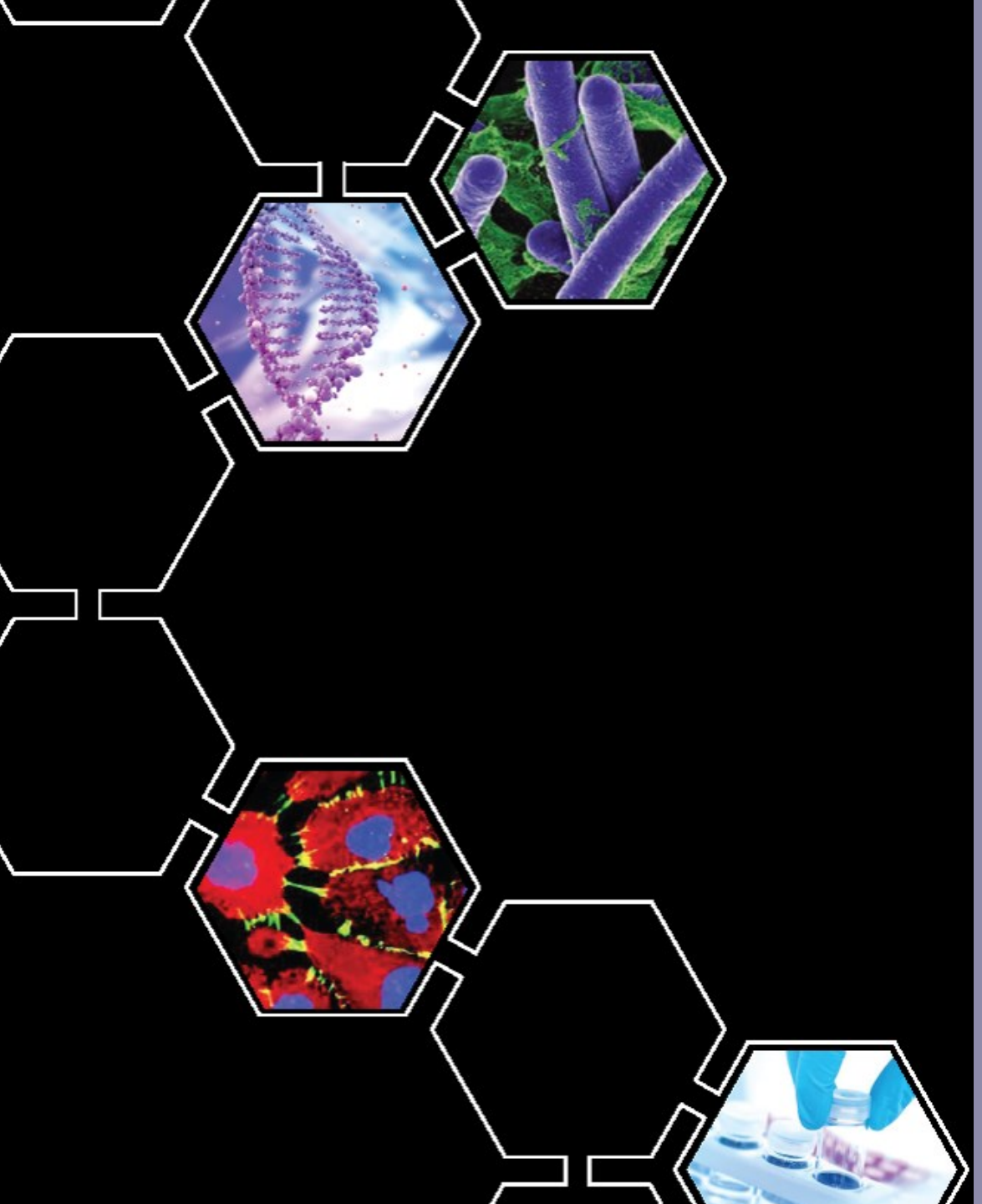


Cell Line Contamination – Mycoplasma Prevention and Detection

Balsam Shawky, M.S., *Senior Biologist*, ATCC
Reed Shabman, Ph.D., *Lead Scientist*, ATCC

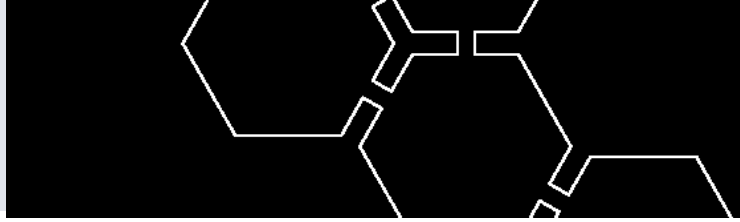
Agenda

- ATCC overview
- Background on mycoplasma
- Historical overview of mycoplasma contamination
- Sources of mycoplasma contamination
- How to protect cell cultures from mycoplasma contamination
  - Prevention
  - Detection methods used in routine testing
- Eradication
- Conclusions

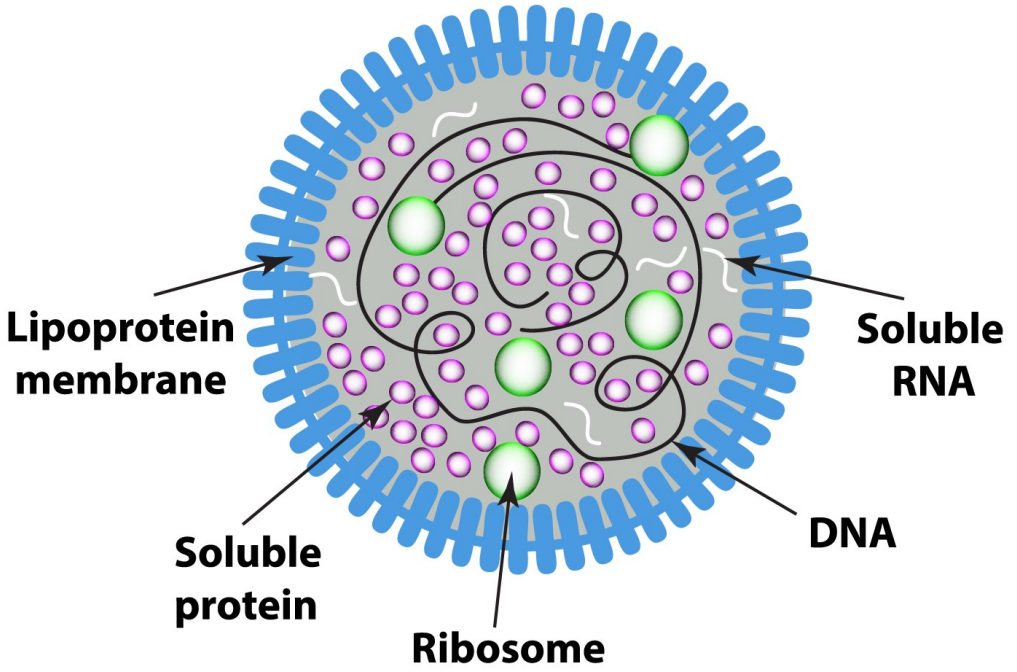
ATCC overview

- Founded in 1925, ATCC is a non-profit organization with HQ in Manassas, VA, and an R&D & Services center in Gaithersburg, MD
- Worldwide brand name and quality recognition
- World's premiere biological materials resource and standards development organization
  - 4,000 cell lines
  - 70,000 microbes
- ATCC collaborates with and supports the scientific community with industry-standard and innovative biological solutions
  - Growing portfolio of products and services
  - Sales and distribution over 140 countries, 15 International distributors
- Talented team of 475+ employees; > one third with advanced degrees
- Multiple accreditations including ISO 9001 and ISO 17025

