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Simple and Effective Routine Mycoplasma Testing with MycoAlert® Biochemical Assays

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Mycoplasma is one of the most common and serious forms of cell culture and media contaminants. It is difficult to detect or treat, and its effects on cell cultures can drastically influence experimental outcomes. Research labs need a fast, reliable method to screen cell cultures for mycoplasma contamination, and the MycoAlert® and MycoAlert® Plus Kits from Lonza are manufactured with these needs in mind. In this study, we performed a comparison of MycoAlert® and MycoAlert® Plus Kits against four other commonly used methods for mycoplasma detection: PCR, qPCR, inoculation in agar, and Hoechst staining.

All methods were tested against three of the most common species of mycoplasma in both a suspension and adherent cell culture. Compared to both PCR and compendial methods, Lonza's MycoAlert® and MycoAlert® Plus Kits offer an advantage to labs in a research use only (RUO) setting because of ease-of-use, minimal hands-on time, minimal time to generate results, and low overall cost. As a result, labs can check cultures on a much more frequent basis than with other methods, ensuring peace of mind that cell cultures remain mycoplasma-free with greater reliability.

Introduction

Mycoplasmas are one of the most common contaminants in cell culture labs. These organisms, which are among the smallest-known self-replicating organisms, lack a cell wall, are undetectable by Gram staining[1], and are not visible under light microscopy. Reports vary as to the general incidence of mycoplasma contamination in cell culture labs, ranging from 15–35% of all continuous cell cultures[1]. In a study of NCBI's RNA-seq archive, Olarerin-George and Hogenesch (2015) showed that 11% of cell cultures might be mycoplasma-positive, and that many of these cell lines had been used to generate data for publications[2].

The widespread prevalence of mycoplasma-contaminated cell cultures is especially concerning given the fact that there are no obvious outward signs of contamination in cell culture (e.g., color changes, changes in pH, turbidity, etc.). Effects of mycoplasma contamination range from retardation of cell growth and proliferation to increased oxidative stress and DNA damage, DNA repair inhibition, altered metabolism, changes in cellular membrane receptors, reduced transfection efficiency and even cell culture collapse[1,3,4]. Changes in gene expression across hundreds of eukaryotic genes as a result of mycoplasma contamination have been recorded[5] (see Chernov *et al*., 2014 and references 40–45 therein). Cast in this light, undetected mycoplasma contamination plays both a harmful and ongoing role in scientific research[2].

Best practices for mycoplasma screening in a research setting vary by the needs of the individual lab. Mycoplasma growth is generally slow[1] and can vary drastically by culture condition[6]. In general, it is recommended that routine testing be carried out on all cell cultures anywhere from every 1–2 weeks to 1 time per month to ensure that cultures are mycoplasma-free. In addition, testing should be carried out 1. anytime new cell lines are brought into the lab, 2. anytime the lab receives primary cells from a patient, 3. prior to freezing backups of long-term cell cultures, and 4. after thawing frozen backups of cell lines/cultures. Researchers in an RUO setting must choose a mycoplasma testing method that balances the frequency of testing with both cost and labor while assuring that their cultures remain mycoplasma-free.

There are a wide variety of testing methods available that vary based on cost, turnaround time, and intended use (e.g., release testing vs. RUO, **Table 1**). Compendial methods, required for release testing of regulated products, are considered the "gold standard" and are required by the FDA, EP, JP, and other regulatory bodies for cell cultures, media, or other components used to manufacture products for use in humans (e.g., vaccines). These methods usually entail a combination of direct inoculation onto agar (both before and after broth culturing) along with Hoechst staining with indicator cells. Other compendial methods exist (e.g., qPCR-based assays) but they must be validated against the standard culture methods. For RUO purposes, compendial methods are likely an unnecessary expense, as they are time consuming and require external, specialized subject matter expertise.

