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Rapid In-Process Testing using MycoSEQ™ and ViralSEQ™ Assays

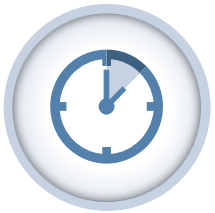
March 11, 2015

The world leader in serving science

In-Process Testing of Cell Cultures in Biopharma Manufacturing

The Challenge

- Cell cultures and bioreactors can become contaminated with difficult to detect Mycoplasma or viruses
- The spread of an undetected contamination event can have a devastating impact
- Potentially high cost per test and long testing cycles as a result of contamination for traditional culture based tests are not practical for routine contamination monitoring
- Development and implementation of rapid methods enabling routine in-process testing require specialized expertise and experience



The Alternative

- Qualify and implement fully integrated, rapid qPCR testing as a risk mitigation tool.
  - Actionable results in hours.

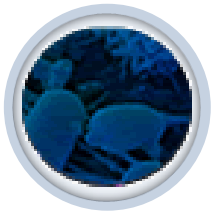
The Solution

- MycoSEQ™ kits: rapid, sensitive qPCR Mycoplasma detection
- ViralSEQ™ kits: rapid, sensitive qPCR MMV and Vesivirus detection

These assays have been implemented globally for use in routine in-process cell culture monitoring.

The Threat Agents

Bacteria



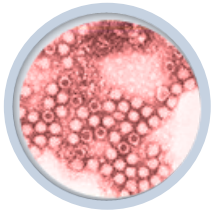
Non-Mycoplasma

- Generally fast growing and easily detectable, often visually, as the Bioreactor becomes turbid and the media changes color
- One exception is *P. acnes*, which grows very slowly in both cell culture and in the traditional Bioburden test

Mycoplasma

- Grow very well in cell culture but are very difficult to detect using traditional culture methods
- Contamination events generally are often undetected until results of the 28-day culture test are analyzed
- Late detection can often result in contamination of subsequent lots either due to shared raw materials or from contaminated equipment

Virus

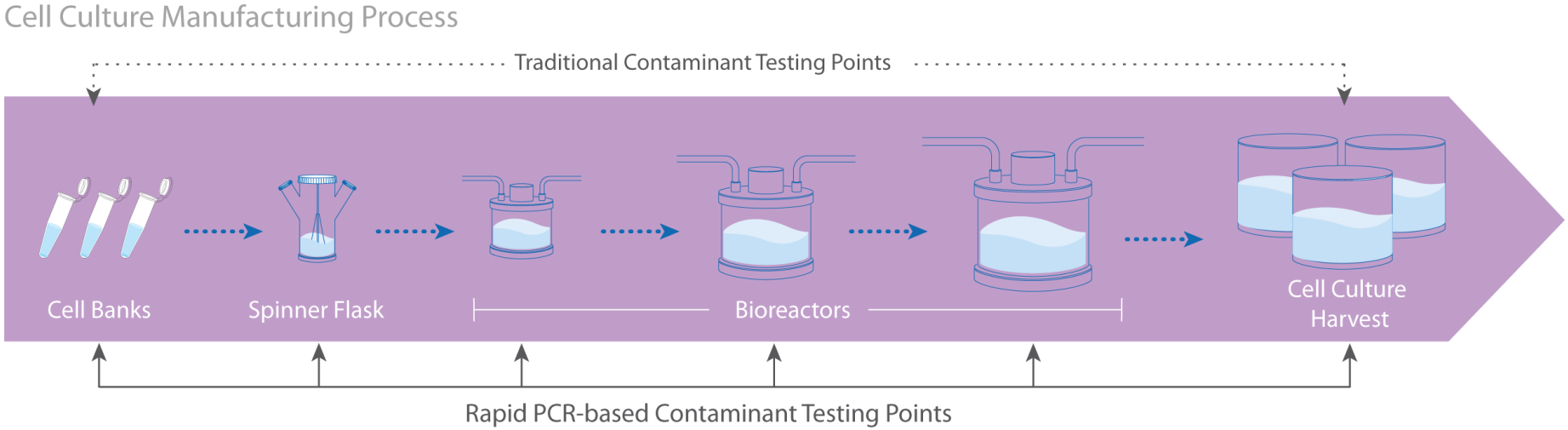


Viral

- Mouse Minute Virus (MMV) and Vesivirus were the causative agents in essentially all known CHO and NS0 viral contamination events
- They infect rodent cell culture very efficiently. Very difficult, perhaps impossible with Vesivirus, to detect using traditional culture test methods

What is in-process testing?

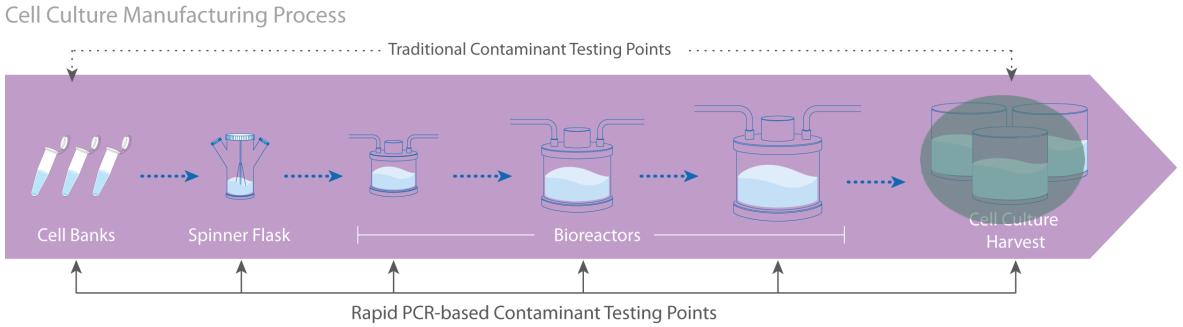
Traditional Culture Testing:
Limited sampling points in cell banks and harvest media only



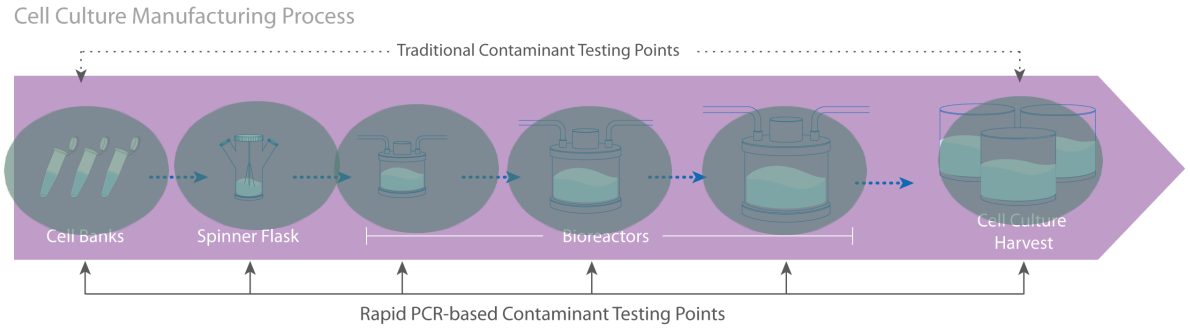
Rapid qPCR Testing:
Sampling, testing and decision making throughout cell culture processes.