Background on mycoplasma

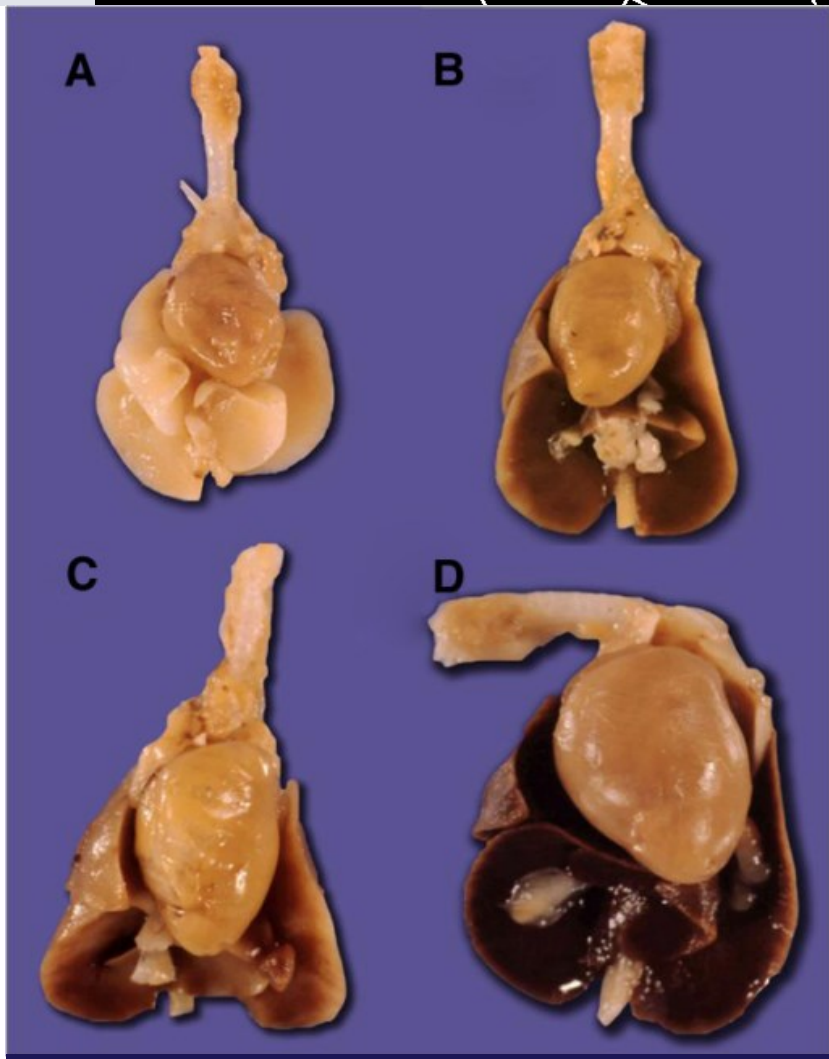


- Bacteria
- Class Mollicutes
- Each species lacks a cell wall but has a simple plasma membrane
- Small size (0.15-0.30 µm)
- Small genome
- Requires the presence of cholesterol, amino acids, fatty acids, vitamins, and other catabolites to survive



Mycoplasma infection is associated with disease and cell culture contamination

- **Mycoplasma are common to the human respiratory and urogenital tracts. Pathogenic mycoplasma examples include:**
  - *Mycoplasma pneumoniae*, which causes atypical pneumonia
  - *Mycoplasma genitalium*, which is linked to pelvic inflammatory disease
- **Over 190 mycoplasma species are known, but only eight are responsible for ~95% of cell culture contamination events.**
  - *Mycoplasma arginini*
  - *Mycoplasma fermentans*
  - *Mycoplasma hominis*
  - *Mycoplasma hyorhinis*
  - *Mycoplasma orale*
  - *Mycoplasma pirum*
  - *Mycoplasma salivarium*
  - *Acholeplasma laidlawii*

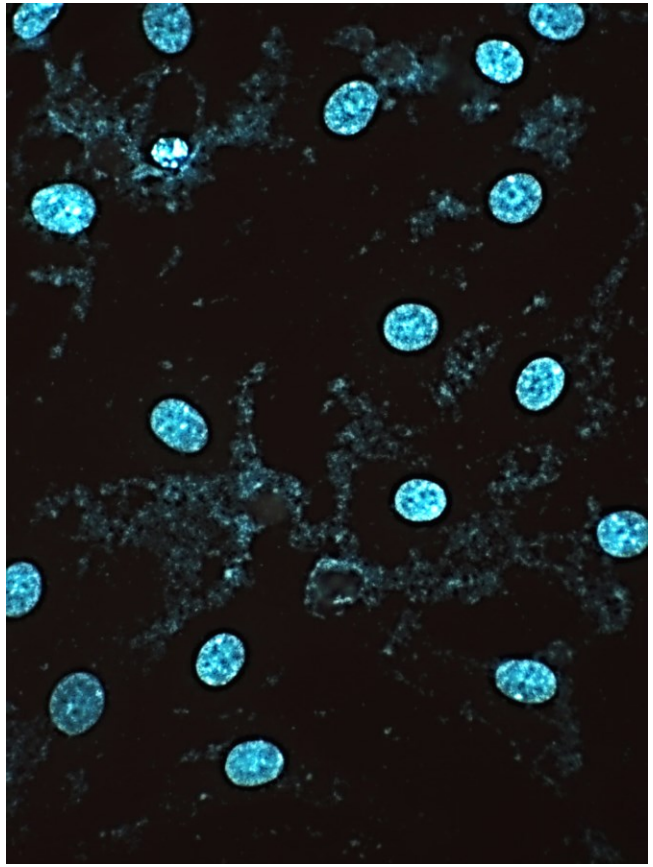


(A) Normal hamster lungs and (B-D) progression of *M. fermentans* infection in hamster lungs. Image courtesy of A. Yáñez.

