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APPLICATION NOTE

Assay Validation of a PCR-based Mycoplasma Detection Kit

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Abstract

Mycoplasma contamination is a persistent issue in laboratories as these organisms are resistant to many common antibiotics and can compromise the integrity of biological products. Routine screening is therefore essential to maintain consistent product quality. This study validates the accuracy, precision, and limit of detection (LOD) of the Universal Mycoplasma Detection Kit (UMDK) (ATCC® 30-1012K™), a PCR-based assay used at ATCC. The assay reliably detected known positive samples with amplicon sizes within ±10% of their expected values. Precision was demonstrated by coefficient of variation (%CV) values below 0.45%, and linearity was shown by proportional band intensity across serial dilutions. In a comparative study of 786 samples, the assay showed consistent performance across users and instruments and produced results concordant with traditional visual culture detection methods. The UMDK detected as little as 20 femtograms of mycoplasma DNA, confirming its high sensitivity. These findings support the UMDK as a reliable tool for routine mycoplasma screening in quality control workflows.

Introduction

Accurate and reliable testing of microbial contamination in biological products is essential for maintaining product quality and safety. Although many types of microorganisms may contaminate biological material, mycoplasmas are among the most common and problematic bacterial contaminants in cell culture.[1] Once mycoplasma contamination has occurred, the bacteria are very difficult to eliminate because these organisms are resistant to most antibiotics commonly employed in cell culture.[1] Therefore, frequent testing by a validated method is critical for early detection and initiation of eradication efforts.[1]

Mycoplasma detection in cell cultures has evolved significantly over the past few decades. Early regulatory guidance, such as the FDA's 1993 Points to Consider memo, emphasized culture-based methods as the gold standard.[2] These included direct culture, which involved growing mycoplasmas on specialized media under multiple conditions, and indicator cell line assays, which used DNA staining to visualize contamination in co-cultured mammalian cells.[3] These approaches were chosen because they were well-characterized, reproducible, and capable of detecting viable organisms.[3] At the time, molecular techniques like PCR were not included because they lacked standardized protocols, robust validation, and regulatory acceptance.[4] Concerns about false positives from contamination and false negatives due to poor primer design further limited their use.[4] Over time, as nucleic acid amplification technologies matured and validation frameworks were established, PCR emerged as a highly sensitive and rapid alternative.[5] Modern guidelines (USP <63>, EP 2.6.7) now allow PCR as an acceptable alternative because it offers rapid turnaround and high sensitivity, provided that the assay is properly validated and contamination controls are in place.[6,7]

At ATCC, the Universal Mycoplasma Detection Kit (UMDK) (ATCC® 30-1012K™), a PCR-based assay, is routinely used to test for the presence of mycoplasma in our cell biology and virology products. The kit amplifies target DNA sequences to detect more than 120 *Mycoplasma* and associated bacterial species. The touchdown PCR protocol implemented enhances assay specificity and sensitivity, enabling the detection of contaminants at low levels.

The objective of this assay validation was to demonstrate that the UMDK performs with acceptable accuracy, precision, robustness, linearity, and repeatability when used to detect mycoplasma. In addition, this assay validation (AV) establishes the limit of detection (LOD) of the assay.

Results with Materials and Methods

To assess the accuracy of the UMDK (ATCC® 30-1012K™) for detecting mycoplasma contamination, established kit protocols were followed to test two mycoplasma-positive cell lines (Items A and B) and a common gram negative bacterium (Item C), with the kit's positive and negative controls included to verify assay performance.

Item A – B6YH4 cells infected with *Mycoplasma fermentans*

Item B – BHK-21 cells infected with *Mycoplasma orale*

Item C – Genomic DNA from *Escherichia coli* strain K-12 (ATCC® 10798D-5™)

The LOD of the assay was evaluated by serially diluting *Mycoplasma hominis* DNA (ATCC® 23114D-5™) and spiking 150 femtogram (fg) of each dilution into human genomic DNA extracted from THP-1 cells (ATCC® TIB-202™). Additional serial dilutions were prepared down to approximately 10 fg and processed using the same procedure. The LOD was defined as the lowest DNA amount that consistently produced a visible amplification band across all nine replicates, as independently assessed by three biologists through visual inspection of gel images captured on the Bio-Rad Gel Doc System under a fixed 2.00-second exposure time.