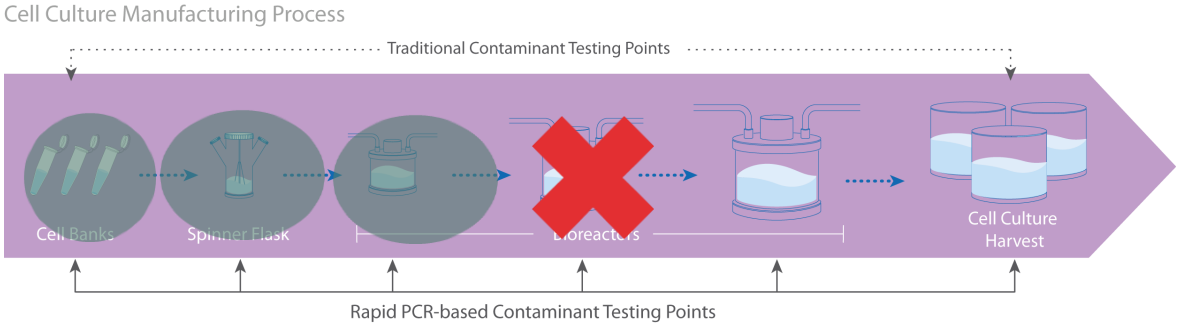
Potential Risks of Undetected Contamination

Impact to Subsequent Lots



PTC test- 28-35 days TTR





Plant Shutdown and Decontamination!

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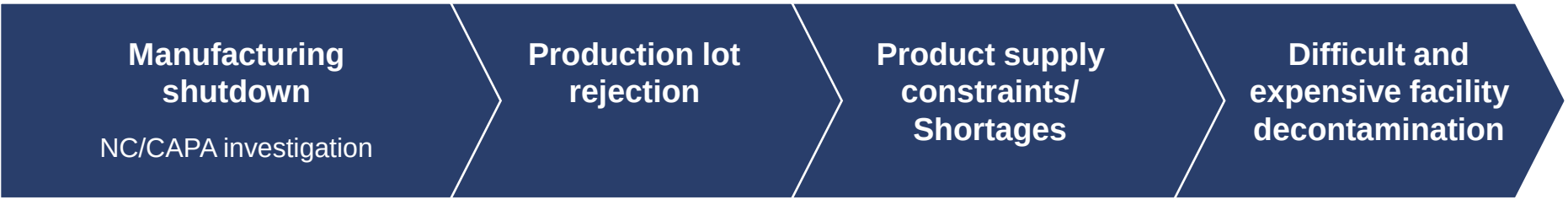
The Impact of Contamination Can Be Minimized

Contamination events can lead to



In-Process Testing allows you to minimize the impact

- Detect contamination at earliest possible point
  - Even more critical for hard-to-detect viruses using adventitious agent test
- Protect facility from the spread of contamination
  - Limit contamination to current lots
  - Avoid introduction of contaminant into downstream processes
  - Quarantine suspect raw materials
- Correct contamination quickly
  - Rapidly investigate and assess scope of contamination
  - Decontamination is much easier if isolated to single bioreactor

Critical Considerations

qPCR for In-Process Testing

Key Attribute	MycoSEQ and ViralSEQ Features
Intended Use	• Assays and protocols designed for in-process testing
Sensitivity	• Consistent across species: 1-10 Genome Copies/PCR reaction • Sensitively not dependant on metabolic state of organism, 1 cell or viral particle contains 1 genome copy.
Specificity	• Specific for organisms targeted • Proven specificity in multiple studies
Results Interpretation	• Objective by using acceptance criteria • Cycle at Threshold (Ct): Input DNA quantity • Melting Temperature (Tm): Amplicon Size • Derivative Value (DV): Amplicon Quantity
Controls	• PCR Inhibition Control • Negative Control • Positive Control, discriminatory • Internal Positive control for ViralSEQ™ assays
Support	• Specialized Field Application Scientists: On-site training • Experience with validation design and regulatory support • Equipment Validation services: IQ/OQ • Results interpretation advice from team with deep domain knowledge.

3 Components of MycoSEQ™ Assay Sensitivity

Rapid sample extraction with optional automation + High Percentage nucleic acid recovery + Detection Using Highest Sensitivity qPCR Assay = **High Sensitivity contaminant Detection typically in under 4 hours**

Rapid Protocols for the following:

- Bioreactors
- Cell Therapy
- Tissue Therapy
- Raw Materials
- Media
- Serum

PrepSEQ™ Magnetic Bead Nucleic Acid Preparation

- High percentage recovery
- Manual or Automated options

MycoSEQ™ or ViralSEQ™ Detection Assay

- 1-10 copy per PCR reaction sensitivity
- Consistent across species or strains

Assay Sensitivity as a Function of Test Sample Volume

For rapid in-process testing, we recommend a 100 uL test sample volume

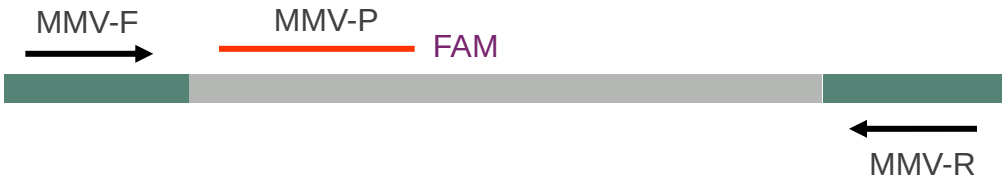
Starting Sample Volume	Sample Prep Elution Vol.	Assay Sensitivity* (GC/Rxn)	Sample Volume (PCR reaction)	Sensitivity (CFU or GC/mL)
10 mL	100 uL	10	1 mL	10
1 mL	100 uL	10	100 uL	100
100 uL	100uL	10	10 uL	1000

***The assay sensitivity stated above is not the lower limit of detection of the assay. We recommend that validation experiments are done at 10 GC or CFU/mL in order to obtain consistent results.**

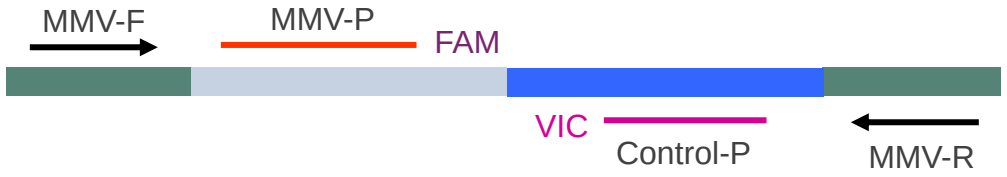
Mouse Minute Virus Assay and Control Design