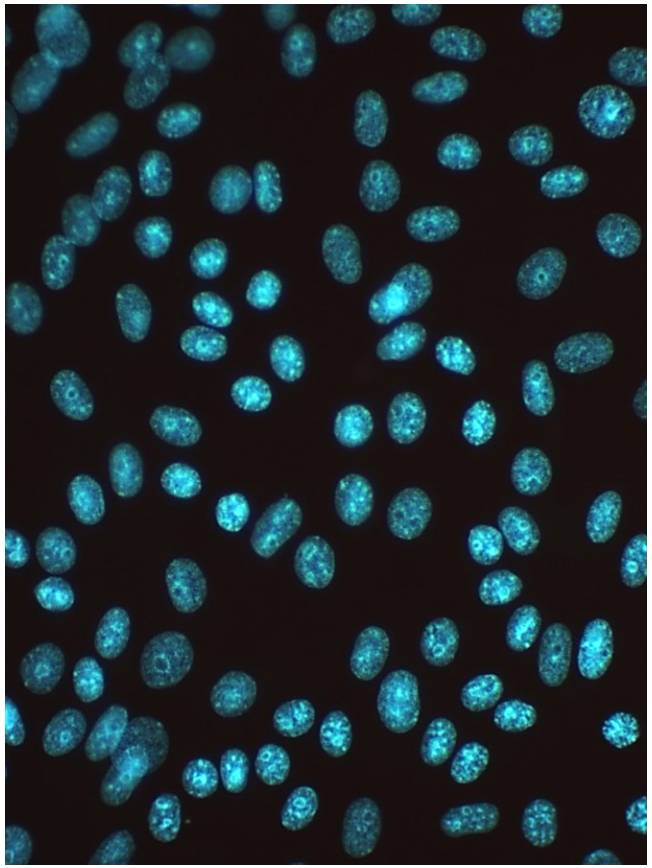
Impact of mycoplasma contamination on scientific research

Contamination results in a number of deleterious effects

- Chromosomal aberrations
- Disruption of nucleic acid synthesis
- Changes in membrane antigenicity
- Inhibition of cell proliferation and metabolism
- Decreased transfection rates
- Changes in gene expression profiles
- Affects virus production
- Cell death



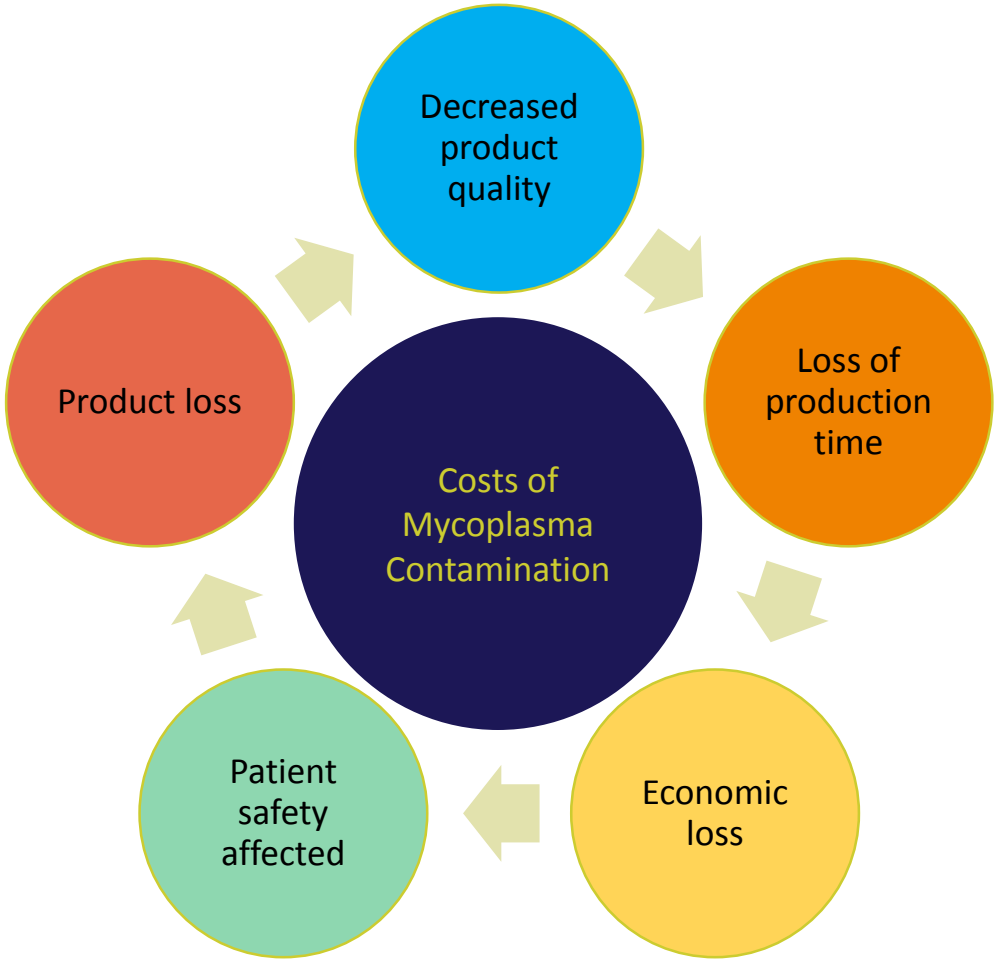
Vero cells infected with *M. hyorhinis*



Uninfected Vero cells

Impact of mycoplasma contamination on cell-derived biopharmaceuticals

Mycoplasma contamination of cell lines used in the production of biopharmaceuticals poses a major safety and economic risk!

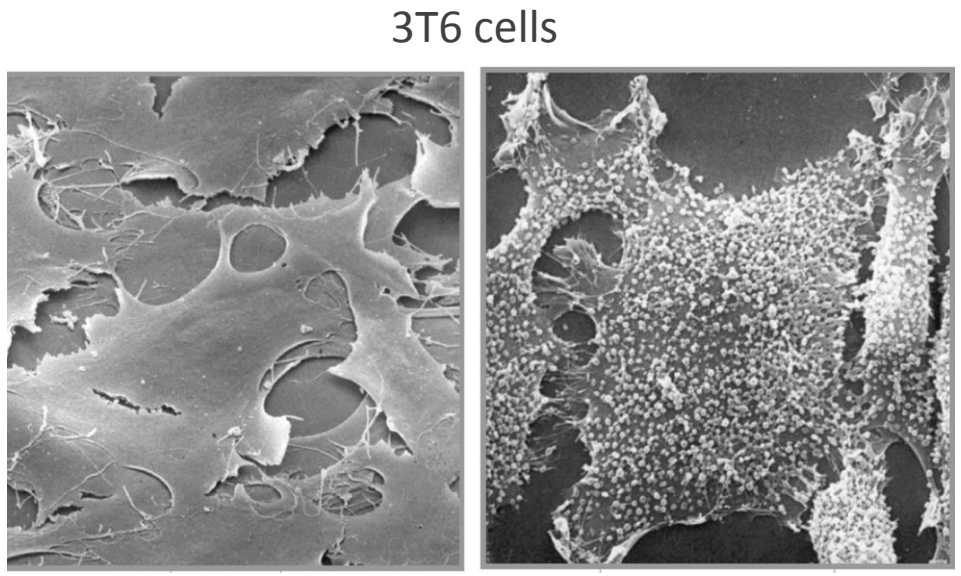


Review: Armstrong SE, *et al.* The scope of mycoplasma contamination within the biopharmaceutical industry. Biologicals. 38(2):211-3, 2010.

