Development of a sensitive, robust, and rapid mycoplasma test method

C. Martino and J. Mariano
Bionique Testing Laboratories, 156 Fay Brook Drive, Saranac Lake, NY 12983

Introduction

The need for a sensitive, robust, and rapid in-process mycoplasma test was self-evident to meet short turnaround time constraints without compromising quality.

Bionique developed a test method that can detect down to 10 CFU/mL for most species, is robust enough to accommodate the wide variety of materials submitted to a contract testing laboratory, and has a testing time of only 3 days.

This validation was designed as an in-process test with consideration for the qualitative assay parameters recommended in EP 2.6.7[1], and PDA Technical Reports 33[2] and 50[3].

Objective

To design and validate a PCR-based method "as good, or better than" the traditional direct and indirect culture methods for detecting viable mycoplasma contamination in a broad range of products.

Primary goals:

- Establish the ability to recover and detect viable mycoplasma, not just previously purified gDNA
- Detect down to 10 CFU/mL for most species with minimal optimization
- Minimize interference from diverse matrices
- Provide system suitability data for different matrices
- Eliminate the potential for false positives and false negatives
- Translatable to a final product release test following a product specific validation, including method optimization if necessary

Study Design

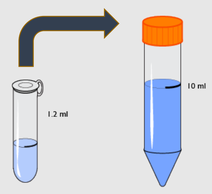
Spike a representative matrix (approx. 10^6/mL CHO) with four molecularly divergent[4] species of viable mycoplasma also identified as common contaminants[5] including a:

- Known aggregator
- Slow grower
- Species historically considered non-cultivable and cell associated

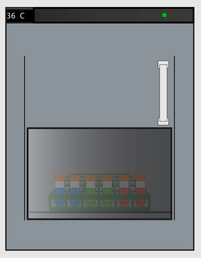
All control stocks were well characterized for CFU/mL, species identity, and had GC/CFU ratios ≤ 10.

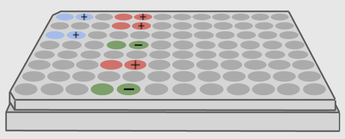
Inoculate these test articles into Bionique's proprietary mycoplasma broth media, enrich for three days, and analyze cultures using an optimized protocol for the MycoSEQ™ assay.

Evaluate the ability to achieve required sensitivity, specificity, repeatability, and ruggedness, as well as comparability with the FDA Points to Consider method[6].

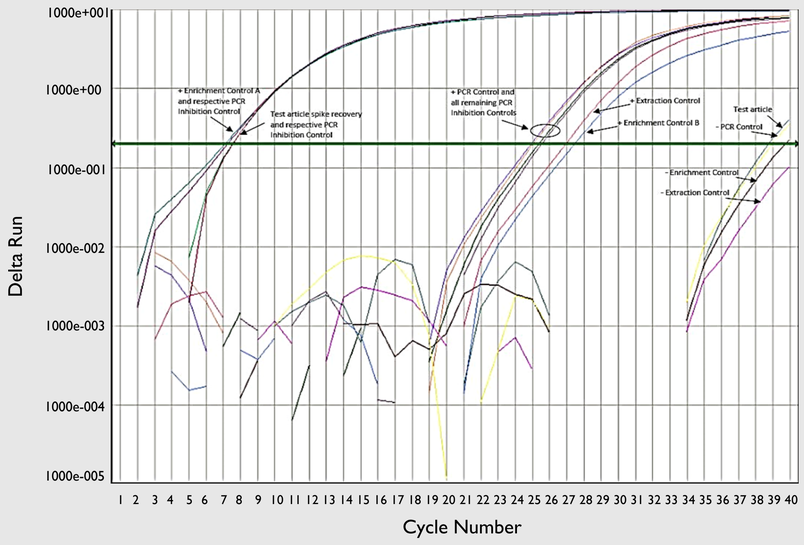


3 Days





Delta Run vs. Cycle



MycoSEQ™ is a trademark of Thermo Fisher Scientific Inc. (Waltham, MA, USA).

