# qPCR Strategies for Rapid Mycoplasma Validation and Contamination Investigation


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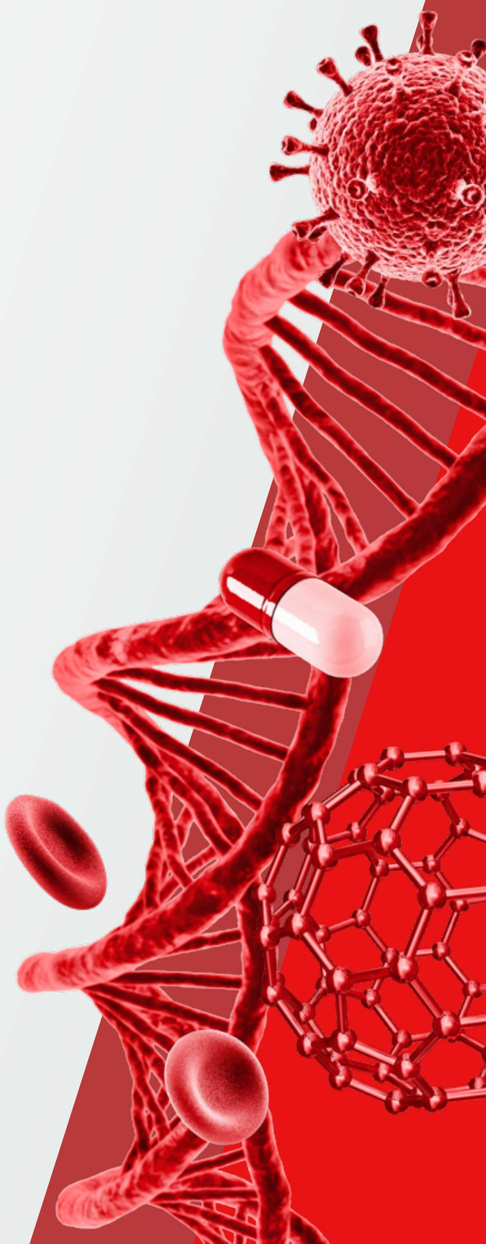

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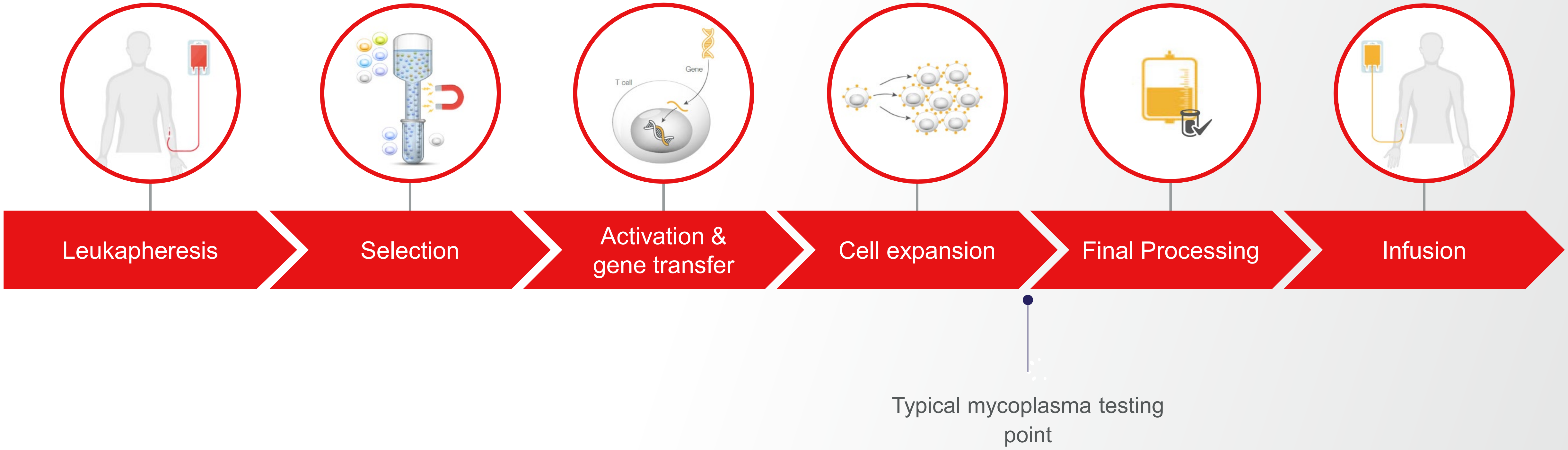
# Rapid Release Testing- Mycoplasma

## Mycoplasma testing in the cell therapy manufacturing process




**Mycoplasma testing is typically done after the cell expansion phase**
Additional testing points may be added based on process specific risk assessment

# FDA, EMA and Japan Guidance on Sample Point for Cell Therapy Mycoplasma Testing

- **FDA:** To maximize the sensitivity of safety testing, it is important that you perform each test at the stage of production at which contamination is most likely to be detected. For example, tests for mycoplasma or adventitious viruses (in vivo or in vitro) should be performed on cell culture harvest material (cells and supernatant) prior to further processing, e.g., prior to clarification, filtration, purification, and inactivation.
  - ***Chemistry, Manufacturing, and Control (CMC) Information for Human Gene Therapy Investigational New Drug Applications (INDs), Guidance for Industry, Center for Biologics Evaluation and Research, January 2020***

- **EMA:** However, testing for mycoplasma should be ideally performed on the harvest of the last cultivation stage prior to further processing e.g. lysis, filtration, washing or purification as post-treatment testing significantly increases the risk to not detect potential contamination with mycoplasma.
  - ***Guideline on quality, non-clinical and clinical requirements for investigational advanced therapy medicinal products in clinical trials, Draft, March 2024***

- **Japan:** Safety may be assured even if virus testing or mycoplasma testing is performed on crude bulks or intermediates, provided that it is justified, e.g., for the reason that, after the process of culturing, amplification of virus or mycoplasma is not expected or can be detected with high sensitivity.
  - ***Ensuring the Quality and Safety of Gene Therapy Products, PSEHB/MDED, July 2023***

# Regulatory guidance continued

**Frequently Asked Questions — Developing Potential Cellular and Gene Therapy Products, Draft Guidance for Industry, November 2024**

- "Some tests are necessary to confirm safety of the product prior to release but are not performed on the final product; such samples should be acquired at the necessary and appropriate manufacturing steps. **For example, tests for mycoplasma and adventitious agents should be performed on cell culture harvest material prior to further processing.** Tests for sterility, endotoxin, and identity should be performed on formulated product in the final labeled container to ensure that microbial contamination and product mix-ups (such as those that may occur during final DP manufacturing steps) do not occur."