RUO methods tend to be much less expensive and time consuming and should be considered as a preferred approach for routine mycoplasma testing. The majority fall into two categories: PCR-based assays and biochemical assays. PCR-based assays are relatively inexpensive, but require more time and a higher level of expertise than biochemical methods. Biochemical methods are inexpensive, rapid (results within 20 minutes), and require no advanced expertise to carry out or interpret. At Lonza, we have developed two biochemical assays, MycoAlert® (e.g., cat. no. LT07-318) and MycoAlert® Plus (e.g., cat. no. LT07-710) Kits, that are designed to deliver rapid and reliable results with routine mycoplasma testing in mind. In this study, we compare the performance of Lonza's MycoAlert® Kits to both the leading RUO PCR testing method as well as the gold-standard compendial release methods. We show that MycoAlert® and MycoAlert® Plus Assay performance, combined with their minimal cost and labor per analysis, makes them the optimal solution for RUO labs that need frequent, accurate testing to maintain peace of mind about their cell cultures.

Method	Time To Result	Relative Cost	For Release?[a]
Direct Inoculation on Agar	14–28 days	Very Expensive	Yes
Hoechst Indicator Cell Staining	3–7 days	Very Expensive	Yes
qPCR Assay	8 days	Very Expensive	Yes
Commercial PCR Test Kit	6 hours	Less Expensive	No
MycoAlert® / MycoAlert® Plus (biochemical)	20 minutes	Least Expensive	No

Table 1: Methods of mycoplasma detection compared in this study. (a) "For Release" indicates that a method complies by FDA, EP, JP and/or other regulatory agency guidelines for testing materials used in the manufacture of products for use in humans (e.g., vaccines).

Cell Line	Origin	Type	Media Used	Conditions
K562	Myelogenous Leukemia	Suspension	Lonza RPMI 1640 + 10% FBS + 1% Pen/Strep	36 (±1)°C and 5–10% CO_2
HeLa	Cervical Cancer	Adherent	DMEM w/high Glucose + pyrGlutaMAX 1 + 10% FBS + 1% Pen/Strep	36 (±1)°C and 5–10% CO_2

Table 2: Cell lines used in this study, their characteristics, and culture conditions.

Methods

The performance of six different mycoplasma detection methods (direct inoculation, Hoechst staining, qPCR, a commercial PCR testing kit, and two Lonza biochemical testing kits: MycoAlert® and MycoAlert® Plus; **Table 1**) was tested by inoculating two different cell lines (K562 suspension cells and HeLa adherent cells; **Table 2**) with three different species of mycoplasma (Mycoplasma hyorhinis, Mycoplasma arginini and Mycoplasma orale; **Table 3**) at two final concentrations (Low i.e., 1 CFU/mL, and High i.e., 20 CFU/mL). K562 is an immortalized myelogenous leukemia suspended cell line, while HeLa is an immortalized, adherent cervical cancer cell line. Both are commonly found in research laboratories and are used in a wide variety of applications.

Cell Culture

K562 cells were cultured using the conditions in **Table 2** for one month and passaged twice weekly. HeLa cells were cultured using the conditions in **Table 2** for two weeks and passaged twice weekly. On Day 0 of the experiment (T=0), T75 flasks were prepared containing 20mL of 1.6×10^5 cells/mL of K562 cells or HeLa cells. For each of the mycoplasma species (Table 3), one T75 flask was inoculated to a final concentration of 1 CFU/mL (Low) and one flask to a final concentration of 20 CFU/mL (High). Inoculum controls were created for each mycoplasma strain by inoculating 50 CFU on agar and incubating for 14 days after which viability at time of inoculation was assessed. A single T75 flask was not inoculated and was used as an untreated culture control during all tests.

Following inoculation, K562 and HeLa cells were further cultured and subpassaged with periodic harvest days (T=3, 7, 14 and 21) in which the supernatant was aliquoted for the five different detection assays. Aliquots for MycoAlert®, MycoAlert® Plus and direct inoculation were stored at 2–8° C and tested later that day; all other aliquots were stored at -60° C until testing.

Species	% of Contamination Cases	Origin	Characteristics	Common Sources
Mycoplasma orale	20–40%	Human	Slow growing, non-fermenting	Saliva droplets, e.g., sneeze
Mycoplasma hyorhinis	10–40%	Swine	Moderately fast growing, fermenting, strongly cell-associated	Porcine-derived supplements e.g. trypsin
Mycoplasma arginini	20–30%	Bovine	Fast growing, non-fermenting	Bovine-derived products such as FBS

Table 3: Species of mycoplasma used in this study, the estimated ranges for the % of contamination cases caused by them, and their origin. Data from Drexler and Uphof (2002)[1].