MMV Detection Assay

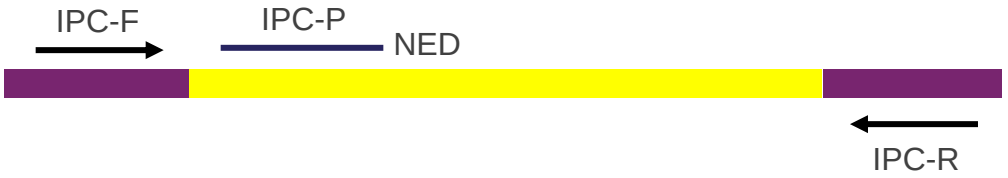
MMV viral target – FAM™ dye



Discriminatory PCR positive & extraction control – VIC™ dye



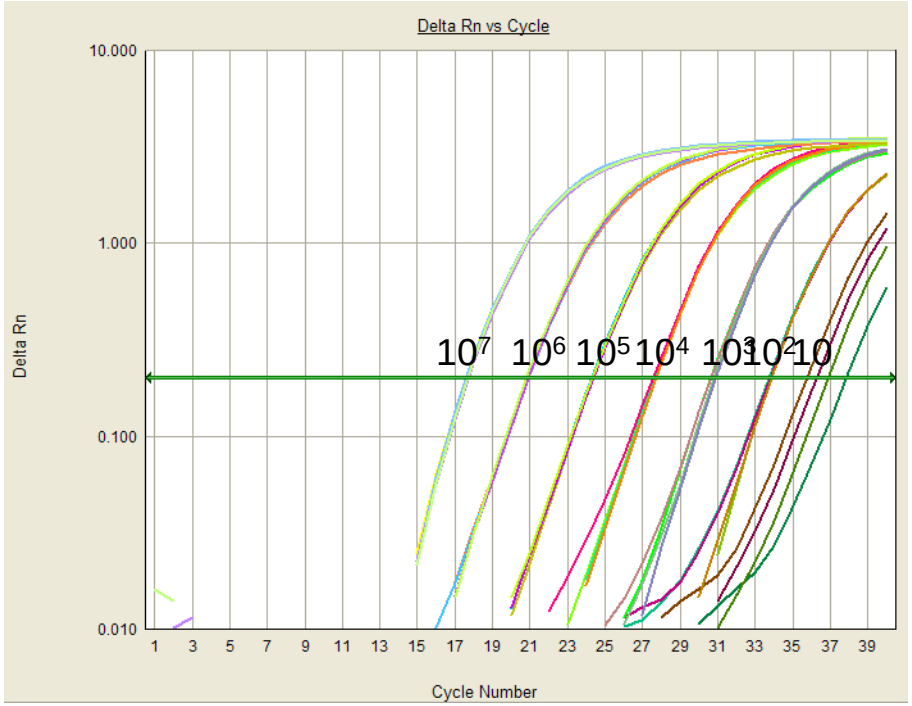
IPC control – NED™ dye

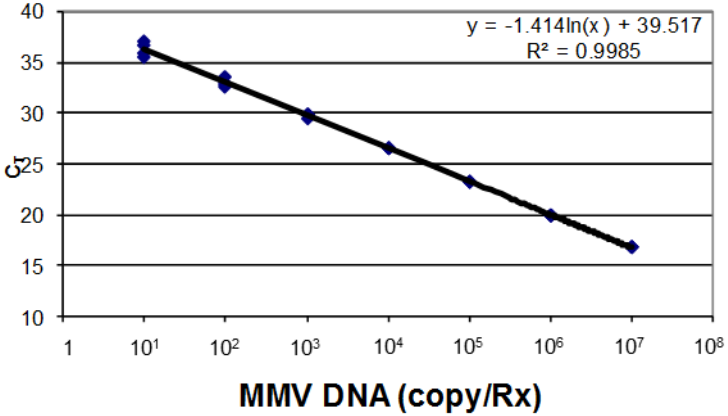


- Unique design includes detection of target, discriminatory control, and internal positive control (IPC)
- Assay targets conserved region of MMV genome, enabling detection of all known MMV strains
- The discriminatory positive control enables monitoring sample prep efficiency and RT-PCR reaction while minimizing the risk of a false positive result due to cross-contamination of samples
- The internal positive control present in every PCR reaction enables assessment of PCR efficiency and detection of potential inhibition in every reaction to help reduce risk of false negative results.

High Sensitivity, Broad Dynamic Range

MMV Detection Assay





- At least 7 orders of linear dynamic range
- Detection limit ~10 genome copies

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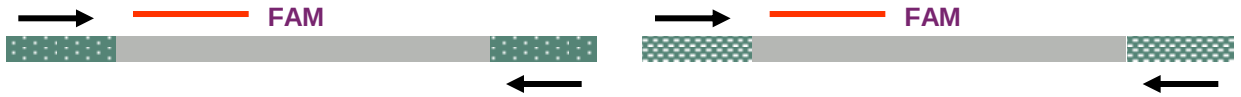
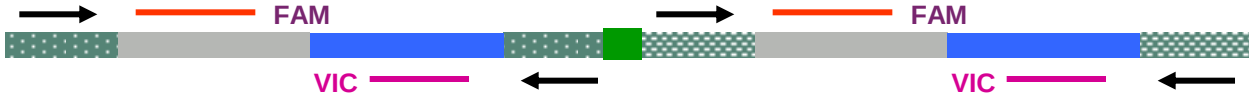
Vesivirus Assay and Control Design