# qPCR-based *Mycoplasma* testing:

## Applications of Cycle at Threshold (Ct) analysis

The Cycle at Threshold (Ct) is a quantitative parameter representing the quantity of DNA present in the test sample at the start of the PCR process.

- Viability assessment
- Acceptance criteria for test and control samples
- Estimation of contamination level in positive samples
- Comparison of results between experiments
- GC/CFU ratio
- Sample preparation protocol optimization
- Validation studies:
  - Compare your Ct to expected values for each species
  - Estimation of LLOD by extrapolation from values obtained at 10 GC or CFU/sample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# Example of using qPCR to monitor *Mycoplasma* growth rate and viability

**In this case, *M. arginini* is the model species and the qPCR testing was done with the MycoSEQ assay**

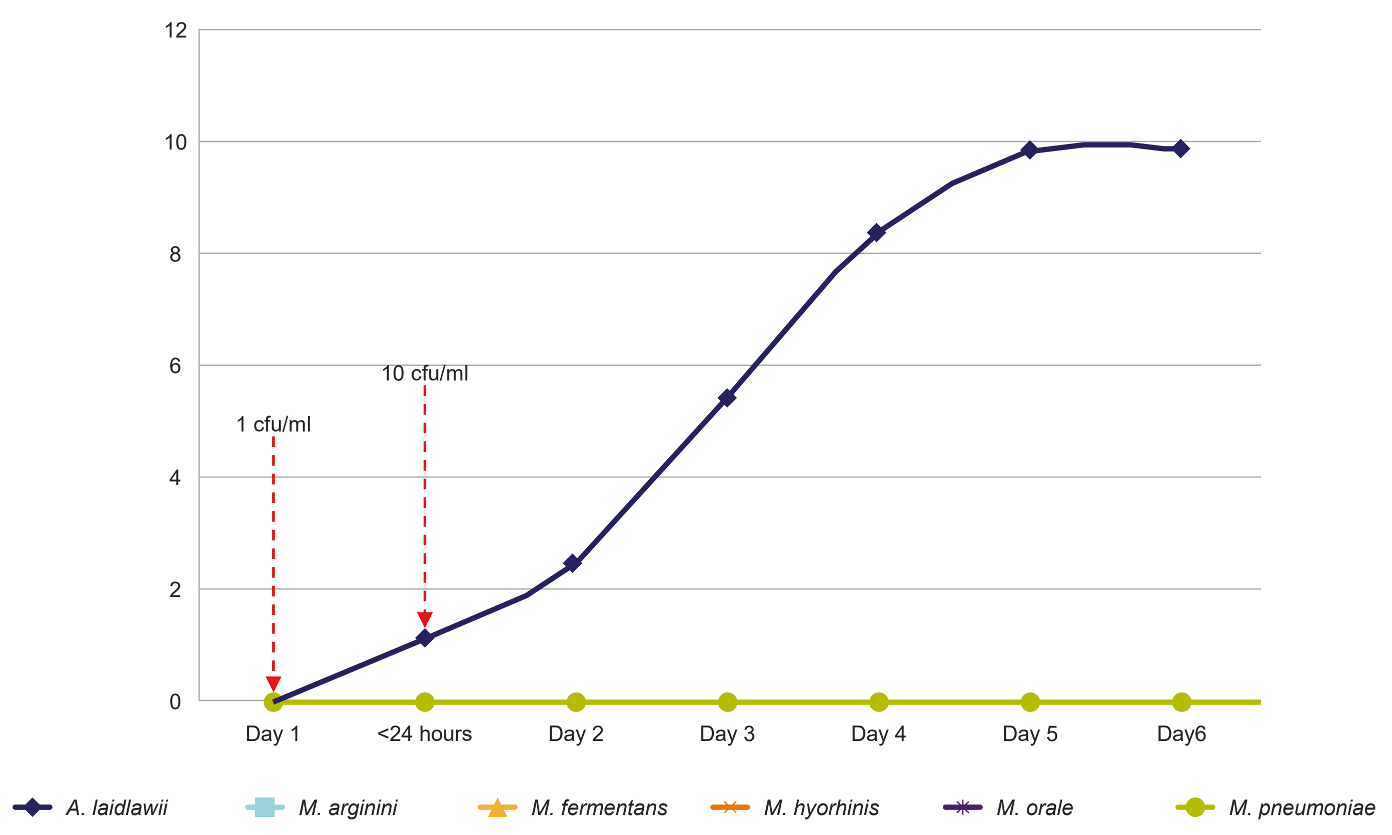
- *Mycoplasma arginini* inoculated into CHO culture at a level near LOD of the MycoSEQ kit
- Samples were tested at 9 timepoints post inoculation
- DNA levels in the samples were estimated using Ct and comparison to a standard curve generated from the qPCR analysis of purified *M. arginini* DNA

| Day | Hours | Positive / Negative | Ct | GC/Rxn | Tm (ºC) | Derivative |
|---|---|---|---|---|---|---|
| 1 | 0 | Low level positive | 35.9 | ~1 | 79.6 | 0.06 |
| | 4 | Low level positive | 35.9 | ~1 | 79.9 | 0.05 |
| | 8 | Low level positive | 36.5 | ~1 | 79.6 | 0.06 |
| 2 | 24 | Positive | 32.0 | ~8 | 79.6 | 0.10 |
| | 28 | Positive | 31.2 | ~16 | 79.6 | 0.12 |
| | 32 | Positive | 28.4 | ~100 | 79.4 | 0.14 |
| 3 | 48 | Positive | 21.4 | ~10,000 | 79.6 | 0.14 |
| | 52 | Positive | 21.9 | ~10,000 | 79.6 | 0.13 |
| | 56 | Positive | 21.7 | ~10,000 | 79.6 | 0.14 |

# Growth kinetic analysis: qPCR time course study

- Test sample: HEK 293 AOF Perfusion Process, conditioned media
- 6 *Mycoplasma* species evaluated
- Inoculation Level: 1 CFU/mL
- Growth monitored over 6 days by MycoSEQ qPCR analysis
- Ct values used to estimate genome copy/CFU level by comparison to standard curve