Historical overview of mycoplasma contamination

Historical findings highlight the need for mycoplasma testing

- 1956: Researchers at Johns Hopkins reported mycoplasma contamination of HeLa cells used in their lab
- 1990s: The US Food and Drug Administration tested over 20,000 cell cultures and found that 15% were contaminated with mycoplasma
- 1991: Researchers from Argentina found that 70% of the 200 samples tested were contaminated
- 2002: Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) in Germany found that 28% of the 440 cell lines tested (mostly leukemialymphoma) were contaminated



References:
Robinson LB, Wichelhausen RH. Contamination of human cell cultures by pleuropneumonialike organisms. Science 124(3232): 1147–1148, 1956.
Rottem S, Barile MF. Beware of mycoplasmas. Trends Biotechnol 11(4): 143–151, 1993.
Coronato S, Coto CE. Prevalence of *Mycoplasma orale* as a contaminant of cell cultures in Argentina. Rev Argent Microbiol 23(3):166–171, 1991.
Drexler HG, Uphoff CC. Mycoplasma contamination of cell cultures: Incidence, sources, effects, detection, elimination, prevention. Cytotechnology 39(2): 75–90, 2002.
Image source: Corning and ATCC: Understanding and Managing Cell Culture Contamination.

2015: Analysis of public sequence data highlights mycoplasma contamination

Assessing the prevalence of mycoplasma contamination in cell culture via a survey of NCBI's RNA-seq archive

Anthony O. Olarerin-George, John B. Hogenesch

Nucleic Acids Research, Volume 43, Issue 5, 11 March 2015, Pages 2535–2542,
https://doi.org/10.1093/nar/gkv136

- This study identified the presence of mycoplasma in public datasets
- Their analysis of next-generation sequencing data in the public domain identified that 884 of the 9395 samples examined (11%) contained mycoplasma-specific reads
- Representative data from the report (right) illustrates publications from high-impact journals that used mycoplasma-contaminated datasets

Table 1.

Publication status of some of the most contaminated series

GEO series ID	Mycoplasma-mapped RPM	Field of study	Journal	Year of publication	Citations
GSE25183	144 281	Prostate cancer	Nat Biotechnol	2011	271
GSE30772	96 083	Mitochondria biology	Cell	2011	137
GSE45982	84 759	B-cell cancer	Cancer Cell	2013	74

Sources of mycoplasma contamination

How do you get mycoplasma contamination in the lab?

Personnel and equipment

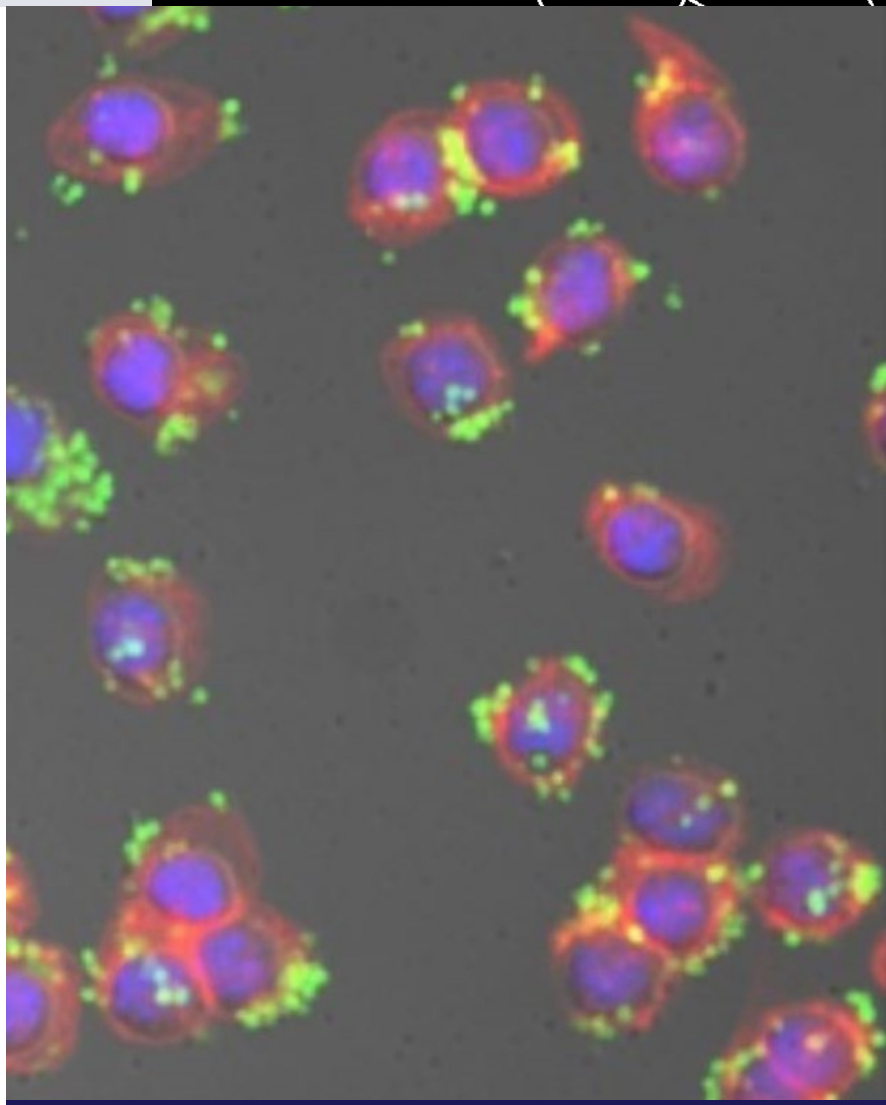
- Poor culturing practices
- Dust and aerosols

Cross contamination

- Aerosol dispersion of contaminated cell cultures
- Broken or faulty laminar flow

Culture reagents

- Sera, media, and reagents



Adult male chicken red blood cells infected with *M. gallisepticum*

How to protect cell cultures from mycoplasma contamination: Prevention methods

Use proper aseptic techniques and practices

- Wear personal protective equipment (PPE)
- Work in a vertical laminar flow hood

Quarantine new cell lines of any origin

Use antibiotics responsibly

- Avoid the indiscriminate use of antibiotics

Employ good cell banking practices

- Only work with one cell line at a time
- Ensure all media, sera, and reagents are obtained from mycoplasma-free sources

How to protect cell cultures from mycoplasma contamination: Routine testing methods