Testing Methods

Direct inoculation tests were performed according to established European and US Pharmacopeia guidelines (EP 2.6.1 and US<63>). Two types of agar were used: a basic agar and a Z+ agar. Z+ agar was used for *Mycoplasma orale* as it was determined to be the most suitable agar for that species; all other species were grown on the basic agar. All plates were incubated in microaerophilic conditions for at least 14 days after which they were microscopically examined for mycoplasma colonies.

Hoechst staining for indicator cells was carried out according to established European and US Pharmacopeia guidelines (EP 2.6.1 and US<63>). In brief, indicator cells (Vero cells) were seeded before inoculation and incubated on 12-well plates. Triplicate wells were inoculated with each test sample, and positive and negative controls established. Cultures were incubated for 4 days, after which supernatant was subpassaged onto fresh indicator cells and then incubated for 3 more days. At the end of this time period, cultures were fixed, stained with DAPI, and observed microscopically for extra-nuclear fluorescence, an indicator of mycoplasma contamination.

The qPCR method used was validated as a qualitative yes/no assay. Briefly, samples were pre-cultured in basic broth for 8 days to support growth of low numbers of mycoplasmas followed by a qPCR reaction.

The PCR assay tested in this study is a leading mycoplasma testing kit and was carried out according to manufacturer kit instructions. Presence of mycoplasma is indicated by a band detected within a size range of 434–469 bp.

Lonza MycoAlert® and MycoAlert® Plus Assays were carried out according to kit instructions. For both assays, the work flow consisted of adding MycoAlert® Reagent to each sample well in a 96 well plate followed by a 5 minute incubation and then a luminescence measurement (Reading A). Next, MycoAlert® Substrate was added to all wells and the plate was incubated for 10 minutes followed by a second luminescence reading (Reading B). The ratio B:A determines if the sample is contaminated, borderline (quarantine and re-test in 24 hours) or clean (**Table 4**)[7,8].

	Negative for mycoplasma	Borderline*	Positive for mycoplasma
MycoAlert®	< 0.9	0.9–1.2	> 1.2
MycoAlert® Plus	< 1.0	1.0–1.2	> 1.2

Table 4: Interpretation of MycoAlert® and MycoAlert® Plus Assay luminescence results. Values reflect the ratio of luminescence reading B (after addition of MycoAlert® Reagent) to luminescence reading A (prior to addition of MycoAlert® Reagent). * = Borderline samples should be quarantined and re-tested in 24 hours[7,8].

Results and Discussion

The K562 and HeLa cell cultures maintained high viability across the entire experiment: over 90% across all treatments and time points except for the untreated HeLa control at T=7 (83.9%). Mycoplasma inoculation did not affect cell viability for either cell type. All three species of mycoplasma passed viability specifications at the start of each experimental trial except for *M. arginini* in the K562 experiment, which did not have an inoculum control. However, this species clearly grew on the agar plates used in the direct inoculum method, demonstrating the integrity of the *M. arginini* inoculum.

We evaluated the five assays by their ability to detect each species of mycoplasma at a given time point for a given initial level of inoculation (Low vs. High). In K562 culture, *M. arginini* was not detected by any assay at any time point when inoculated at an initial level of 1 CFU/mL (**Fig 1a**). However, *M. hyorhinis* and *M. orale* were both detected by MycoAlert® and MycoAlert® Plus Assays between 3 and 14 days after initial inoculation at 1 CFU/mL.