**_Mycoplasma_ and _Acholeplasma_ growth kinetics in serum-free HEK293 perfusion process**





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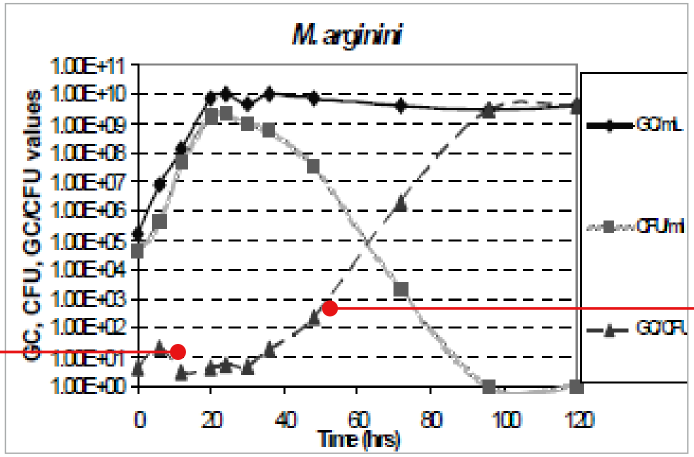
# Use of qPCR analysis for Genomic Copy (GC) to Colony Forming Unit (CFU) ratio assessment

- Mycoplasma can quickly reach log phase growth in liquid media (typically measured with qPCR)
- Stationary phase can be reached quickly, followed by the death phase or a decline in viability
- When measured in log or stationary phase, the GC:CFU ratio is close to 1:1
  At death phase, viable cells (CFU) decrease but GC remains stable
- If CFU assessment (agar plating) is not done at the appropriate time point, PCR or qPCR-based LOD results could be very misleading
- Mycoplasma stocks used for LOD validation must be confirmed to have low GC:CFU ratio
- qPCR sensitivity for DNA is not impacted by viability loss

MycoSEQ enables detection of 10 CFU/mL at 1:1 GC:CFU ratio

MycoSEQ enables detection of 10 GC/mL purified DNA



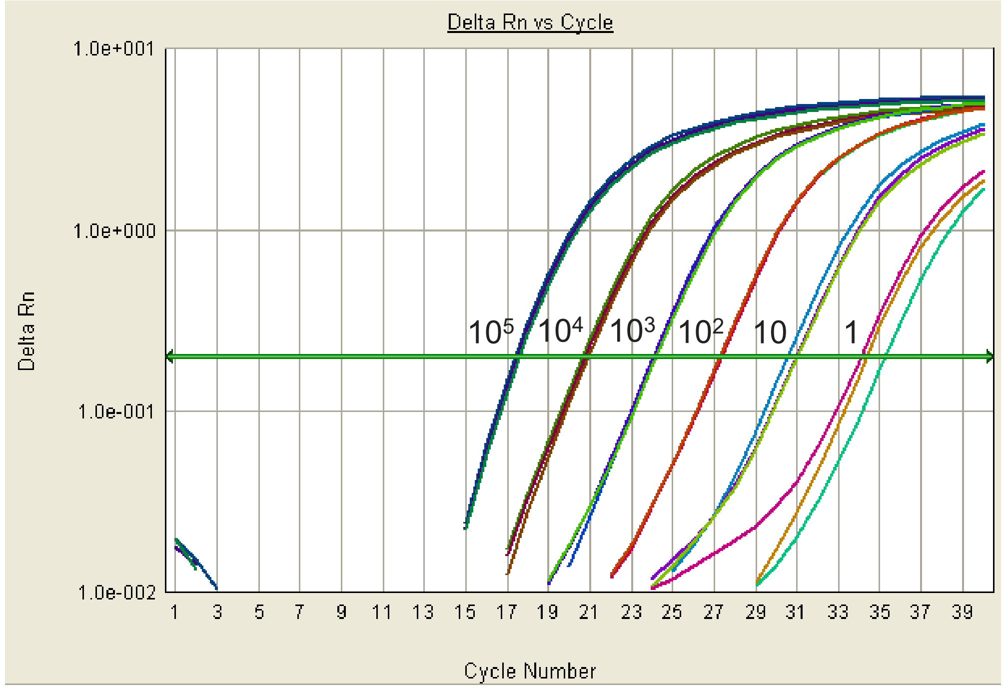

LOD results can be misleading if mycoplasma stocks with high GC: CFU ratios are used in validation

Dabrazhynetskaya A et al. (2011) J Appl Microbiol 111, 904–914.

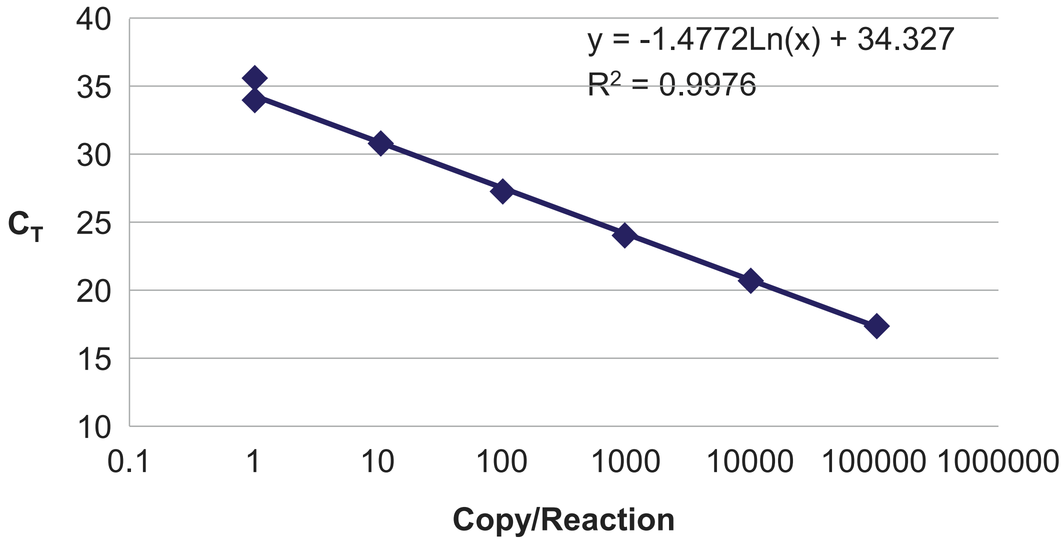
# MycoSEQ assay range and linearity analysis

## *M. arginini*

### Analysis of a 10-fold dilution series of purified *M. arginini* DNA




### Curve generated from 10-fold dilution series





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# Results comparison: $C_t$ Data can be compared from experiment to experiment and lab to lab

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10 mL test samples, spiked with *M. arginini* as purified DNA (GC/mL) or cells (CFU/mL)

**Results from Mycosafe validation, 2009, 10 GC/mL:**

| Mycoplasma species (type strain) | Total number tests/positive reactions | Percent positive | Mean $C_t$ (n=24) |
|---|---|---|---|
| *M. arginini* G230$^T$ | 24/24 | 100 | 30.9 |

**Results from Mycosafe validation, 2009, 10 CFU/mL:**

| Mycoplasma species (type strain) | Total number tests/positive reactions | Percent positive | Mean $C_t$ (n=24) |
|---|---|---|---|
| *M. arginini* G230$^T$ | 4/4 | 100 | 32.8 |

