medium was used as filtration fluid (Figure 3B). Using the challenge level and the number of mycoplasmas that could penetrate the filter membrane, it was possible to calculate the LRV (see equation below). Since we could not detect any passage of mycoplasmas in these experiments, it was only possible to determine the minimum LRV. The true LRV will in fact be higher. The minimum LRV was 9.0 (RPMI + 10% FBS) and 8.9 (DMEM, F17, phosphate buffer) for the samples with *A. laidlawii* and 8.3 (RPMI + 10% FBS) and 8.6 (DMEM, F17) for the samples with *M. hyorhinis* (Figure 3C).

$$LRV = \log_{10}\left(\frac{challenge\ suspension\ titer\,[CFU/mL]}{filtrate\ titer\,[CFU/mL]}\right)$$

For the evaluation of the results of the tests with *A. laidlawii,* the PDA Technical Report No. 75 can be used, which describes industry standard methods and criteria to evaluate filters according to their retention capacity against mycoplasmas.[13] In this standard, *A. laidlawii* was defined as a challenge micro-organism. In this study, Sartolab® RF 250 with a 0.1-µm PES membrane met the criteria and retained *A. laidlawii* with a minimum challenge level of ≥ $1 \times 10^7$ CFU/cm². Although for *M. hyorhinis,* the PDA Technical Report No. 75 oriented minimum challenge level of ≥ $1 \times 10^7$ CFU/cm² could not be achieved, a 100% retention could be determined at a challenge level of at least $0.5 \times 10^7$ CFU/cm², suggesting that *M. hyorhinis* in this magnitude can be reliably removed from cell culture media by filtration. The chosen low differential pressure of minus 0.5 bar by vacuum filtration also supports a reduction of mycoplasmas, especially since the pressure dependence of the passage of *A. laidlawii*—even through 0.1-µm membranes—is known.[10]

In addition to the impact of differential pressure, the temperature of the medium to be filtered also plays an important role in the retention of mycoplasmas. For example, at a differential pressure of 2 bar and 0.1-µm membranes with rather open pores, it was shown that at a media temperature of 3 °C *M. orale* was retained with an LRV of 2 and at 38 °C with an LRV of 1.[12] Despite the known property for *M. orale* to penetrate 0.1-µm filters, this is of minor relevance for filtration applications, since new *M. orale* infections are most likely to have occurred from cross-contamination and this organism is unlikely to be present in media components that are filtered during processing.

To ensure that the challenge micro-organisms are small enough, the PDA Technical Report No. 26 requires *A. laidlawii* to pass through a 0.22-µm-rated membrane.[13] In our tests, all of 0.22-µm-rated control filters allowed penetration of both *A. laidlawii* and *M. hyorhinis* cells.

## Conclusion

In order to mitigate the contamination risk of cell cultures with mycoplasmas, various measures such as aseptic working, use of mycoplasma-free tested cell cultures and media, and regular mycoplasma testing must be integrated into the workflow. Here, filtration of cell culture media, especially media prepared from powder, represents a cornerstone of a comprehensive prevention strategy. We have been able to show that the Sartolab® RF vacuum filters with a 0.1-µm PES membrane can effectively remove mycoplasmas from cell culture media as demonstrated for *A. laidlawii* and *M. hyorhinis.* In addition, to further minimize the risk of mycoplasma penetration through the filter, it is recommended to keep the differential pressure and media temperature as low as possible during filtration.

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## Abbreviations

| | |
|---|---|
| CFU | Colony Forming Units |
| DMEM | Dulbecco Modified Eagle Medium |
| DNA | Deoxyribonucleic Acid |
| FBS | Fetal Bovine Serum |
| EFA | Effective Filtration Area |
| LRV | Logarithmic Reduction Value |
| MES | Mycoplasma Experience solid medium |
| PDA | Pharmaceutical Drug Association |
| PES | Polyethersulfon |
| RNA | Ribonucleic Acid |
| RF\|BT | Receiver Flask and Bottle Top |
| RPMI | Roswell Park Memorial Institute |


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