MYCOPLASMA RELEASE TEST WITH LOW VOLUME PROTOCOL: FROM SAMPLE TO RESULTS IN LESS THAN 1-HOUR

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INTRODUCTION

Testing for mycoplasma contamination is a required release test for Cell and Gene Therapy (CGT) products as specified in the USP, EP and JP[1-3]. Current compendial methods require 28 days of testing to generate results and are not suitable for short shelf-life products. Alternative nucleic acid test (NAT) methods are available and can reduce the time to results to hours instead of days.

The BIOFIRE® Mycoplasma test is a closed system sample-to-answer nucleic acid test that is designed to report the presence/absence of over 130 mycoplasma species in less than an hour and thereby suitable as a release test for short shelf-life CGT products.

A validated protocol involving pre-processing of 10 mL cell samples allows release testing of CGT products at 1x10^5 cells/mL, following regulatory guidelines with a limit of detection (LOD) of ≤ 10 CFU/mL[4]. As the availability of sample for testing is limited in CGT manufacturing, a protocol which minimizes the sample volume while retaining cells to comply with pharmacopeial guidance is needed.

PURPOSE

Present a new Low Volume protocol that allows the inclusion of mammalian cells from high cell density products thus aligning with the upcoming EP guideline[5] whilst providing the required level of detection (≤ 10 CFU/mL) as a mycoplasma release test.

MATERIALS AND METHOD

BIOFIRE® FILMARRAY® 2.0 Industry System

The BIOFIRE FILMARRAY 2.0 Industry system utilizes the FILMARRAY® 2.0 instrument and closed 'lab-in-a-pouch' concept to detect the presence of over 130 different mycoplasma species (Figures 1 and 2). The disposable BIOFIRE® Mycoplasma pouch contains all the reagents for automated cell lysis, nucleic acid purification, reverse transcription, first and second stage nested PCR and analyte detection (Figure 2). Several controls are integrated into the pouch to ensure the quality of the results including a total process control, reverse transcription control, and PCR I and II controls. The instrument & software (21 CFR Part 11 compliance ready) process the pouch and provide presence/absence results in less than an hour.

Figure 1. FILMARRAY 2.0 Industry standard configuration with 2 instruments; up to 8 instruments can be connected to a single PC.

2A

2B

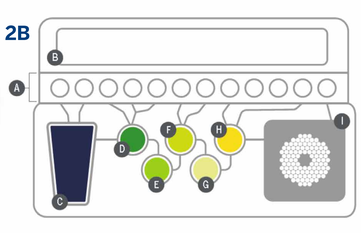

Figure 2A: BIOFIRE Mycoplasma pouch.
Figure 2B: Pouch diagram. (A) Fitment with freeze-dried reagents (B) Plungers-deliver reagents to blisters (C) Sample lysis and bead collection (D) Wash (E) Magnetic bead collection blister (F) Elution (G) Multiplex Outer PCR blister (H) Dilution blister (I) Inner Nested PCR array.

Sample Pre-Processing Protocol

The Low Volume protocol comprises 2 steps: a first step to normalize the cell density and a second step to concentrate the sample prior to loading into the pouch (Figure 3).

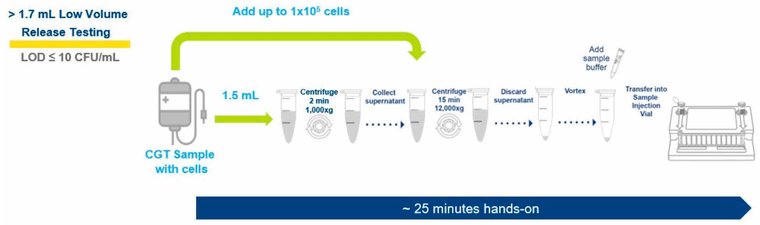

Figure 3: BIOFIRE FILMARRAY 2.0 Industry system CGT release protocol for mycoplasma testing. Low volume pre-processing for 1.7 mL samples – Double centrifugation.

Study outline

Freshly grown Jurkat cells (Clone E6-1) prepared at 1x10^7 cells/mL and stored at +4°C for maximum 48 hours were used as a representative cell matrix for CGT.

The performance of the Low Volume protocol was assessed by evaluating the Limit of Detection, Specificity (including Inclusivity & Exclusivity) as well as Robustness and Ruggedness.

Mycoplasma inoculation was carried out with viable titrated reference stocks displaying a low ratio GC/CFU (Genome Copy/ Colony Forming Unit) below 10, from either the American Type Culture Collection (ATCC) or Mycosafe. Bacterial strains were sourced from our internal stocks.

RESULTS

Limit Of Detection

A Pre-LOD study was run by testing mycoplasma strains at concentrations spanning 10 CFU/mL. The LOD confirmation was then performed on 24 replicates with expected detection of at least 95% (≥ 23/24 detected) (Table 1).