A. Accuracy

Accuracy of the UMDK was demonstrated by the positive detection of *Mycoplasma* for Items A and B and no detection of *Mycoplasma* for Item C. Accuracy was also evaluated by measuring the amplicon size of Items A and B using the Agilent 2100 Bioanalyzer Instrument with the Agilent 1000 DNA kit, which has a manufacturer-specified amplicon sizing accuracy of ±10%. The expected amplicon size of Items A and B are 465 bp and 463 bp, respectively (Table 1).

Table 1: Expected amplicon size of Items A and B

Item	Expected Size (bp)	Minus 10% cut-off	Plus 10% cut-off
Item A	465	419	512
Item B	463	417	509

Items A and B, along with the positive control, produced a single positive amplicon band when tested by three independent biologists. In contrast, all known mycoplasma-negative samples, Item C, and the negative control showed no detectable amplicon across all evaluations. Amplicon sizes for Items A and B were confirmed for each replicate using the Agilent 1000 DNA kit; each AV run represents a different biologist (Table 2, Table 3).

Table 2: Estimated amplicon size of Item A replicates

AV Run	Actual Size (bp)	Replicate	Estimated size by Agilent (bp)	Within ±10% range
1	465	1	475	Yes
		2	473	Yes
		3	471	Yes
2	465	1	470	Yes
		2	470	Yes
		3	472	Yes
3	465	1	470	Yes
		2	471	Yes
		3	470	Yes

Table 3: Estimated amplicon size of Item B replicates

AV Run	Actual Size (bp)	Replicate	Estimated size by Agilent (bp)	Within ±10% range
1	463	1	482	Yes
		2	479	Yes
		3	477	Yes
2	463	1	476	Yes
		2	476	Yes
		3	478	Yes
3	463	1	478	Yes
		2	477	Yes
		3	477	Yes

To further evaluate the assay's accuracy, eight of the most commonly encountered mycoplasma contaminants in cell culture were tested in triplicate.[1] These included *Mycoplasma arginine* DNA (ATCC® 23383D™), *M. fermentans* (ATCC® 19989D™), *M. hominis* (ATCC® 23114D™), M. *hyorhinis* (ATCC® 17981D™), *M. orale*(ATCC® 23714D™), *M. pirum* (ATCC® 25960D™), *M. salivarium* (ATCC® 23046D™), and *Acholeplasma laidlawii* (ATCC® 23206D™). For each species, 15 µL of purified DNA was spiked into the UMDK assay reaction and the experiment was performed in triplicate. All species produced a visible band at approximately 464 base pairs, confirming successful amplification of the target sequence. Consistent detection across all three replicates demonstrates the assay's broad applicability and reliability for identifying diverse mycoplasma species.

Accuracy results met predefined acceptance criteria. The UMDK successfully amplified known mycoplasma-positive samples and showed no amplification of the gram-negative bacterial DNA, confirming assay specificity. All three biologists correctly identified positive and negative samples upon gel visualization. Amplicon sizing was within the manufacturer's specification tolerance and confirmed amplification of the intended mycoplasma target based on sequencing data. Within this AV, the sizing accuracy was within ±2.5% of the expected size range *Mycoplasma fermentans* and within ±4.5% of the expected size range for *Mycoplasma orale*.

B. Precision & Repeatability

To assess the precision and repeatability of the UMDK, the %CV (standard deviation/mean x 100) was calculated for the estimated amplicon sizes for Items A and B within a single analytical run (Table 4 and Table 5). Each experiment was repeated in triplicate by three independent biologists (AV Run). Based on the manufacturer's specification, the acceptance criterion for %CV was <5%CV for within-run testing.