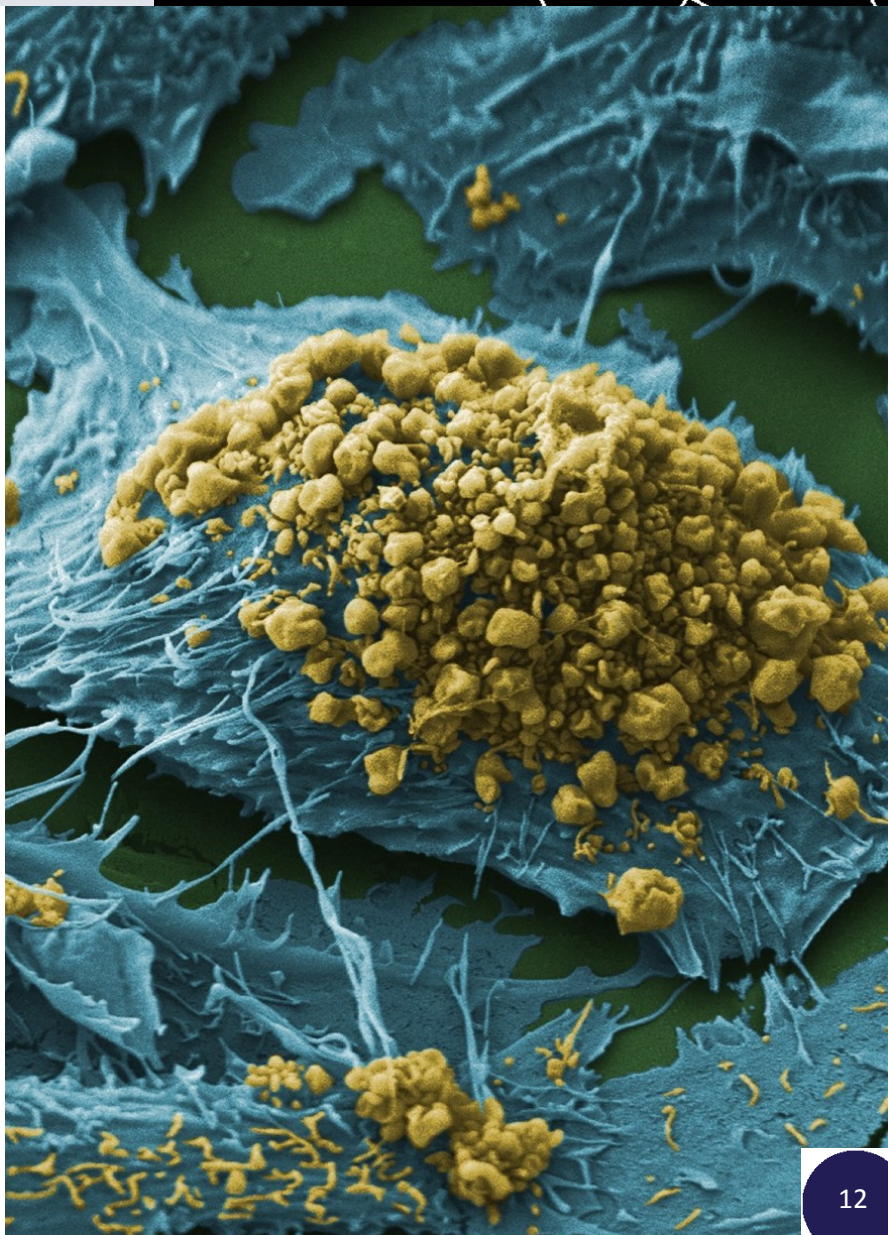
Why is routine testing is important?

Mycoplasma contamination is not easily detected

- Does not cause media turbidity
- Does not alter the pH of the media
- Few metabolic byproducts
- Cannot be detected by microscopy

Common testing methods

- Direct agar culture
- Indirect Hoechst DNA staining
- PCR-based testing



Direct agar culture

Advantages

- Considered the “gold standard” for testing
- Easy to perform
- Detects viable cells
- Meets FDA Points to Consider

Disadvantages

- Time intensive
- Laborious
- Not all mycoplasma are culturable *in vitro*
- May require expert interpretation
- Requires selective media