Mycoplasma stocks and DNA prepared by **Mycosafe**

**Results from customer validation, 2011, 8 GC/mL:**

| Mycoplasma species (type strain) | Total number tests/positive reactions | Percent positive | Mean $C_t$ (n=24) |
|---|---|---|---|
| *M. arginini* | 24/24 | 100 | 31.1 |

**Results from customer validation, 2011, 8 CFU/mL:**

| Mycoplasma species (type strain) | Total number tests/positive reactions | Percent positive | Mean $C_t$ (n=24) |
|---|---|---|---|
| *M. arginini* | 24/24 | 100 | 31.5 |

Mycoplasma stocks and DNA prepared by **Bionique**

**Results from Bionique validation, 2023, 10 CFU/mL:**

| Mycoplasma species (type strain) | Total number tests/positive reactions | Percent positive | Mean $C_t$ (n=24) |
|---|---|---|---|
| *M. arginini* | 24/24 | 100 | 30.42 |

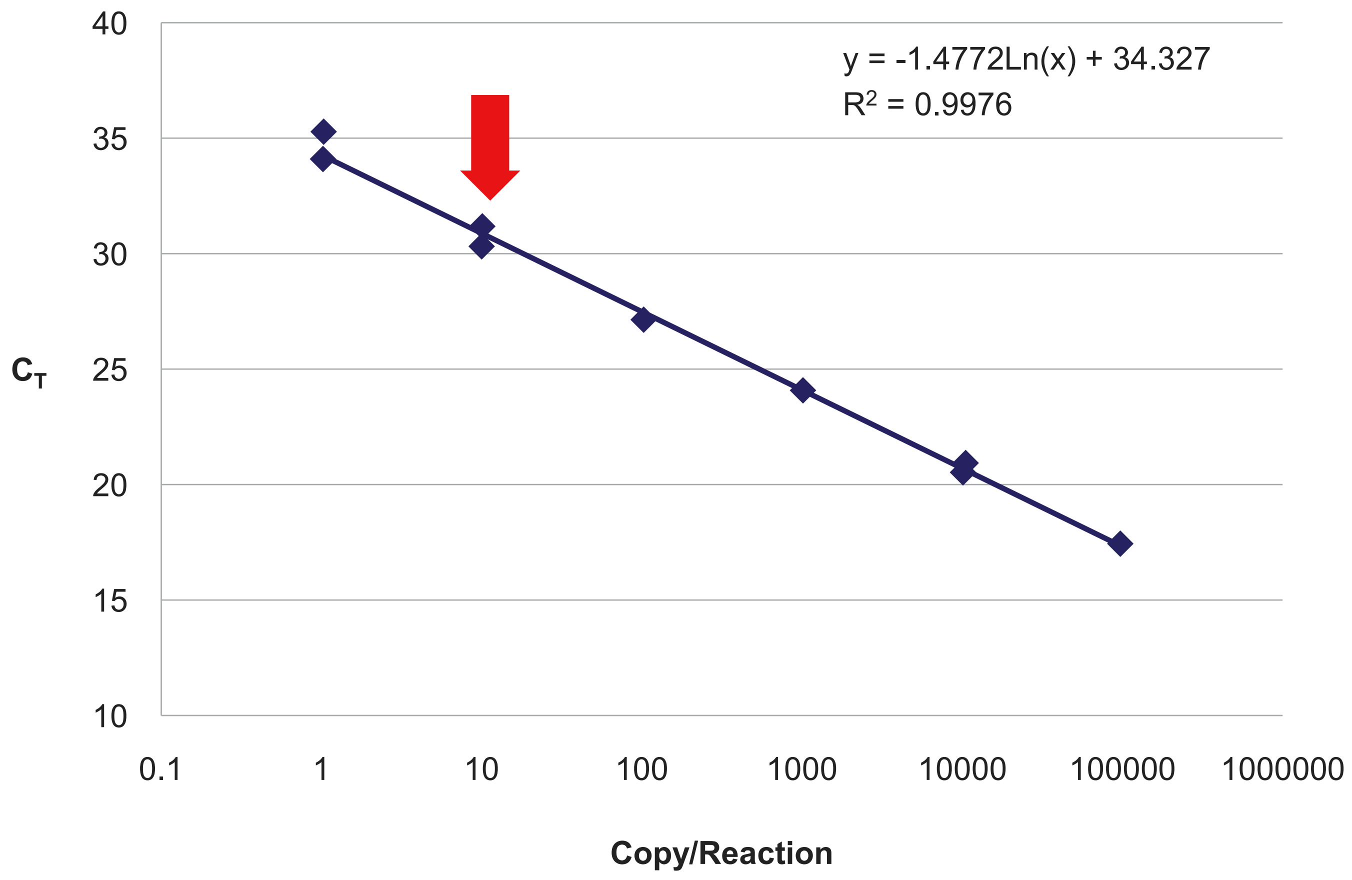
Mycoplasma stock prepared by **ATCC**

T = Type strain

# Validation study results comparison: *M. arginini*

- Ct value from studies in previous slide compared to standard curve, *M. arginini* DNA