Execution

Validation performed across…

→ 2 analysts
→ 4 days
→ 4 enrichment cultures / biological replicates per species
→ 8 isolations / process replicates per species
→ 24 reactions / technical replicates per species

Acceptance Criteria

Test	Ct	Tm	D.V.
Positive test article	≤ 35	75°C - 81°C	≥ 0.1
Positive PCR control	24-26	~ 84°C	≥ 0.1
Positive extraction control	25-27	~ 84°C	≥ 0.1
PCR Inhibition control	No more than 2 Ct above the Positive PCR control	N/A	N/A
All negative test articles and controls	≥ 36 and/or	< 75°C or >81°C	N/A

Results

Challenge	Hybrid PCR Validation	Points-to-Consider Direct Culture	Points-to-Consider Indicator Cell Assay
10 CFU/mL A. laidlawii in CHO	24 out of 24 replicates +	+	+
10 CFU/mL M. hyorhinis in CHO	24 out of 24 replicates +	+	+
10 CFU/mL M. orale in CHO	24 out of 24 replicates +	+	-
80 CFU/mL M. pneumoniea in CHO	24 out of 24 replicates +	+	-

Day	Species spiked into CHO	Analyst	Enrichment Culture	Isolation	Reaction	Ct
Day 2	A. laidlawii	A	1	1	1	7.2
	A. laidlawii	A	1	1	2	7.5
	A. laidlawii	A	1	1	3	7.6
	A. laidlawii	A	1	2	1	8.8
	A. laidlawii	A	1	2	2	9.5
	A. laidlawii	A	1	2	3	9.3
	A. laidlawii	A	2	1	1	10.4
	A. laidlawii	A	2	1	2	10.8
	A. laidlawii	A	2	1	3	11.0
	A. laidlawii	A	2	2	1	8.4
	A. laidlawii	A	2	2	2	8.8
	A. laidlawii	A	2	2	3	8.9
Day 4	A. laidlawii	B	3	1	1	7.9
	A. laidlawii	B	3	1	2	8.2
	A. laidlawii	B	3	1	3	8.2
	A. laidlawii	B	3	2	1	8.2
	A. laidlawii	B	3	2	2	8.5
	A. laidlawii	B	3	2	3	8.4
	A. laidlawii	B	4	1	1	8.1
	A. laidlawii	B	4	1	2	8.3
	A. laidlawii	B	4	1	3	8.4
	A. laidlawii	B	4	2	1	8.0
	A. laidlawii	B	4	2	2	8.3
	A. laidlawii	B	4	2	3	8.3
Day 2	M. hyorhinis	A	1	1	1	15.7
	M. hyorhinis	A	1	1	2	15.7
	M. hyorhinis	A	1	1	3	15.7
	M. hyorhinis	A	1	2	1	15.6
	M. hyorhinis	A	1	2	2	15.8
	M. hyorhinis	A	1	2	3	15.9
	M. hyorhinis	A	2	1	1	16.8
	M. hyorhinis	A	2	1	2	16.8
	M. hyorhinis	A	2	1	3	16.8
	M. hyorhinis	A	2	2	1	16.8
	M. hyorhinis	A	2	2	2	16.8
	M. hyorhinis	A	2	2	3	16.8
Day 4	M. hyorhinis	B	3	1	1	17.1
	M. hyorhinis	B	3	1	2	17.1
	M. hyorhinis	B	3	1	3	17.1
	M. hyorhinis	B	3	2	1	17.1
	M. hyorhinis	B	3	2	2	17.1
	M. hyorhinis	B	3	2	3	17.1
	M. hyorhinis	B	4	1	1	15.5
	M. hyorhinis	B	4	1	2	15.6
	M. hyorhinis	B	4	1	3	15.5
	M. hyorhinis	B	4	2	1	15.6
	M. hyorhinis	B	4	2	2	15.6
	M. hyorhinis	B	4	2	3	15.6