Vesivirus targets



Discriminatory RT-PCR positive control

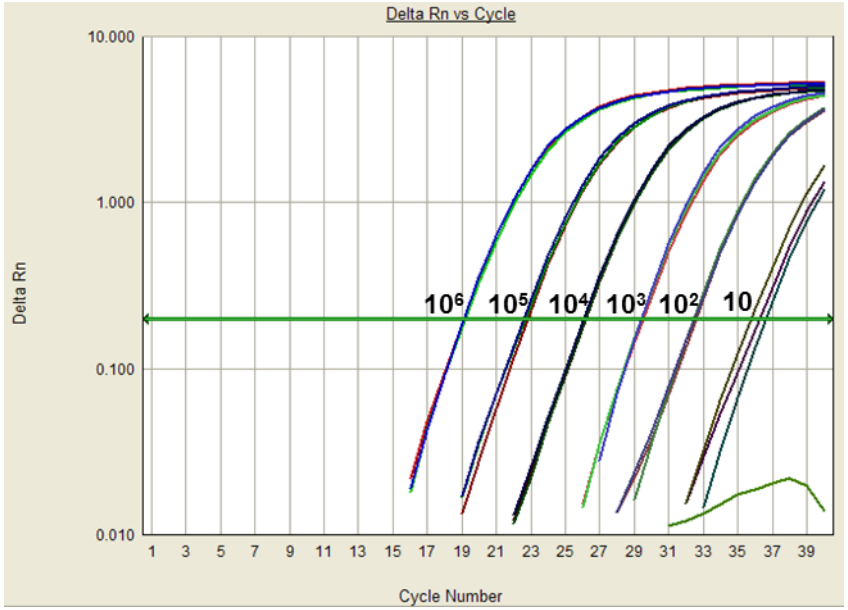


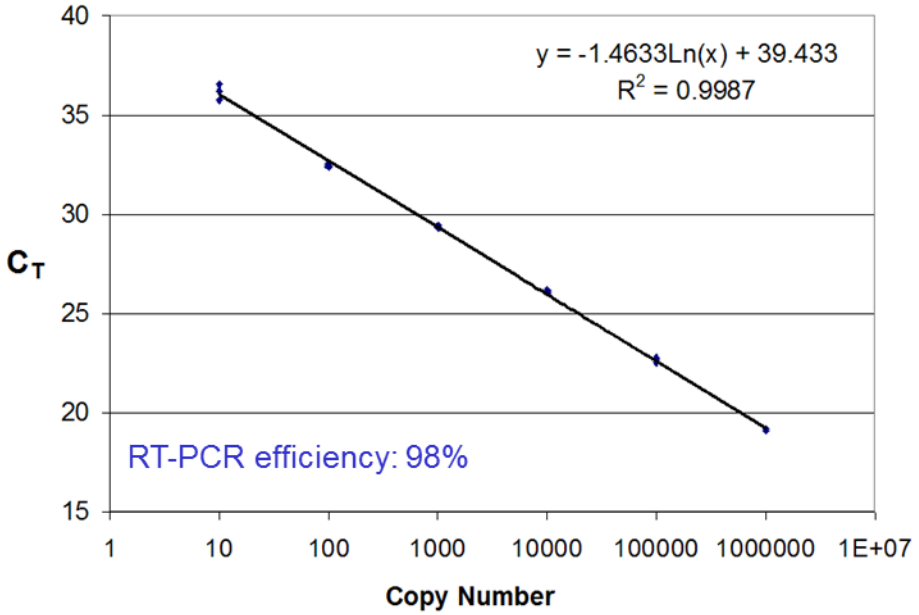
Internal positive Control

- Unique design, 2 independent viral targets, helps reduce risk of false negative result due to genetic drift
- Assay targets conserved regions of Vesivirus genome, enabling detection of all known Vesivirus strains
- The discriminatory positive control enables monitoring sample prep efficiency and RT-PCR reaction while minimizing the risk of false positive result due to cross-contamination of samples by positive control template.
- The Internal Positive Control (IPC) present in every PCR reaction enables assessment of PCR efficiency and detection of potential inhibition in every reaction to help reduce risk of false negative results.

High Sensitivity, Broad Dynamic Range

Vesivirus Detection Assay





- At least 6 orders of linear dynamic range
- Detection limit ~10 genome copies

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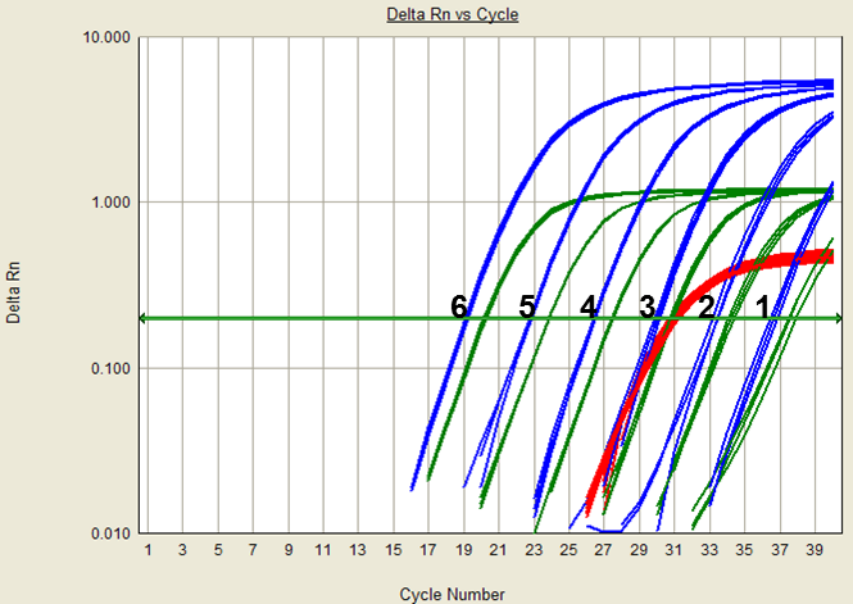
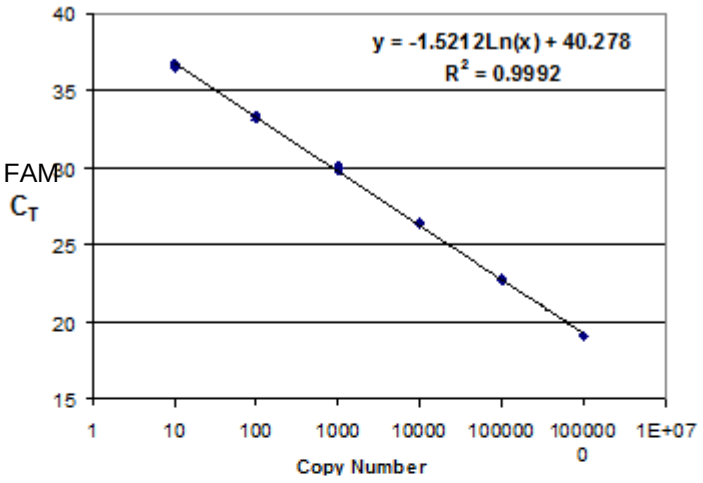
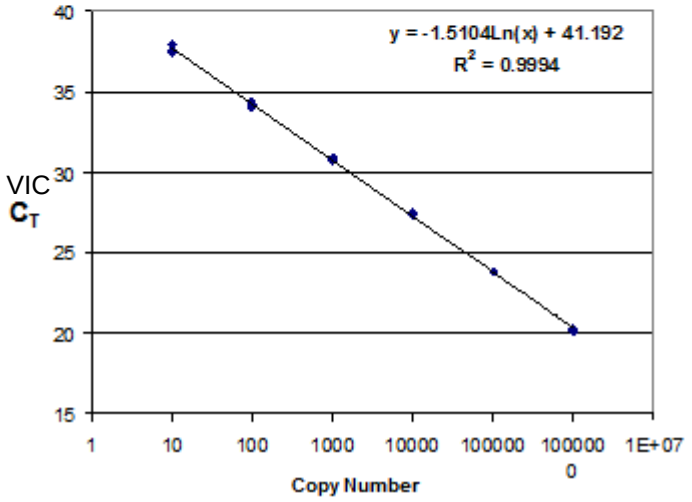
High Sensitivity, Broad Dynamic Range Positive Control Template

10 to 10^6 copies of RNA per RT-PCR reaction

- Blue: Vesivirus probe
- Green: Discriminatory positive control probe
- Red: Internal positive control







- At least 6 orders of linear dynamic range
- Detection limit ~10 copies of control