Observed precision and repeatability results met this criterion. Observed %CVs were ≤0.45%, with an overall mean %CV of 0.23% across all items and replicates. These results demonstrate that the UMDK produces highly consistent amplicon sizing and exhibits excellent precision and repeatability for amplification of the target mycoplasma sequence.

Table 4: Precision and repeatability assessment of Item A

AV Run	Actual Size (bp)	Replicate	Agilent Size (bp)	Standard Deviation (STDEV.P)	Mean	%CV (Std.Dev/Mean) x 100
1	465	1	475	1.633	473.0	0.35%
		2	473			
		3	471			
2	465	1	470	0.943	470.7	0.20%
		2	470			
		3	472			
3	465	1	470	0.471	470.3	0.10%
		2	471			
		3	470			

Table 5: Precision and repeatability assessment of Item B

AV Run	Actual Size (bp)	Replicate	Agilent Size (bp)	Standard Deviation (STDEV.P)	Mean	%CV (Std.Dev/Mean) x 100
1	463	1	482	2.055	479.3	0.43%
		2	479			
		3	477			
2	463	1	476	0.943	476.7	0.20%
		2	476			
		3	478			
3	463	1	478	0.471	477.3	0.10%
		2	477			
		3	477			

C. Linearity

Linearity is demonstrated when assay results are directly proportional to analyte concentration. Here, linearity was assessed by visual evaluation of LOD samples, with decreasing mycoplasma concentrations expected to produce correspondingly reduced band intensities. Linearity was established by the consistent decrease in band intensity observed across the dilution series, spanning approximately 150 fg to 10 fg of mycoplasma DNA per sample reaction (Figure 1).

These results demonstrate that the UMDK exhibits linear performance, with assay signal intensity directly correlated with mycoplasma concentration. Higher concentrations consistently produced stronger bands on the visualized gel, while lower concentrations yielded proportionally reduced band intensity, confirming linear assay response across the tested range.

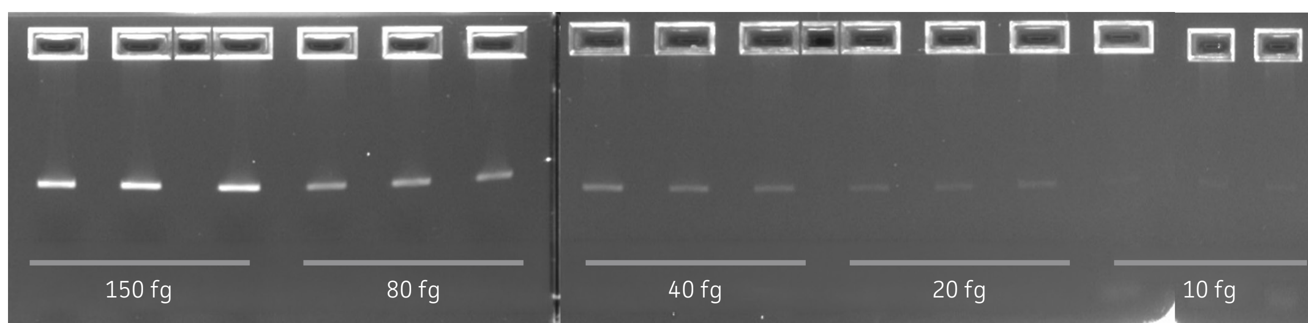

Figure 1: Representative LOD dilution series demonstrating assay linearity. Reactions were spiked with decreasing amounts of *M. hominis* DNA (150 fg to 10 fg).

D. Robustness and Reproducibility

Robustness and reproducibility of the assay were demonstrated through deliberate variation of key parameters, including operators, run days, and run times. Minimal to no variability was observed among replicates for all test items and LOD samples, both within individual runs and across different operators. Consistent and identical results were obtained across all runs and replicates for Items A through C and the controls.