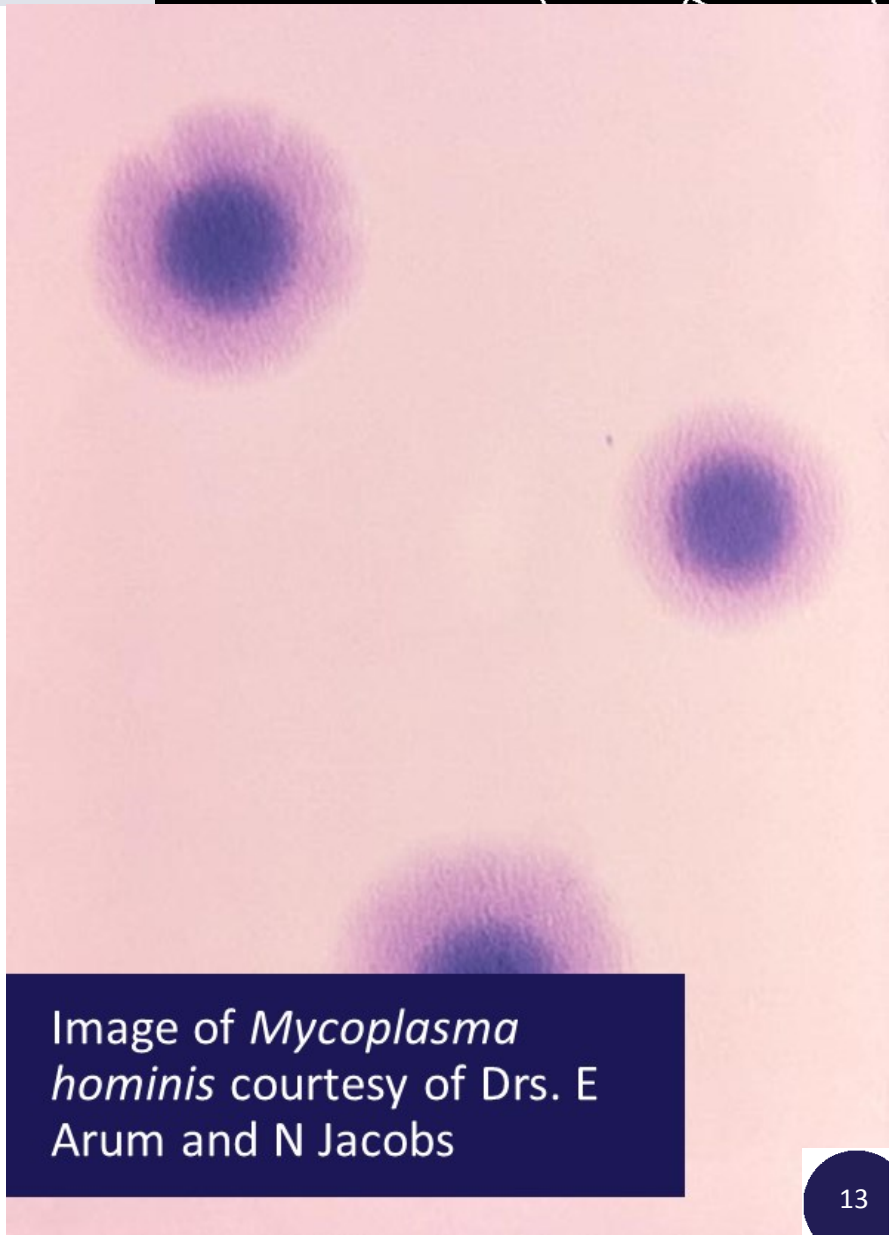


Image of *Mycoplasma hominis* courtesy of Drs. E Arum and N Jacobs

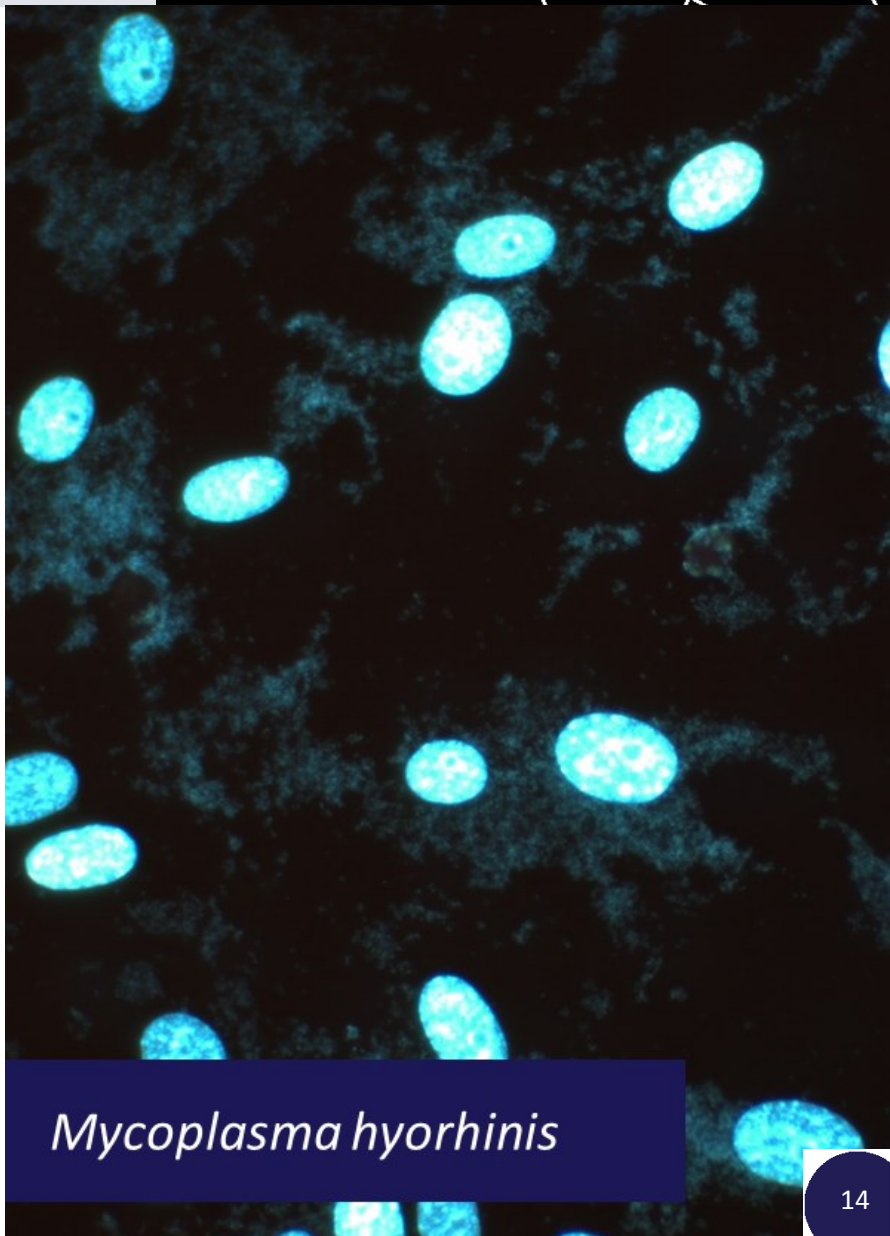
Indirect Hoechst DNA staining

Advantages

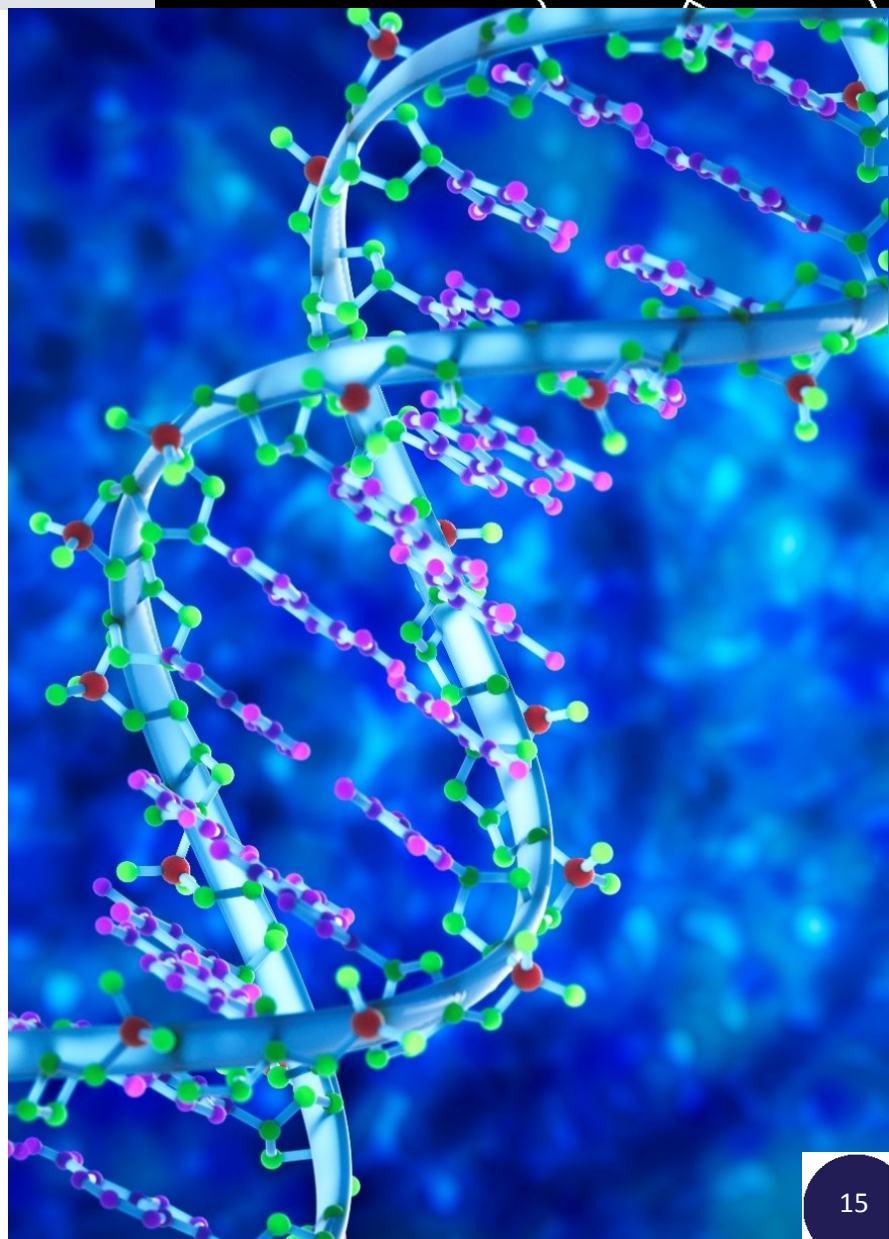
- Easy to perform
- Rapid analysis
- Cost effective