Individually inoculated samples from at least three independent stock dilution events, 2 operators and three different days were used. In addition, up to 8 instruments and 13 lots of pouches were used.

Table 1: Results of Pre-LOD and LOD study performed on sample of 1x10^7 Jurkat cells/mL using the Low Volume protocol.

Organism	Titrated Stock Reference	Pre-LoD (CFU/mL)	Confirmed LOD (CFU/mL)	Detection rate	
				Number of positive replicates	Percent %
Unspiked sample	NA	NA	NA	0/22	0
Acholeplasma laidlawii	ATCC / 23206-TTR	3	3	23/24	95
Mycoplasma arginini	ATCC / 23838-TTR	0.1	0.1	24/24	100
Mycoplasma fermentans	ATCC / 19989-TTR	3	3	24/24	100
M. hyorhinis BTS7	ATCC / 17981-TTR	3	3	24/24	100
M. hyorhinis Alpha	Mycosafe / ATCC 29052	10	10	23/24	95
Mycoplasma orale	ATCC / 23714-TTR	1	1	23/24	95
Mycoplasma salivarium	ATCC / 23064-TTR	3	3	24/24	100
Mycoplasma pneumoniae	Mycosafe / ATCC 15531	10	10	24/24	100

No false positive result was reported and appropriate sensitivity (10 CFU/mL or below) was reached for all mycoplasma strains tested with a detection rate of 95% or above on 24 replicates, as per Pharmacopeia requirements[2].

Specificity (Inclusivity and exclusivity)

The inclusivity study evaluated the detection of three additional mycoplasma strains *Mycoplasma bovis, Mycoplasma hominis* and *Ureaplasma urealyticum* performed at 10 or 100 CFU/mL in triplicate (Table 2).

Table 2: Results of the inclusivity study performed at 10 CFU/mL on sample of 1x10^7 Jurkat cells/mL using the Low Volume protocol.

Organism	Titrated Stock Reference	Test Concentration (CFU/mL)	Detection rate
Mycoplasma bovis	Mycosafe / ATCC 25523	10	3/3
Mycoplasma hominis	ATCC / 27545-TTR	10	3/3
Ureaplasma urealitycum	Mycosafe / ATCC 27618	10	0/3
		100	3/3

Inclusivity at 10 CFU/mL was demonstrated for both *M. hominis* and *M. bovis* while U. urealyticum was inclusive at a lower sensitivity of 100 CFU/mL.

The exclusivity study evaluated on triplicate samples whether unexpected amplification occurred of organisms from the Bacilli and Clostridia classes. The organisms used represent closely phylogenetically related species to mycoplasmas and were tested at high concentrations (Table 3).

Table 3: Results of the exclusivity study performed at different concentrations of microorganisms on sample of 1x10^7 Jurkat cells/mL using the Low Volume protocol.

Organism Class	Organism	Source	Test Concentration (CFU/mL)	Mycoplasma Test Result	
				Detection Rate	Summary
Bacilli	*Bacillus cereus*	ATCC 14579	1.39x10^5	0/3	Not Detected
	Bacillus thuringiensis	ATCC 35646	1.16x10^5	0/3	Not Detected
	Lactobacillus plantarum	ATCC 8014	7.70x10^6	0/3	Not Detected
	Streptococcus pneumoniae	ATCC 49619	7.87x10^4	0/3	Not Detected
	Streptococcus pyogenes	ATCC 19615	2.64x10^5	0/3	Not Detected
Clostridia	*Clostridium perfringens*	ATCC 13124	1.30x10^7	0/3	Not Detected

Robustness and ruggedness

The robustness evaluation of the Low Volume protocol was performed using *A. laidlawii* and *M. hyorhinis* cultivable strains used at 10 CFU/mL. All triplicates of fifteen variations in method parameters, such as variations in sample pre-processing steps, sample buffer ratio or different features of cell matrices (e.g., cellular state, cell density) were positive, supporting robustness of this protocol.

The ruggedness evaluation verified that testing of the same sample by different analysts, different instruments, different reagent lots and several test days did not cause greater than expected variability in system performance with percent agreement between test results and expected results ≥ 95% for each sample.

CONCLUSION

The performance of the new Low Volume protocol was successfully evaluated on the BIOFIRE FILMARRAY 2.0 Industry System by performing LOD, Specificity, Robustness and Ruggedness studies. The results of these studies confirm this new protocol is suitable for mycoplasma release testing according to Pharmacopeia requirements[1-3] with a sensitivity of 10 CFU/mL. As this new protocol requires limited sample volume for testing and provides flexibility to include mammalian cells in the test article, it is ideal for cell and gene therapy applications.

ACKNOWLEDGEMENTS:

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