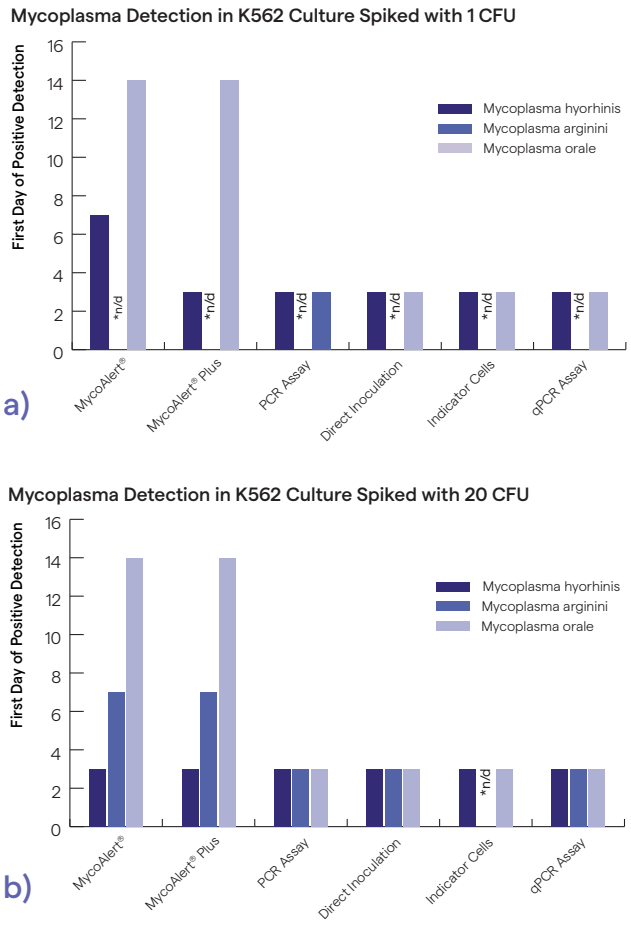

Fig 1: First day of positive mycoplasma detection in K562 suspension culture compared across the six assays used in this study. (a). Initial inoculation at a level of 1 CFU/mL culture. (b). Initial inoculation at a level of 20 CFU/mL culture. "Indicator Cells" = Hoechst stained indicator cell assay. *n/d = *M. arginini* not detected by the indicated methods.

With initial inoculation levels of 20 CFU/mL, all methods detected *M. arginini* in addition to *M. hyorhinis* and *M. orale* except for the Hoechst indicator cells method, which failed to detect *M. arginini* at any time point despite preculturing in broth (**Fig 1b**). Once again, MycoAlert® and MycoAlert® Plus Assays were able to detect mycoplasma between 3 and 14 days after initial inoculation.

In HeLa culture, *M. orale* was not detected at any time point by either PCR or biochemical assays and was detected inconsistently by the other methods (**Fig 2 a-b**). This was likely due to culture conditions not being optimal for sustained growth of *M. orale*, causing it to die out or become diluted out of culture over time. All methods detected *M. hyorhinis* and *M. arginini* in both Low and High inoculation treatments (**Fig 2**). MycoAlert® and MycoAlert® Plus Assays detected mycoplasma between 3 and 21 days post contamination depending on species and level of inoculation (**Fig 2**).