- 10 GC/PCR reaction, $C_t$ = 30.5



# Lower Limit of Detection (LLOD) assessment using principles of qPCR

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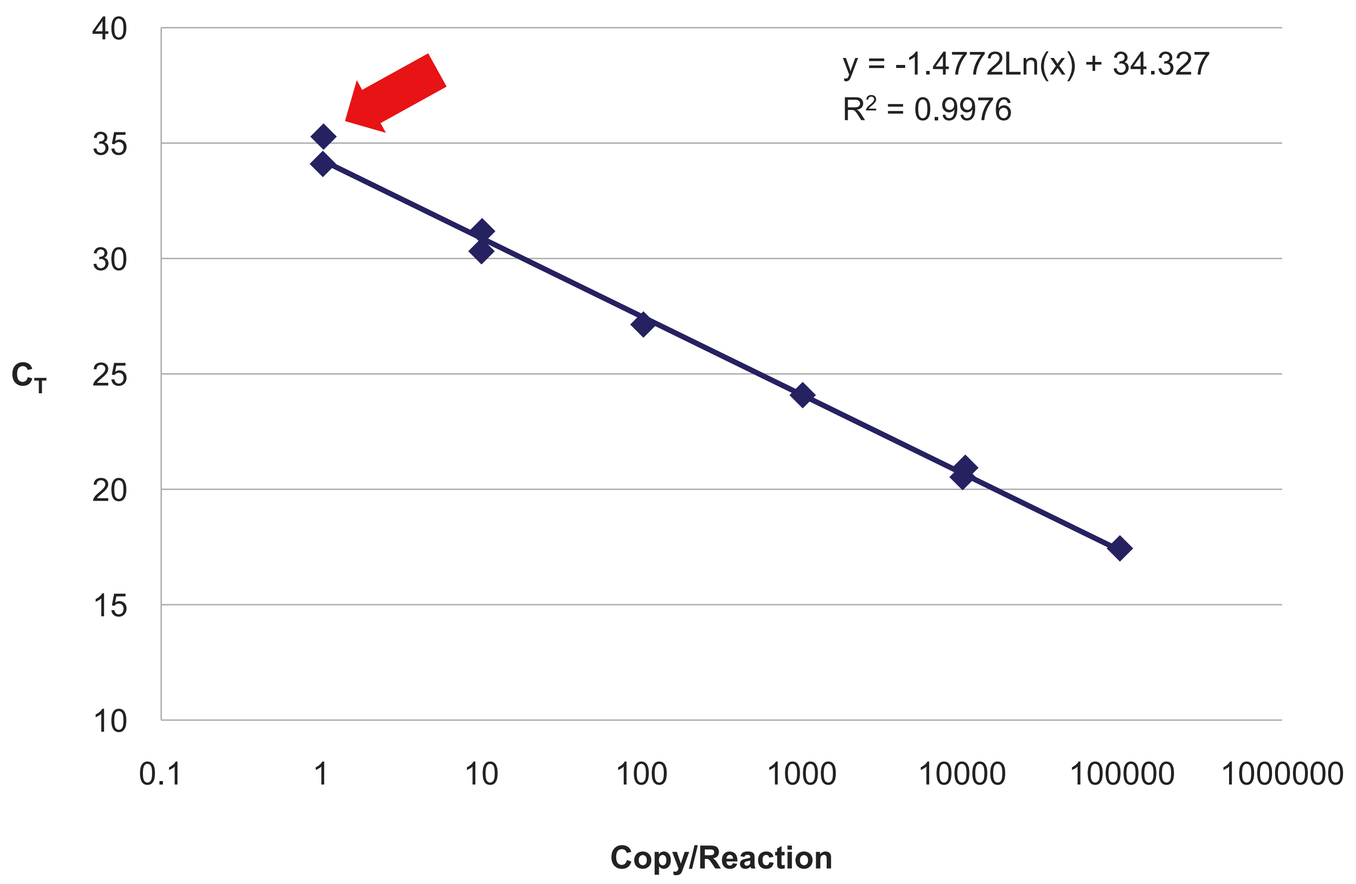
- For efficient, linear qPCR assays, 1 $C_t$ difference represents a 2-fold difference in the starting quantity of the target DNA
- The Applied Biosystems™ MycoSEQ™ assay is highly efficient and linear for all species tested

**Example calculation of LLOD:**
The mean Ct of *M. arginini* at 10 GC/PCR reaction from the previous examples = 31

| | |
|---|---|
| 5 GC/PCR reaction | **32** |
| 2-3 GC/PCR reaction | **33** |
| 1 GC/PCR reaction | **34*** |

*- less than 1 copy of qPCR target is not detectable




**This approach to LLOD estimation is supported by experimental results.**

# Guidance on qualification of spiking stocks for LOD testing

- Using the titer data in the vendor Certificate of Analysis, calculate a dilution plan to dilute to a level of 10 CFU (*Mycoplasma* stocks) or Genome Copies (*Mycoplasma* DNA)/µL
- Dilute the *Mycoplasma* or *Mycoplasma* DNA in DNA Dilution Buffer to 10 CFU or Genome Copies/µL
- Prepare a 1/100 dilution of Discriminatory Positive Control, to a final concentration of 10 copies/µL. To 990 µL of DNA Dilution Buffer, add 10 µL DPC and mix well.
- Test 10 µL of diluted stock or DPC (100 copies total) directly in each qPCR reaction
- Compare the Ct values obtained to the values in this table:

| *Mycoplasma* species | Approximate Ct value, 100 copies/qPCR reaction |
|---|---|
| ***M. arginini*** | 30 |
| ***M. hyorhinis*** | 33 |
| ***M. pneumoniae*** | 31 |
| ***M. gallisepticum*** | 30 |
| ***M. orale*** | 31 |
| ***M. fermentans*** | 31 |
| ***M. synoviae*** | 31 |
| ***M. salivarium*** | 32 |
| ***M. hominus*** | 31 |
| ***A. laidlawii*** | 32 |
| **Discriminatory Positive Control DNA (DPC)** | 33 |

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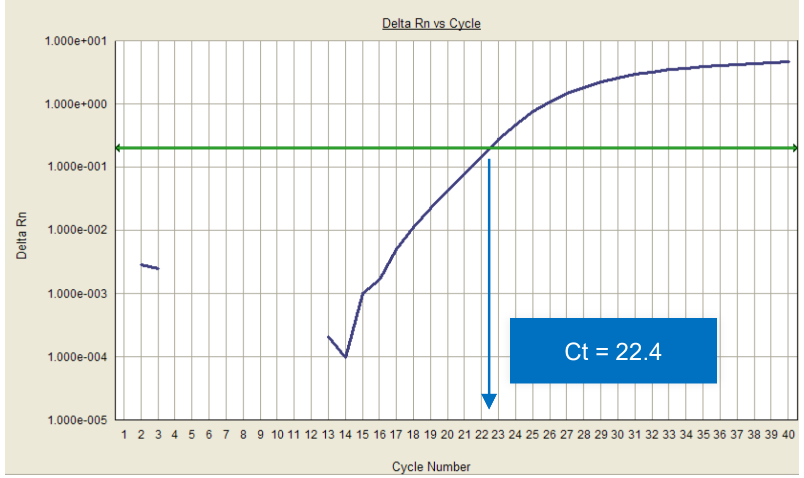
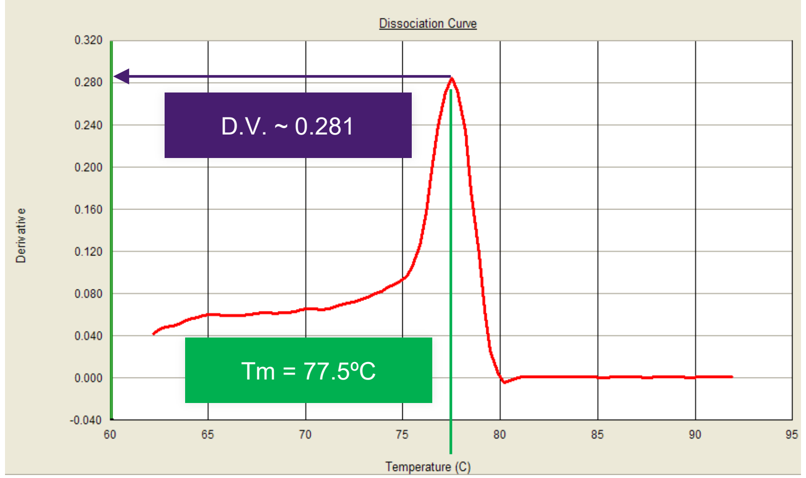
# *Mycoplasma* detection and identification:

## Example from Customer A:

**MycoSEQ assay data**




**MycoSEQ assay result:**
CT = 22.4, Tm = 77.5, D.V = 0.281,
**Positive for *Mycoplasma***

| Well | Sample Name | Detector | Task | Ct | StdDev Ct | Quantity | Mean Qty | StdDev Qty | Filtered | Tm |
|---|---|---|---|---|---|---|---|---|---|---|
| B3 | 2B | Mycoplasma | Unknown | 22.4426 | | | | | | 77.5 °C |

**Reserve of Applied Biosystems™ PrepSEQ™ assay eluate analyzed with the Applied Biosystems™ MicroSEQ™ 500 Bacterial Identification kit:**

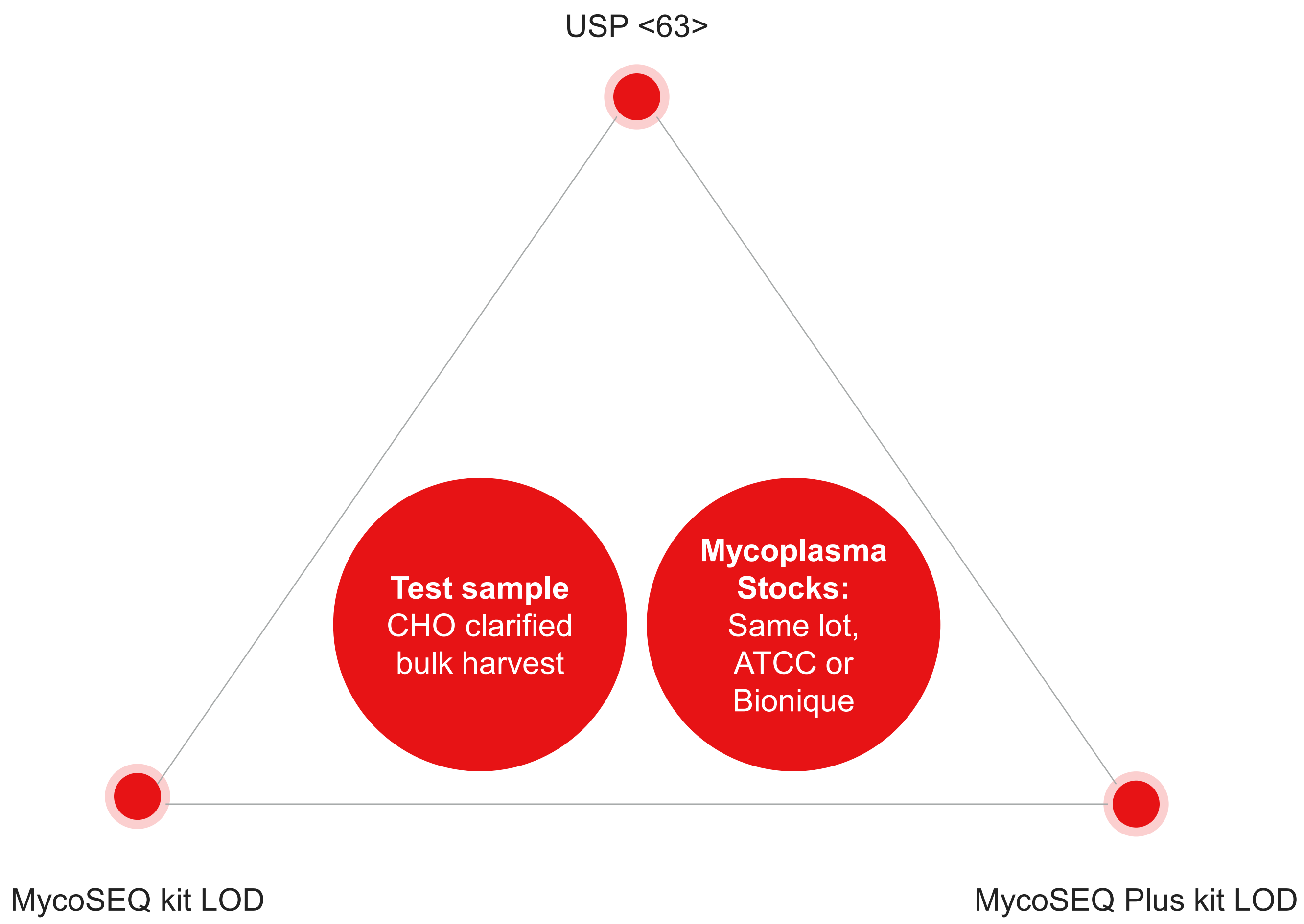
| Specimen | # Samples | Basecalling | Filter | Assembly | Specimen Score | Top Match | % Match | Consensus Length | Library Entry Length | Comments |
|---|---|---|---|---|---|---|---|---|---|---|
| 2-(1.10) | 2 | | | | 30 | Mycoplasma hyorhinis (ATCC=17981) | 99.82 | 466 | 485 | |

# MycoSEQ comparability study

Performed by Bionique Testing Labs, 2023-24

# 3 Armed Study: MycoSEQ kit, Applied Biosystems™ MycoSEQ™ Plus kit, and USP <63>

Results can be compared for all **3 methods**



# Study details

Test sample: CHO Bioreactor Bulk Rituximab Clarified Harvest spiked at 10 CFU/mL

**A spike stock verification protocol was executed prior to LOD testing**

| | MycoSEQ assay | MycoSEQ Plus assay | USP <63> protocol |
|---|---|---|---|
| | **SYBR Green, Dye intercalation, 32 primer multiplex** | **TaqMan, Dye release, 22 primer/probe multiplex** | • **Broth culture with agar plating**<br>• **Indicator cell culture** |
| **Workflow:** | • Low speed spin to remove cellular material<br>• Treat supernatant with DNase/RNase and Proteinase K.<br>• Recover Mycoplasma by high-speed centrifugation<br>• Suspend pellet in Lysis Buffer and extract DNA with the Applied Biosystems™ AutoMate™ Express instrument | • Low speed spin to remove cellular material<br>• Recover Mycoplasma by high-speed centrifugation<br>• Suspend pellet Lysis Buffer and extract DNA with AutoMate Express instrument | • Test sample is frozen following Mycoplasma spike, thawed prior to inoculation of test |
| **Results parameters:** | • Ct, DNA quantity<br>• Tm, amplicon size<br>• D.V., amplicon quantity | • Ct, DNA quantity<br>• Delta Rn, magnitude of normalized fluorescent signal (if needed) | • Culture: Visible growth of Mycoplasma colonies<br>• Indicator: Fluorescence under microscopy |
| **Readout: positive/negative** | | | Either culture or indicator cell |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# Validation study plan

Test type: Limit test for impurity per ICH, Q2 (R2) validation of analytical procedures

LOD evaluated: 10 CFU/mL test sample per Ph. Eur. 2.6.7, *Mycoplasmas*, 2023

## Specificity

- **Part 1:**

  No detection of non-mycoplasma bacteria or other off target species.