Disadvantages

- Interpreting results can be challenging
- Stains all nucleic acids, so you cannot differentiate between:
  - Eukaryotes vs. prokaryotes
  - Mycoplasma vs. other bacteria



PCR-based methods



Advantages

- Easy to perform
- Reproducible
- High sensitivity and specificity
- Efficient
- Cost effective

Disadvantages

- Cannot distinguish viable and non-viable cells
- Requires primers that are broad enough to amplify different mycoplasma, but specific enough to not amplify other bacterial contaminants
- Requires optimization

ATCC products and services

Direct and indirect culture (bundled service)

- Direct culture – Uses both broth and agar
- Indirect culture – Hoechst DNA stain

PCR-based testing service – *New!*

- Sample spotting on FTA paper and mycoplasma detection by using the ATCC Universal Mycoplasma Detection Kit

ATCC Universal Mycoplasma Detection Kit

- A PCR-based assay that can be purchased and run in the researcher's laboratory

Check out these services by clicking here!

Direct and indirect culture (bundled service)

ATCC® 119-X™

- Meets FDA Points to Consider requirements
- Schedule a test through our customer support team
- Submit a live cell culture flask for testing
- Results obtained within 4 to 5 weeks

Direct and indirect culture sample reports

ATCC®

Mycoplasma Testing Service

Customer Name:	Balsam Shawky
Organization:	ATCC
Sales Order Number:	SO00000
Testing Initiated:	01Sep2018
Results Date:	29Sep2018

Hoechst DNA Staining Results

Sample Designation	Result	Comments
Negative Control	None Detected	Vero cells
Positive Control	Positive	Infected with *Mycoplasma fermentans*
Positive Control	Positive	Infected with *Mycoplasma orale*
Positive Control	Positive	*Mycoplasma hyorhinis*
CRL-2273	None Detected	None

NOTES: ATCC® offers *Mycoplasma* Testing Services for research purposes only. These services are not to be used for clinical diagnosis or applications involving humans.

An "indeterminate" Hoechst DNA Staining result indicates that some extra-cellular or particulate staining is evident; it is unclear whether the staining is consistent with that of *Mycoplasma*.

A "positive" Hoechst DNA Staining result indicates that extranuclear, perinuclear, cytoplasmic, and/or extracellular staining was observed. This type of staining is usually indicative of *Mycoplasma* contamination, but can also be caused by other types of contaminants or culture conditions. Additional testing by the customer may be useful to determine the source and/or identity of any observed contaminant(s).

In order to detect all strains of *Mycoplasma*, the Hoechst DNA Staining method must be used in conjunction with the Direct Culture method. Please note that Direct Culture results are reported separately from Hoechst DNA Staining results.

PCR-based mycoplasma testing service (FTA paper)

ATCC® 136-XV™

FTA paper contains chemicals that lyse cells on contact, tightly binds and protects DNA from degradation, and prevents bacterial growth.

FTA cards provide for easy sample handling and shipping.

- ✓ ISO/IEC 17025:2005 certified
- ✓ Uses the ATCC Universal Mycoplasma Detection Kit
- ✓ Results obtained within 3 to 5 business days

PCR-based mycoplasma testing service (FTA paper)

ATCC® 136-XV™

1. Place your order for the service to receive an FTA Kit with a unique barcode
2. Spot your cells onto the FTA card following sample preparation instructions and fill out your contact information
3. Mail the dried sample card to ATCC in the provided envelope
4. Receive an email notification once your sample is received and is in processing
5. Receive your testing results by email

ATCC® | CELL AUTHENTICATION TESTING SERVICE
MYCOPLASMA TESTING

Sample Preparation Instructions

Note: Cells should be spotted at a target density of 1 x 10^6 cells/mL. Cell counting is recommended as cells spotted at a density less than 0.8 x 10^6 cells/mL may not yield acceptable results.

1. Fill out a separate Cell Authentication Sample Submission Form for each sample submitted. Be sure the Barcode Number on the top of the Sample Submission Form matches the Barcode Number on the Indicating FTA™ Micro Card (Sample Collection Card).
2. Prepare the samples one at a time at an optimal target cell density of 1 x 10^6 cells/mL.
   a. For adherent cells: Scrape the cells into the existing culture media and suspend. **Important:** *Do not treat cells with trypsin or EDTA as these agents disrupt mycoplasma.*
   b. For suspension cells: Harvest and count the cells. If cell density is less than 1 x 10^6 cells/mL, centrifuge cells and resuspend them in a volume of existing cell culture media that will result in a cell density of 1 x 10^6 cells/mL.
3. Before handling the Sample Collection Card, thoroughly clean the work surface. With gloved hands, carefully open the Sample Collection Kit and remove the Sample Collection Card.

Sample Submission Form

Place Barcode Label

Thank you for placing an online order for the Mycoplasma PCR Testing Service.
Please read this form in its entirety and follow all steps accurately.

Peace of mind in 3 easy steps

1 **The Mycoplasma PCR Testing Results will be emailed by ATCC to the email address provided below.**
Customer Information (All fields are required. Please print information to ensure it is legible)

Name ____________________

Institution/Company ____________________

Address ____________________

City ________ State ________ Zip code ________ Country ________

Email ____________________

(Please print clearly; authentication results will be sent to the email address above)

Phone ____________________ Ext. ________
(Include Country Code)

SAMPLE

Mycoplasma PCR sample report

ATCC®

Cell Authentication Service

Mycoplasma PCR Testing Report

Sample Submitted By:	Balsam Shawky
E-mail Address:	bshawky@atcc.org
Sales Order Number:	SO0169872
FTA Barcode:	MYCA0001
Cell Line Designation:	CRL-1658
Date Sample Received:	Tuesday, May 08, 2018
Report Date:	Friday, May 11, 2018

The Mycoplasma Testing Service from Whatman® FTA® paper offers a quick and sensitive PCR-based test to detect mycoplasma contaminants using ATCC's Universal Mycoplasma Detection Kit technology. The kit detects over 60 species of Mycoplasma, including the top eight most likely to contaminate cell cultures. Samples that are positive for mycoplasma are recognized by a distinct PCR product ranging in size from 434 to 468 base pairs (bp) on an agarose gel.

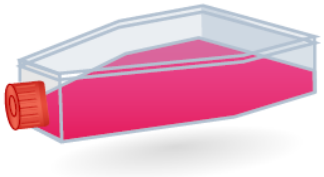
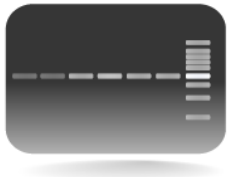
PCR Testing Results

Sample Designation	Result	Comments
Negative Control	NEGATIVE	No visible band present in the negative control lane.
Positive Control	POSITIVE	A visible band is present at 464-bp for *M.arginini DNA*.
MYCA0001-CRL-1658	**NEGATIVE**	No visible band present in the mycoplasma detection range.