Day	Species spiked into CHO	Analyst	Enrichment Culture	Isolation	Reaction	Ct
Day 1	M. orale	A	1	1	1	13.6
	M. orale	A	1	1	2	13.8
	M. orale	A	1	1	3	13.7
	M. orale	A	1	2	1	12.5
	M. orale	A	1	2	2	13.8
	M. orale	A	1	2	3	13.8
	M. orale	A	2	1	1	14.3
	M. orale	A	2	1	2	14.4
	M. orale	A	2	1	3	14.4
	M. orale	A	2	2	1	14.4
	M. orale	A	2	2	2	14.5
	M. orale	A	2	2	3	14.5
Day 3	M. orale	B	3	1	1	15.5
	M. orale	B	3	1	2	15.6
	M. orale	B	3	1	3	15.7
	M. orale	B	3	2	1	15.2
	M. orale	B	3	2	2	15.4
	M. orale	B	3	2	3	15.4
	M. orale	B	4	1	1	16.6
	M. orale	B	4	1	2	16.6
	M. orale	B	4	1	3	16.6
	M. orale	B	4	2	1	16.6
	M. orale	B	4	2	2	16.6
	M. orale	B	4	2	3	16.7
Day 1	M. pneumoniae	A	1	1	1	23.2
	M. pneumoniae	A	1	1	2	23.2
	M. pneumoniae	A	1	1	3	23.2
	M. pneumoniae	A	1	2	1	26.7
	M. pneumoniae	A	1	2	2	26.7
	M. pneumoniae	A	1	2	3	26.7
	M. pneumoniae	A	2	1	1	23.9
	M. pneumoniae	A	2	1	2	24.1
	M. pneumoniae	A	2	1	3	24.1
	M. pneumoniae	A	2	2	1	31.4
	M. pneumoniae	A	2	2	2	32.3
	M. pneumoniae	A	2	2	3	32.1
Day 3	M. pneumoniae	B	3	1	1	26.6
	M. pneumoniae	B	3	1	2	26.6
	M. pneumoniae	B	3	1	3	26.7
	M. pneumoniae	B	3	2	1	26.8
	M. pneumoniae	B	3	2	2	26.8
	M. pneumoniae	B	3	2	3	26.8
	M. pneumoniae	B	4	1	1	23.1
	M. pneumoniae	B	4	1	2	23.2
	M. pneumoniae	B	4	1	3	23.3
	M. pneumoniae	B	4	2	1	33.6
	M. pneumoniae	B	4	2	2	33.7
	M. pneumoniae	B	4	2	3	34.1

Study Conclusions

This validation study confirmed the ability of the hybrid method described herein to reliably recover and detect viable mycoplasma down to 10 CFU/mL from a representative matrix as well, or better than, the FDA Points to Consider method.

In routine application, a spike recovery qualification is performed on each new matrix to demonstrate system suitability. If a positive signal for mycoplasma is detected, a secondary time point of the enrichment culture can be analyzed to determine whether a viable contamination exist. These two measures, combined with a comprehensive control panel, eliminate the potential for false positives and false negatives.

Matrices that have experienced recovery or PCR inhibition were successfully addressed by applying small remediation measures.

Scaling and optimizing this type of method for final product release testing has proven successful, including the ability to achieve sensitivity of 10 CFU/mL for all species.

- ☑ Required Sensitivity
- ☑ Specificity
- ☑ Repeatability
- ☑ Ruggedness
- ☑ Comparability

References

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4. Volokhov, D.V., Simonyan V., Davidson M.K., Chizhikov V.E. (2012) RNA polymerase beta subunit (rpoB) gene and the 16S-23S rRNA intergenic transcribed spacer region (ITS) as complimentary molecular markers in addition to the 16S rRNA gene for phylogenetic analysis and identification of the species of the family Mycoplasmataceae. *Molecular Phylogenetics and Evolution* 62:515-528.
5. Drexler, H.G., Uphoff, C.C. (2002) Mycoplasma contamination of cell cultures: Incidence, sources, effects, detection, elimination, prevention. *Cytotechnology* 39:75-90.
6. CBER, FDA, Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals, Attachment #2 - Recommended Procedures for Detection of Mycoplasma Contamination in Biological Products in Cell Substrates (1993).