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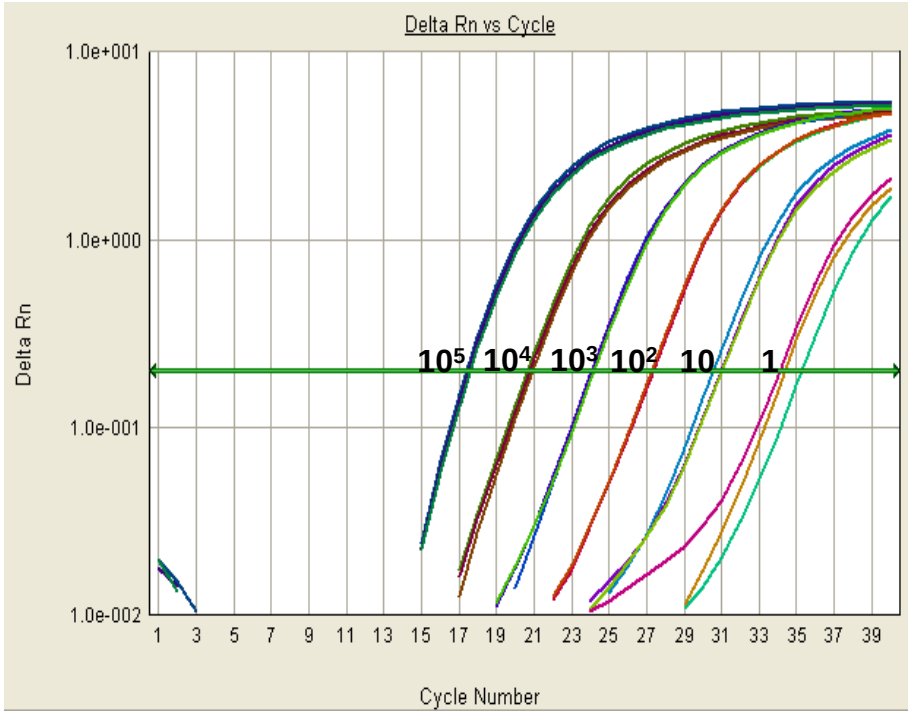
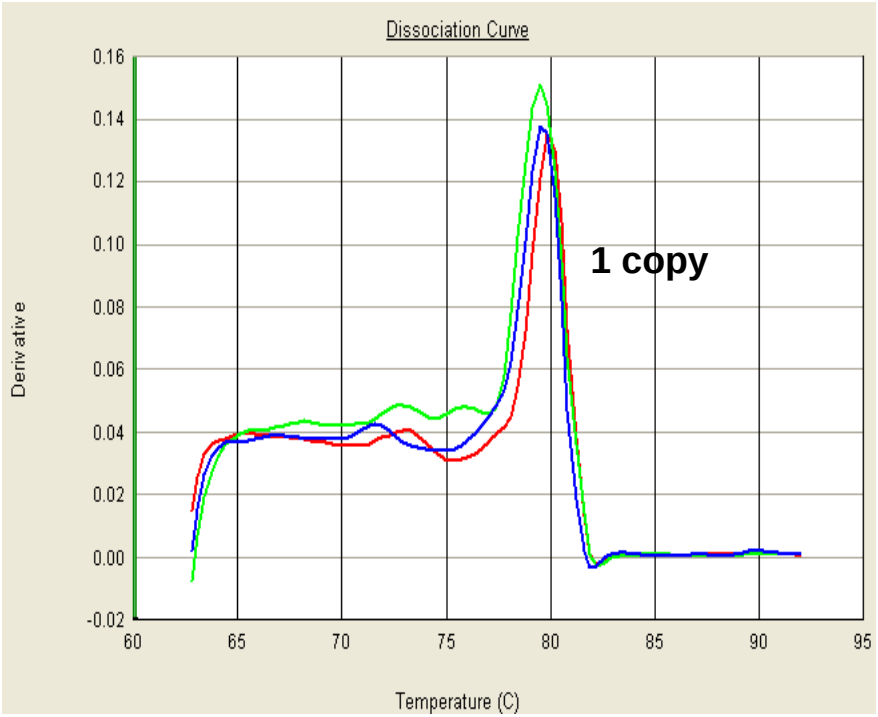
Mycoplasma Assay Sensitivity

Mycoplasma arginini





- At least 6 orders of linear dynamic range
- LOD: 1 genome copy/ PCR reaction
- Amplicon melt analysis: Additional confirmation of positive result.

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Broad Detection of *Mycoplasma* Species

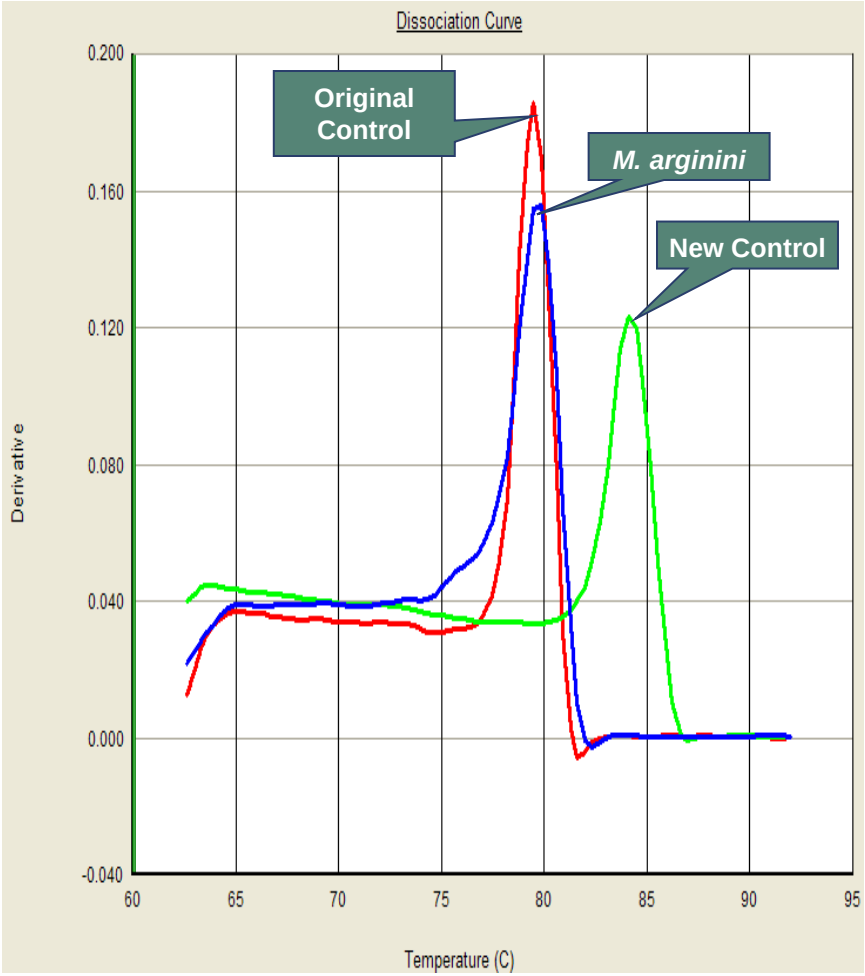
Inclusion Panel (Partial)

Acholeplasma granularum
Acholeplasma laidlawii
Acholeplasma pleciae
Mycoplasma alkalescens
Mycoplasma alvi
Mycoplasma anseris
Mycoplasma arginini
Mycoplasma auris
Mycoplasma buccale
Mycoplasma californicum
Mycoplasma canadense
Mycoplasma capricolum
Mycoplasma caviae
Mycoplasma collis
Mycoplasma cricetuli
Mycoplasma equirhinis
Mycoplasma fermentans
Mycoplasma gallinaceum
Mycoplasma gallisepticum
Mycoplasma gateae
Mycoplasma genitalium
Mycoplasma gypis
Mycoplasma hominis
Mycoplasma hyorhinis
Mycoplasma imitans
Mycoplasma indiense
Mycoplasma lagogenitalium
Mycoplasma lipofaciens
Mycoplasma mobile
Mycoplasma molare
Mycoplasma mycoides
Mycoplasma neurolyticum
Mycoplasma orale
Mycoplasma phocidae
Mycoplasma pirum
Mycoplasma pneumoniae
Mycoplasma salivarium
Mycoplasma simbae
Mycoplasma sp.
Mycoplasma spumans
Mycoplasma synoviae
Mycoplasma testudinis
Mycoplasma timone
Spiroplasma citri
Spiroplasma endosymbiont
Spiroplasma insolitum
Spiroplasma kunkelii
Spiroplasma melliferum
Spiroplasma mirum
Spiroplasma phoeniceum
Spiroplasma poulsonii
Spiroplasma sp.
Mycoplasma bovirhinis
Mycoplasma bovis
Mycoplasma bovigenitalium
Mycoplasma canis
Mycoplasma felis
Mycoplasma fastidiosum
Mycoplasma muris
Mycoplasma pulmonis