In addition, we conducted side-by-side quality control testing of biological products using both the PCR-based UMDK and traditional direct and indirect culture methods (PTC). This comparative analysis further demonstrates the reliability and robustness of the PCR assay. Here, a total of 786 cell line samples were tested using both PCR and PTC methods. Among these samples, 3 that tested positive by PTC also tested positive by PCR, and 783 samples that tested negative by PTC also tested negative by PCR, demonstrating concordant detection.

Collectively, these results demonstrate that the performance of the PCR-based UMDK is comparable to that of traditional culture methods while offering the advantages of faster turnaround time and higher sensitivity. The high level of agreement between the two methods supports the use of PCR for routine mycoplasma screening.

E. Limit of Detection (LOD)

To determine the LOD of the UMDK, a mycoplasma DNA spike-in dilution series was prepared in a human genomic DNA background. Here, human DNA was extracted from the THP-1 cell line (ATCC® TIB-202™). *M. hominis* DNA (ATCC® 23114D-5™) was quantified using the Qubit dsDNA Broad Range Assay Kit (Thermo Fisher Scientific) and serially diluted 1:2 down to 0.0000381 ng/µL (= 38.1 fg/µL). The five lowest *M. hominis* DNA dilutions were then spiked into the human DNA at a 1:10 (v/v) ratio (Table 6). Five separate spike mixtures were prepared and tested, yielding a total of 15 PCR reactions across three runs.

Table 6: Limit of detection – DNA dilution

Sample No.	Mycoplasma DNA Concentration (ng/µL)	Final Mycoplasma DNA Concentration after 1:10 dilution in Human DNA (fg/µL)	Final Mycoplasma DNA Concentration in 25 µL PCR Reaction (fg)
1	0.0006104	61.04	152.59
2	0.0003052	30.52	76.29
3	0.0001526	15.26	38.15
4	0.0000763	7.63	19.07
5	0.0000381	3.81	9.54

All LOD samples were run in triplicate alongside Items A-C and the assay controls. Following gel visualization, each biologist independently reviewed the image and classified each sample as positive or negative based on the presence or absence of a visible band. All three biologists observed a positive band present for all three replicates for LOD sample numbers 1 through 4, corresponding to mycoplasma DNA inputs ranging from 153 fg to 19 fg. For LOD sample 5 (10 fg), one biologist reported no visible band in one of the three replicates (Table 7). Based on the collective visual assessment across analysts and replicates, the LOD of the UMDK under the conditions tested in this AV was determined to be 20 fg of mycoplasma DNA per reaction.

Table 7: Limit of detection – Experimental results

LOD Sample No.	Final Mycoplasma DNA Concentration in 25 µL PCR Reaction (fg)	AV 1 Visual Band Detection Result (positive/total)[1]	AV 2 Visual Band Detection Result (positive/total)	AV 2 Visual Band Detection Result (positive/total)
1	152.59	3/3	3/3	3/3
2	76.29	3/3	3/3	3/3
3	38.15	3/3	3/3	3/3
4	19.07	3/3	3/3	3/3
5	9.54	3/3	3/3	2/3

Samples were independently classified as positive or negative by three biologists based on visual detection of band presence.

Conclusions

The UMDK PCR assay demonstrated high accuracy, correctly detecting mycoplasma-positive samples and showing no amplification in negative controls. Amplicon sizes were consistently within manufacturer specified tolerance and confirmed across three independent biologists and multiple mycoplasma species. The assay showed excellent precision, with %CV values well below the 5% acceptance criterion and exhibited clear linearity from 150 fg to 10 fg of mycoplasma DNA.

Robustness and reproducibility were confirmed through consistent results across operators, run days, and test conditions. Comparative testing of 786 samples also showed complete concordance with traditional culture methods. The LOD was established at 20 fg per reaction. Together, these results verify that the UMDK is a sensitive, specific, and reliable assay suitable for routine mycoplasma screening.

References

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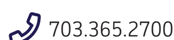

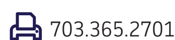

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