  (Conferred by primer design, not evaluated in this study)

- **Part 2:**

  No interference from test sample matrix.

## LOD

**For each mycoplasma species included in the study:**

- 6 samples with 150 CFU (10 CFU/mL) live mycoplasma spiked directly into 15 mL samples
- Process samples and extract DNA with Automate Express
- Run 4 qPCR reactions per extraction (24 total qPCR reactions)

## Comparability to USP <63>

**For each mycoplasma species included in the study:**

- 1 sample with 100 CFU (10 CFU/mL) live mycoplasma spiked directly into 10 mL sample
- Test per USP <63>

# LOD results: MycoSEQ assay

Level of detection, live *Mycoplasma*, 10 CFU/mL LOD using 15 mL test sample, AME extraction:

**Summary of results**

| *Mycoplasma* Species (strain type) | Total number tests/ positive reactions | % Positive | Mean CT (n=24) | SD | CV (%) |
|---|---|---|---|---|---|
| ***M. arginini* 23206-TTR** | 24/24 | 100 | **30.42** | 0.32 | 1.1 |
| ***M. pneumoniae* 15531-TTR** | 24/24 | 100 | **30.99** | 0.18 | 0.6 |
| ***M. hyorhinis* BTS7$^T$** | 24/24 | 100 | **33.28** | 0.23 | 0.7 |
| ***M. fermentans* 19989-TTR** | 24/24 | 100 | **29.28** | 0.11 | 0.4 |
| ***A. laidlawii* 23206-TTR** | 24/24 | 100 | **31.64** | 0.25 | 0.8 |
| ***M. orale* 15531-TTR** | 24/24 | 100 | **34.24** | 0.14 | 0.4 |
| ***M. salivarium* 23064** | 24/24 | 100 | **31.73** | 0.19 | 0.6 |

All strains were procured from ATCC except for *M. salivarium* 23064 and *M. hyorhinis* BTS7$^T$ that was prepared and supplied by Bionique.

# LOD results: MycoSEQ Plus Kit

Level of detection, live mycoplasma, 10 CFU/mL LOD* using 15 mL test sample, AME extraction:

**Summary of results**

| *Mycoplasma* species (strain type) | Total number tests/ positive reactions | % Positive | Mean CT (n=24) | SD | CV (%) |
|---|---|---|---|---|---|
| ***M. arginini* 23206-TTR** | 24/24 | 100 | **31.81** | 1.09 | 3.4 |
| ***M. pneumoniae* 15531-TTR** | 24/24 | 100 | **31.81** | 1.06 | 3.3 |
| ***M. hyorhinis* BTS7$^T$** | 24/24 | 100 | **34.10** | 1.08 | 3.2 |
| ***M. fermentans* 19989-TTR** | 24/24 | 100 | **32.01** | 1.05 | 3.3 |
| ***A. laidlawii* 23206-TTR** | 24/24 | 100 | **32.25** | **0.94** | **2.9** |
| ***M. orale* 15531-TTR** | 24/24 | 100 | **34.00** | 1.00 | 3.0 |
| ***M. salivarium* 23064** | 24/24 | 100 | **32.72** | 1.09 | 3.3 |

- *M. pneumoniae* spike amount was adjusted to 1 CFU/mL due to evidence of excess GC/CFU
- *M. fermentans* spike amount was adjusted to 2-3 CFU/mL due to evidence of excess GC/CFU

All strains were procured from ATCC except for *M. hyorhinis* BTS7$^T$ that was supplied by Bionique.

# Comparability study: USP <63> results

**We performed a comparability study to assess performance between USP <63>, MycoSEQ and MycoSEQ Plus Mycoplasma Detection methods.**

Live *Mycoplasma* spiked into clarified bulk harvest, CHO production bioreactor, mAb manufacturing process at LOD (10 CFU/mL)

| *Mycoplasma* species (strain type) | Source | Overall USP <63> results | Culture test arm | Indicator cell arm |
|---|---|---|---|---|
| ***A. laidlawii* PG8** | ATCC 23206-TTR | + | + | + |
| ***M. arginini* G230** | ATCC 23838-TTR | + | + | + |
| ***M. fermentans* PG18** | ATCC 19989-TTR | + | + | + |
| ***M. hyorhinis* BTS7** | Bionique | + | + | + |
| ***M. orale* CH19299** | ATCC 23714-TTR™ | + | + | - |
| ***M. pneumoniae* FH** | ATCC 15531-TTR | + | + | + |
| ***M. salivarium* PG20** | Bionique | + | + | - |

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# *M. salivarium* and *M. orale* were not detected in the indicator cell arm of the USP <63> test

Demonstrates risk of false negative result with USP <63> test

**The indictor cell culture test is in USP <63> to enable detection of *Mycoplasma* species or strains that are not culturable in liquid and/or do not produce colonies on agar plates.**

**The USP <63> *Mycoplasma* test can be affected by the test sample matrix.**
From previous comparability studies that were conducted as part of customer validation studies, there is data that indicates that some *Mycoplasma* species or strains are not detectable at 10 CFU/mL with the USP <63> test and that is sample matrix dependent.

**Autologous Cell Therapy Product Release**
**US Approval of Three Rapid Microbiological Methods**
J Duguid, Vericel
BioProcess International, 2017

**Equivalence (comparability) data:**

- *Mycoplasma orale*, 7 CFU/mL
- NAT positive ≥ PTC positive

**NAT 6/6 positive** **PTC 0/6 positive**

# Lessons learned from the Bionique study

## Live *Mycoplasma* can present challenges

### Spiking stock verification must be performed prior to LOD validation

- ATCC *M. salivarium* had 20-fold lower titer than reported in Certificate of Analysis
- Switched to Bionique *M. salivarium* stock, spike stock verification confirmed expected GC level

### Aggregation or clumping of *Mycoplasma* cells can impact results

- Variability observed in *M. pneumoniae* Ct values in LOD validation
- Variability observed in *M. salivarium* Ct values in LOD validation- root cause determined to be due to aggregation during overnight storage of spiking stock

### Spiking stock preparation and verification procedure required optimization

- **Version 1:** Day 1: Prepare diluted spiking stock, spike one test sample at 10 CFU/mL, perform sample prep and test 3 replicates with qPCR protocol, compare Ct values to Ct values expected for 10 GC/qPCR reaction (10 GC/mL tests sample). Day 2: Use stored spiking stock to spike 6 test samples and perform LOD validation.
- **Version 2:** Day 1: Prepared diluted spiking stock, spike 9 test samples at 10 CFU/mL, perform sample prep on 3 samples, test 6 qPCR replicates per sample, compare Ct values to Ct values expected for 10 GC/qPCR reaction (10 GC/mL tests sample). Day 2: Test remaining 6 samples in LOD validation.