- >90 species
- Related *Acholeplasma laidlawii* and *Spiroplasma citri*
- Common isolated species recommended for testing and validation (listed in red)

Discriminatory Positive / Extraction Control

- Positive control DNA made with *Mycoplasma* amplicon modified to have melting temperature (Tm) outside range of *Mycoplasma* amplicons
- Allows additional level of confirmation of positive test results.
- Higher Tm allows discrimination between true *Mycoplasma* and accidental contamination of test sample with positive control.
- Enables simple extraction control spiking of test samples.



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Sample Preparation Automation

- Same chemistry as the well established PrepSEQ™ kits
- Fully-automated DNA extraction from broad sample types
- Enables high percentage nucleic acid recovery
- Low to medium throughput – 13 samples per 30-45 min
- Minimal hands-on time required
- Easy to use, set up and walk away
- Closed system: all reagents packaged in sealed cartridges

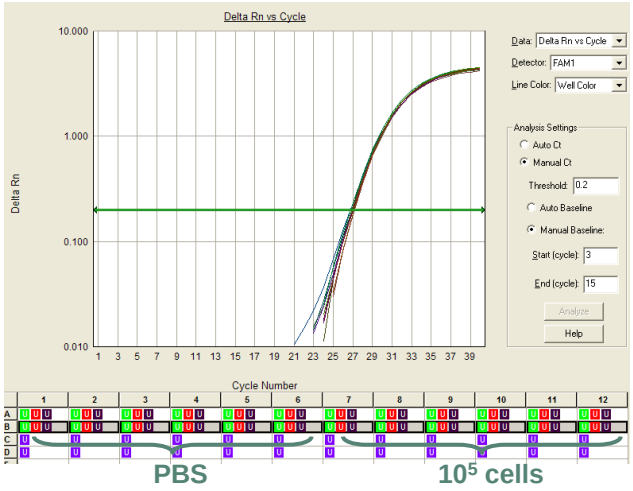
Simultaneous Extraction of *Mycoplasma*, MMV, and Vesivirus Nucleic Acid

Mycoplasma	MMV	Vesivirus
Bacteria (no cell wall)	Non-enveloped DNA virus	Non-enveloped RNA virus
Double-stranded DNA genome	Single-stranded DNA genome	Single stranded RNA genome
~ 1 Mbp	~ 5 kb	7 - 8 kb
SYBR™ Green assay	TaqMan™ assay	TaqMan™ assay
	1 $TCID_{50}$ = ~2x10^4 genomes	
Culture >21 days (10 cfu/mL from Bioreactor harvest)	Culture >21 days 10 $TCID_{50}$/mL	Culture >21 days 10 $TCID_{50}$/mL

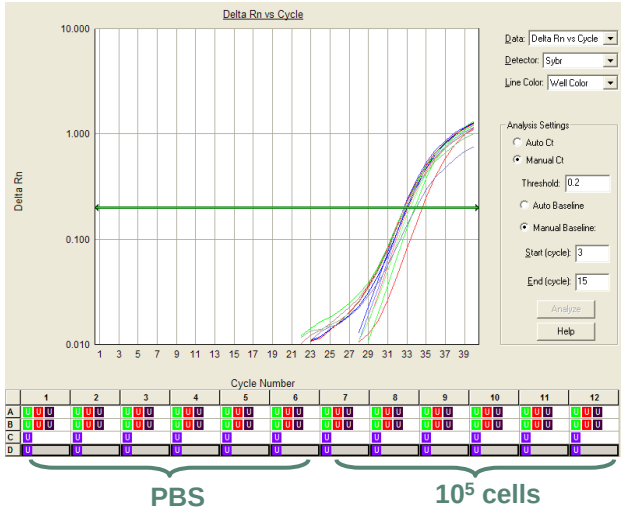
- *M. arginini*, MMV, and Vesivirus (FCV) were spiked into 100 uL test samples, either PBS or 10^5 CHO cells.
- Nucleic Acid was extracted using PrepSEQ 1-2-3 extraction procedure.
- Nucleic acid eluted in 100 uL, 10 uL used per PCR reaction.
- Mycoplasma SYBR™ green assay, Vesivirus RT-PCR assay and MMV TaqMan™ assay were run on the same plate using a single thermocycling protocol.