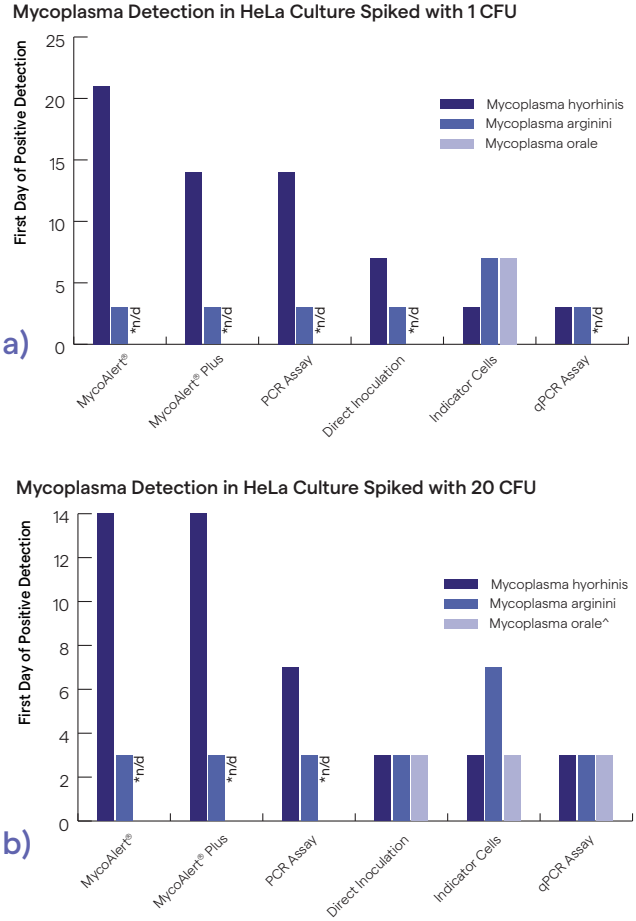

Fig 2: First day of positive mycoplasma detection in HeLa adherent culture compared across the six assays used in this study. (a). Initial inoculation at a level of 1 CFU/mL culture. (b). Initial inoculation at a level of 20 CFU/mL culture. "Indicator Cells" = Hoechst stained indicator cell assay. *n/d = *M. orale* not detected by the indicated methods. ^ = *M. orale* was detected only at T=3 for each of the direct inoculation, Hoechst stained indicator cells, and qPCR Assay screening methods, then not detected by any assay afterwards, indicating the mycoplasma went dormant and was diluted out of culture by passaging.

The comparisons performed in this study focused on the first time point in which an assay method was capable of detecting mycoplasma in cell culture. Except for biochemical methods such as the MycoAlert® Assays, detection of mycoplasma contamination relies on some degree of signal amplification to detect trace levels of contaminants. Direct inoculation onto agar, Hoechst staining, and the qPCR method all rely on culturing samples for up to two weeks before collecting data, while the PCR method amplifies the signal by replicating trace amounts of DNA when mycoplasma populations are small. MycoAlert® and MycoAlert® Plus Assays do not use an amplification step. Our results suggest that the ability of MycoAlert® Assays to detect mycoplasma contamination relies on the size of the mycoplasma population in culture and the growth rate of those cultures, which can vary drastically by culture conditions (**Fig 1 vs Fig 2**). Because they lack an amplification step, MycoAlert® and MycoAlert® Plus Kits are much easier assays to use, deliver results in the quickest timeframe, and are overall much less expensive, especially when cost of labor is factored in. A routine testing regimen will not miss any contaminated cultures where mycoplasma growth may be very slow, catching contamination at a later date as the population expands. The time, cost and effort required to carry out any of the other testing methods considered here restrict the frequency of testing. Any advantage gained through signal amplification is only realized if testing happens to occur within a few days of initial contamination, which would be unlikely under an infrequent testing regimen. Therefore, when establishing a culture of routine monitoring for mycoplasma contamination, the MycoAlert® and MycoAlert® Plus Kits provide an excellent and reliable option for any research lab.

Our study shows that growth of mycoplasma species depends heavily on the interaction between contaminant species and growing media/conditions. A single, standalone negative result using any test method may not be accurate, depending on the growth rate of the contaminating mycoplasma species. The advantage of MycoAlert® Products lie in their ease-of-use and cost-efficiency, allowing them to be seamlessly adopted into a general lab management program employing frequent, routine testing to ensure maintained quality and sterility of cells and media with minimal disruption to lab work. Repeated negative results over time provide a much better guarantee that a given sample is free of contamination, regardless of the testing method used. We recommend testing with Lonza's MycoAlert® Kits at least once per month, as well as checking new cell lines as they come into the lab from other labs or are produced from patient tissues. Further tips, troubleshooting, and best practices can be found in the MycoAlert® and MycoAlert® Plus Manuals on the Lonza website[7,8].

Conclusions

Mycoplasma contamination is a common and serious threat to research labs that handle cell cultures, media, tissues, and animal-derived supplements. Mycoplasma contamination has no outward signs, such as turbidity, and cannot be readily detected outside of dedicated assays designed for mycoplasma detection. Mycoplasma contamination can drastically change the physiology and gene expression of cells in culture and, if undetected, strongly influence the outcome of experiments unbeknownst to the experimenter. Best practices recommend a frequent, routine laboratory testing program to ensure cell cultures, media and supplements are mycoplasma-free. Here at Lonza we offer MycoAlert® and MycoAlert® Plus Mycoplasma Detection Kits: easy-to-use, cost-effective biochemical assays that minimize the disruption of routine testing, providing assurance and peace-of-mind while allowing researchers to focus more of their valuable time on what matters: advancing our knowledge of human health and diseases.

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CD-SP096 09/23

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