### Treatment of spiked sample with Nucleases (Dnase/Rnase) and Proteinase K per MycoSEQ assay protocol can reduce free genome copies in *Mycoplasma* stocks

- Our protocols select for intact *Mycoplasma* cells, reducing impact of excess GC/CFU

# Mycoplasma contamination investigation

# Case study: Production bioreactor contamination investigation

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- CHO production bioreactor
- All operating parameters within normal control limits at harvest

- 28-day *Mycoplasma* test positive
- *A. laidlawii* identified as contaminant species by 16S rRNA sequencing

- Equipment and facility decontamination initiated
- Root cause investigation initiated

- Reserve in-process bioreactor samples submitted for MycoSEQ qPCR testing

**Bioreactor**

**Day 1**

**Day 2**

**Day 3** – Feed 1

**Day 4**

**Day 5** – **MycoSEQ qPCR** Undetected

**Day 6**

**Day 7** – **MycoSEQ qPCR** Undetected

**Day 8**

**Day 9** – Feed 2

**Day 10**

**Day 11** – **MycoSEQ qPCR** Detected, Ct = 34.3, est, titer 60 CFU/mL

**Day 12**

**Day 13** – **MycoSEQ qPCR** Detected, Ct = 26.1, est, 20,0000 CFU/mL

**Day 14** – **MycoSEQ qPCR** Detected, Ct = 14.0 est, 800,0000 CFU/mL

**Harvest** → **Purification**

28-day culture-based mycoplasma test - **Positive**
**MicroSEQ ID** – A.Laidlawii

## Outcome and Conclusions:

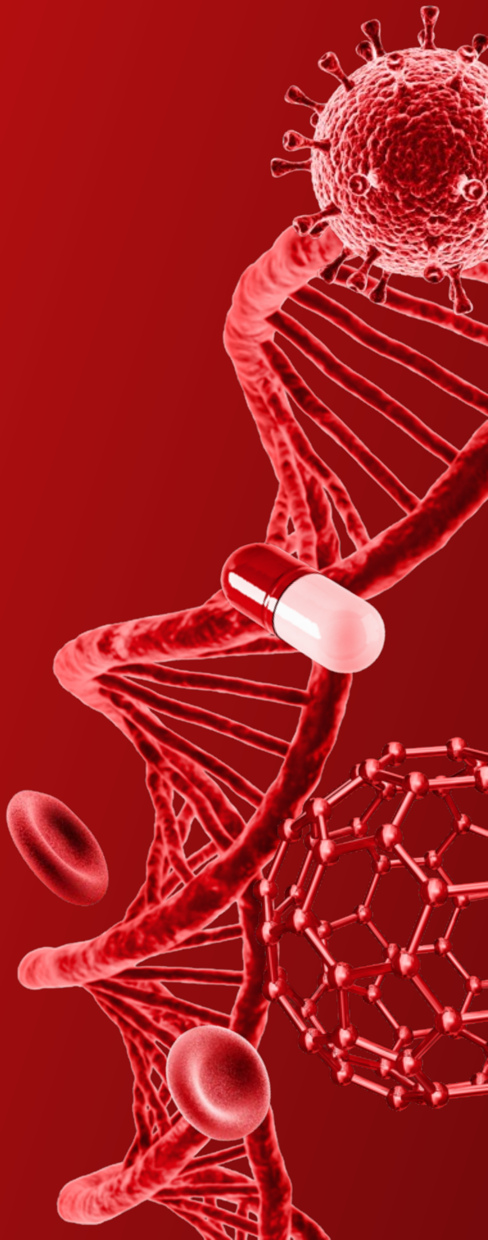
- First MycoSEQ assay qPCR positive result at day 11
- Ct values used to estimate contamination level

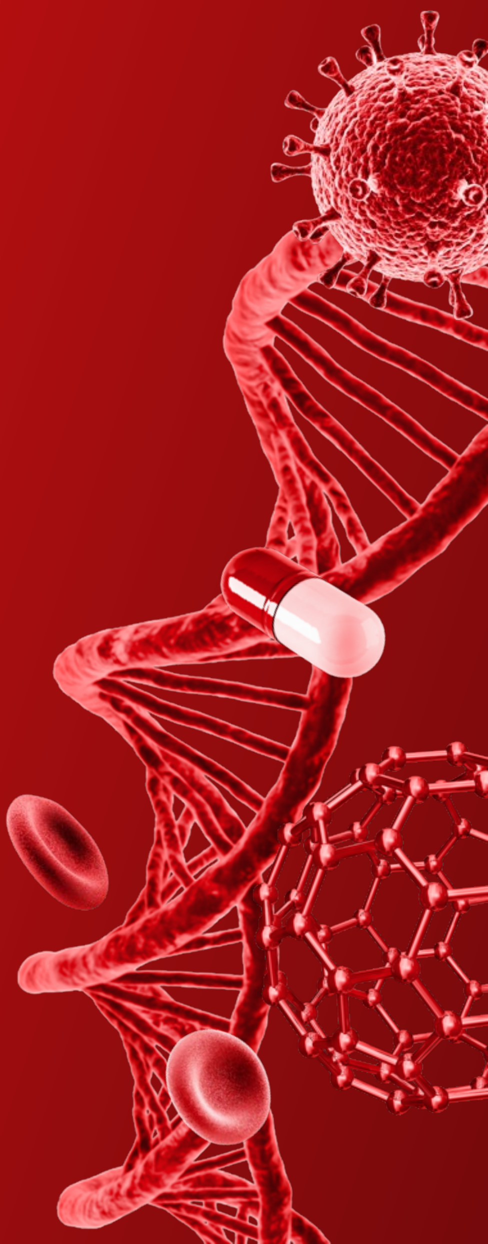
- Decreasing Ct values at day 13 and day 14 confirmed viability and growth rate

- MycoSEQ assay qPCR used to assess impact of contamination on downstream process

- Recommendation that MycoSEQ assay qPCR pre-harvest testing be applied as a conditional release test

# Questions?





# Thank you!

**For Research Use Only. Not for use in diagnostic procedures.**