Detection of MMV, Mycoplasma and Vesivirus extracted from the same test sample

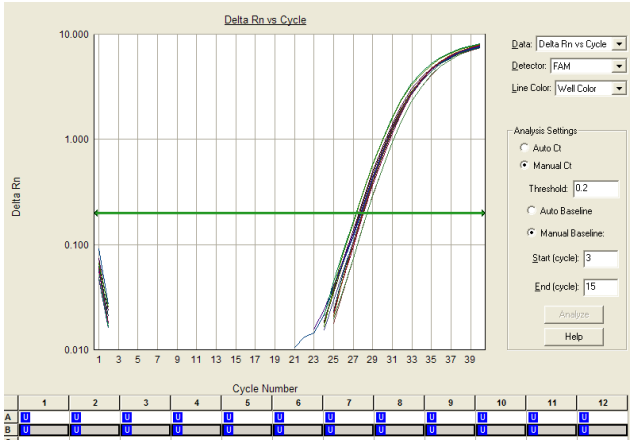
MMV



Mycoplasma

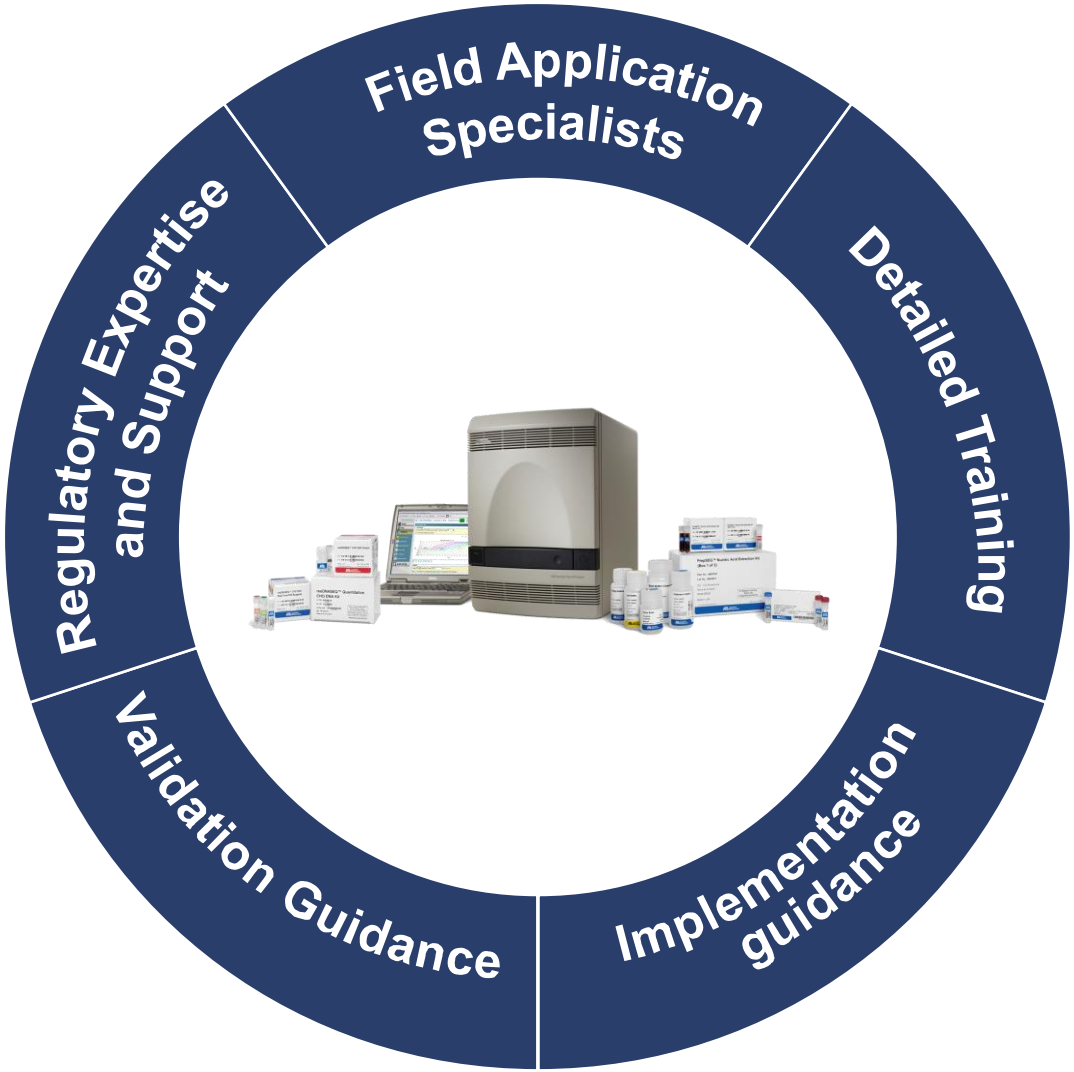


FCV (Vesivirus)



- All three organisms were detected with high efficiency
- No difference in recovery and detection from either PBS or 10^5 CHO cells

Worldwide Support Network



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We Provide Comprehensive Support

- Training by Field Applications Scientist
- Instrument IQ/OQ services
- Sample prep optimization
- Validation study design
- Support with regulatory submissions including attendance at meetings and support answering agency questions
- Data Interpretation advice from scientists with extensive backgrounds in mammalian cell culture manufacturing and contamination events

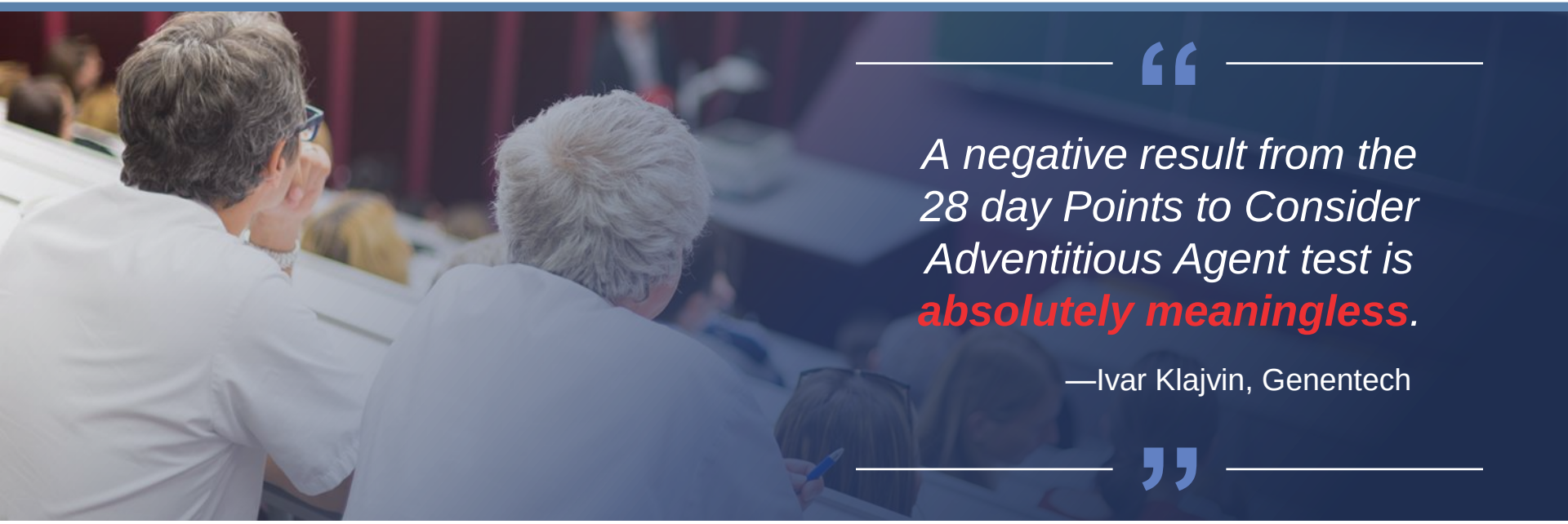
Appendix

Impact of Viral Contamination

NEW YORK, June 16 (Reuters)

A major bio pharmaceutical company has **HALTED PRODUCTION** of two of its top-selling drugs after detecting a virus at one of their plants that U.S. regulators had already cited for deficiencies. The company, whose shares fell 4.2 percent after making the announcement on Tuesday, said current inventories of several key orphan disease treatments were not sufficient to meet global demand. The virus strain, Vesivirus 2117, has not been shown to cause human infection but is known to interfere with the growth of cells used to produce biologic drugs, the biotechnology company said.

PDA/FDA Adventitious Agents Conference, December 2010



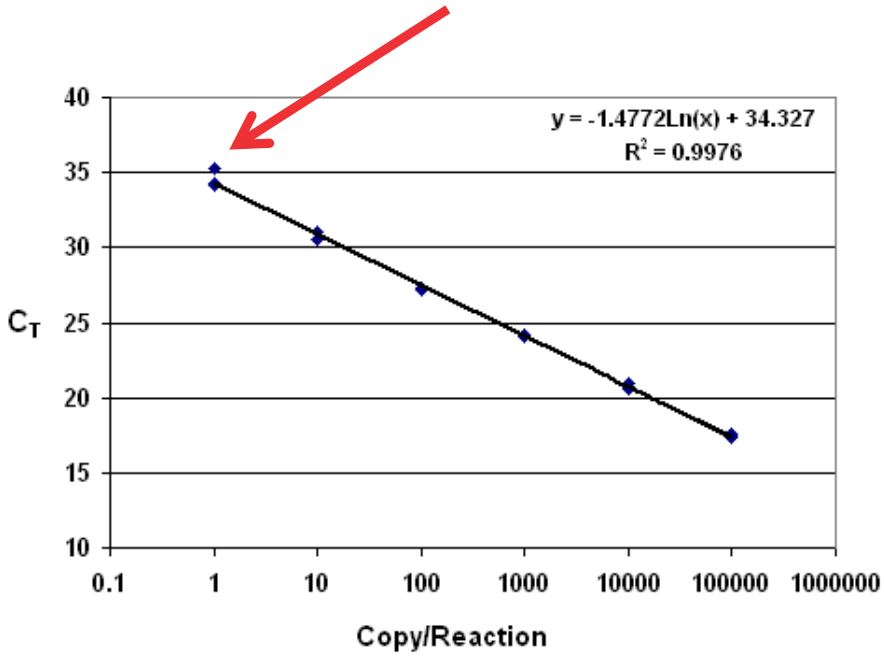
- Presentations on Contamination Events:
  - Genentech, 2, MMV
  - Amgen, 3, MMV
  - Genzyme, 1, Vesivirus
  - Merrimack, 1, MMV
- None of these contaminations were first detected by the 28 day Points to Consider Adventitious Agent test.
- PCR was the key analytical tool used in all phases of these events / investigations / decontaminations.

Applications of Ct using the MycoSEQ™ and ViralSEQ™ Assays

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 or infectious titer ratios
- Sample preparation protocol optimization
- Validation Studies
  - Compare your Ct to expected values for each organism or species
  - Estimation of LLOD by extrapolation from values obtained at 10 GC or CFU/sample

Lower Limit of Detection Assessment Using Principles of qPCR



- For efficient, linear qPCR assays, 1 Ct difference represents a 2-fold difference in the starting quantity of the target DNA.
- The 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
  - 2.5 GC/PCR reaction = 33
  - 1.25 GC/PCR reaction= 34
  - 0.6 GC/PCR reaction =35

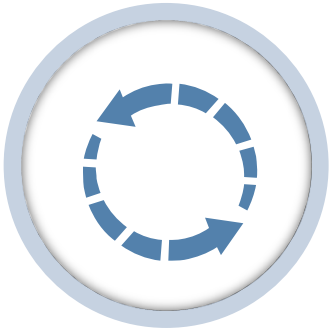
This approach to LLOD estimation is supported by experimental results

Results from External Validation Study

Level of Detection, Part 1, 10 GC/mL from 10 mL sample: Summary of Results

Mycoplasma Species (Type Strain)	Total Number Tests/Positive Reactions	% Positive	Mean C_T (n=24)	SD	CV (%)
A. laidlawii PG8^T	24/24	100	**33.87**	0.625	1.8
M. arginini G230^T	24/24	100	**30.90**	0.99	3.2
M. fermentans PG18^T	24/24	100	**32.21**	1.68	5.2
M. hominis PG21^T	24/24	100	**29.53**	0.86	2.9
M. hyorhinis BTS7^T	24/24	100	**29.22**	0.85	2.9
M. orale CH19299^T	24/24	100	**31.85**	1.81	5.7
M. pneumoniae FH^T	24/24	100	**33.03**	0.73	2.2
M. salivarium PG20^T	24/24	100	**31.14**	0.87	2.8
M. synoviae WVU 1853 ^{T}	24/24	100	**33.25**	0.89	2.7
S. citri R8A2^T	24/24	100	**32.79**	1.65	5.0

Results Interpretation/Acceptance Criteria



Cycle at Threshold (Ct)

- A measure of target DNA level at the beginning of the PCR reaction.
- Acceptance criteria:
  - For Routine Testing: Ct less than or equal to 36.

Derivative Value (DV)

- A measure of specific amplicon quantity generated during PCR reaction.
- Acceptance Criteria: DV greater than 0.08.

Melting Temperature (Tm)

- A measure of amplicon size and base composition (known for Mycoplasma using this assay).
- Acceptance Criteria: Tm between 75 and 81 degrees C.

All three criteria must be met for a test sample to be positive for the presence of *Mycoplasma* DNA.

?

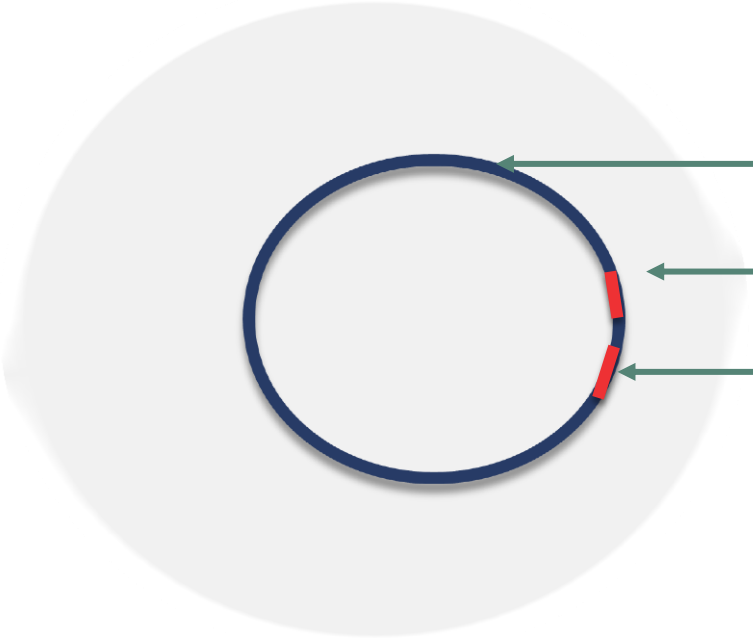
Is there a risk of a false positive result due to detection of DNA from non-viable organisms?

Contaminant Detection Assay Features

Highly Sensitive	• *Mycoplasma*: 1-3 genome copies/PCR reaction • Vesivirus: 10 genome copies/PCR reaction • Mouse Minute Virus: 10 genome copies/PCR reaction
Specific	• No detection of organisms, verified by testing panel of genetically related organisms
Comprehensive	• Detection of all known species or strains
Quantitative PCR Technology	• Enables viability assessment • Closed tube analysis, no handling amplified target DNA
Proprietary Discriminatory Positive/Extraction Controls	• Helps minimize risk of false positive test result from accidental cross contamination with positive control. • Enables extraction/detection control analysis
Internal Positive Control (IPC)	• For Viral assays, present in every PCR reaction. Enables assessment of PCR efficiency and detection of potential PCR inhibition in every reaction to help reduce risk of false negative results.
Sample Preparation	• Single, rapid sample prep for isolation of Nucleic Acid from all organisms (DS-DNA, SS-DNA and RNA) • Versatile, protocols available for small or large test sample volumes • Fully Automated option: increase throughput, improve efficiencies, help reduce risk of analyst error

Mycoplasma

The relationship between a Colony Forming Unit (CFU) and a Genome Copy (GC)



Mycoplasma Cell

Mycoplasma chromosome, 1 circular chromosome per cell, 650Kbp -1900Kbp.

16S rRNA gene, the target of the MycoSEQ™ assay.

Additional copy of 16S rRNA gene, Some species have 2 copies per genome.

Ideally, 1 GC equals 1 CFU, but that must be verified. If the ratio changes because the sample or stock has cells that contain DNA, but do not grow and represent as a CFU….

The sensitivity of an NAT test may be misleading…

Legal Disclaimer

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