- Order information with FTA barcode number
- Test results with analysis
- Expert review and signatures

ATCC Universal Mycoplasma Detection Kit

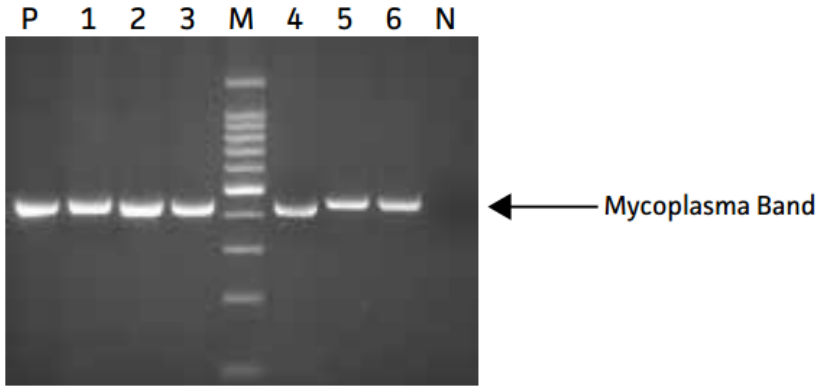
ATCC® 30-1012K™

Grow cells	Scrape and suspend cells, or count	Add Lysis Buffer	Perform PCR	Run gel
				
Works with adherent and suspension cells. Use 10^4 to 10^5 cells per assay.	Transfer cell suspension to Sample Lysis Tubes. Pellet cells. Cell pellet can be stored until ready to assay.	Incubate. Heat at 95˚. Spin. Transfer cell lysate to new microfuge tube. Cell lysate can be stored until ready to assay.	Perform optimized touchdown PCR using Universal PCR + Primers Mix.	Clear results in less than 3 hours.

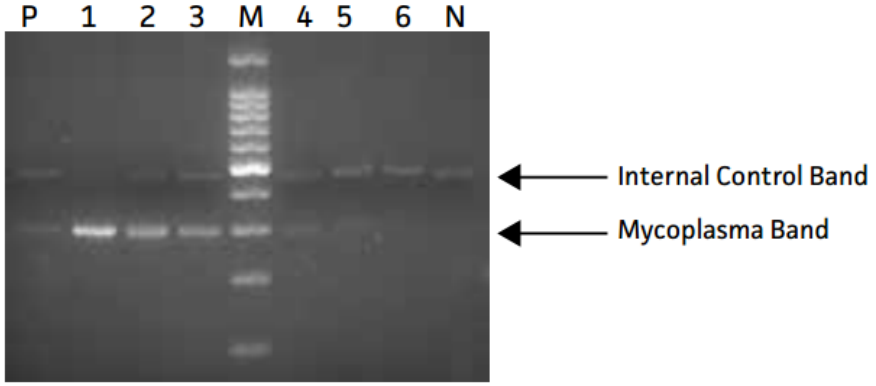
- ✓ Detects over 60 species of *Mycoplasma, Acholeplasma, Spiroplasma,* and *Ureaplasma*
- ✓ All components for the PCR reaction are provided and optimized for amplification

The ATCC Universal Mycoplasma Detection Kit demonstrates high sensitivity

ATCC Universal Mycoplasma Detection



Supplier 1



P=Positive control N=Negative control M= 100 bp Ladder Lanes labeled 1 through 6 correspond to the dilution series

Summary of Results for BHK Cells Infected with *M. orale*

	Dilution					
	1	**2**	**3**	**4**	**5**	**6**
Number of Cells/mL	500,000	50,000	5,000	500	50	5
CFU/mL	2,208	154	13	2	0	0
ATCC	+++	+++	++	++	+	+
Supplier 1	++	+	+	+	+/-	+/-
Supplier 2*	+++	++	++	-	-	-
Supplier 3*	+	++	++	++	-	-

* Positive and negative scores were determined by following the Suppliers' instructions. Supplier 2 provides a metabolic assay for the detection of mycoplasma; Supplier 3 provides a PCR-ELISA test kit.

Benefits of using ATCC's mycoplasma detection services

- ATCC has been providing the mycoplasma detection kit since 2010
- ATCC has multiple years of experience performing direct & indirect mycoplasma testing
- Before distribution, ATCC confirms that all cultures are mycoplasma-free using our direct, indirect, and PCR-based methods
- Accredited service ISO/IEC 17025:2005
- The ATCC website summarizes the advantages of both the direct/indirect culture testing service and the PCR-based mycoplasma detection service

	Option 1: Mycoplasma PCR Testing Service	Option 2: Mycoplasma Direct & Indirect Culture Testing Service
ATCC Number	ATCC® 136-XV™ Order Service	ATCC® 119-X™ Schedule Testing
Sample Format	FTA collection card and kit provided	Live cell culture
Schedule Testing	No	Yes
Test Method	Universal Mycoplasma Detection Kit (ATCC® 30-1012K™) used to amplify material eluted from FTA paper	Direct culture method uses broth and agar Indirect culture method uses Hoechst DNA staining
Protocol	PCR	FDA Points to Consider
Result Type	Qualitative (Positive/Negative)	Qualitative (Positive/Negative)
Delivery	PDF reports emailed	PDF reports emailed
Report within	5 business days	Direct culture results in 4 to 5 weeks Indirect culture results in 7 to 10 business days
Advantages	Rapid, Reproducible, Sensitive Can detect a broad range of mycoplasma species, including non-culturable strains Cost-effective	Sensitive Detects viable cells, indicating an active infection Indirect culture enables the detection of non-culturable mycoplasma strains
	Mycoplasma Submission Instructions Form	

Eradicating mycoplasma contamination

Historical mycoplasma recommendations from ATCC

nature
International journal of science

Product Review | Published: 08 June 1989

Mycoplasma infection of cultured cells

R. J. Hay, M. L. Macy & T. R. Chen

Nature **339**, 487–488 (08 June 1989) | Download Citation

Mycoplasma contamination is tough to detect and even more difficult to eradicate. It is best to start over fresh from clean cell stocks, but several elimination options are available.

R. J. Hay

Cell Culture Department American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852, USA.

M. L. Macy

Cell Culture Department American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852, USA.

T. R. Chen

Cell Culture Department American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852, USA.

Eradicating mycoplasma contamination

Cell culture

- Destroy contaminated cell cultures
- Antibiotic therapy
- Acquire fresh cells

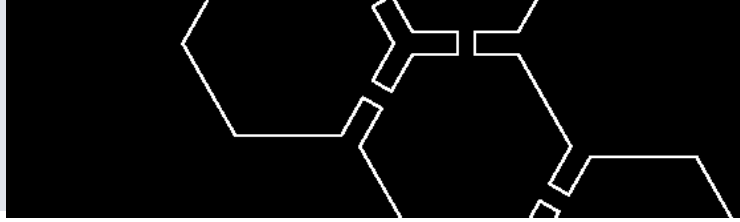
Media

- Destroy contaminated media
- Use media guaranteed to be mycoplasma-free
- Sterilize media via filtration or UV irradiation

Laboratory

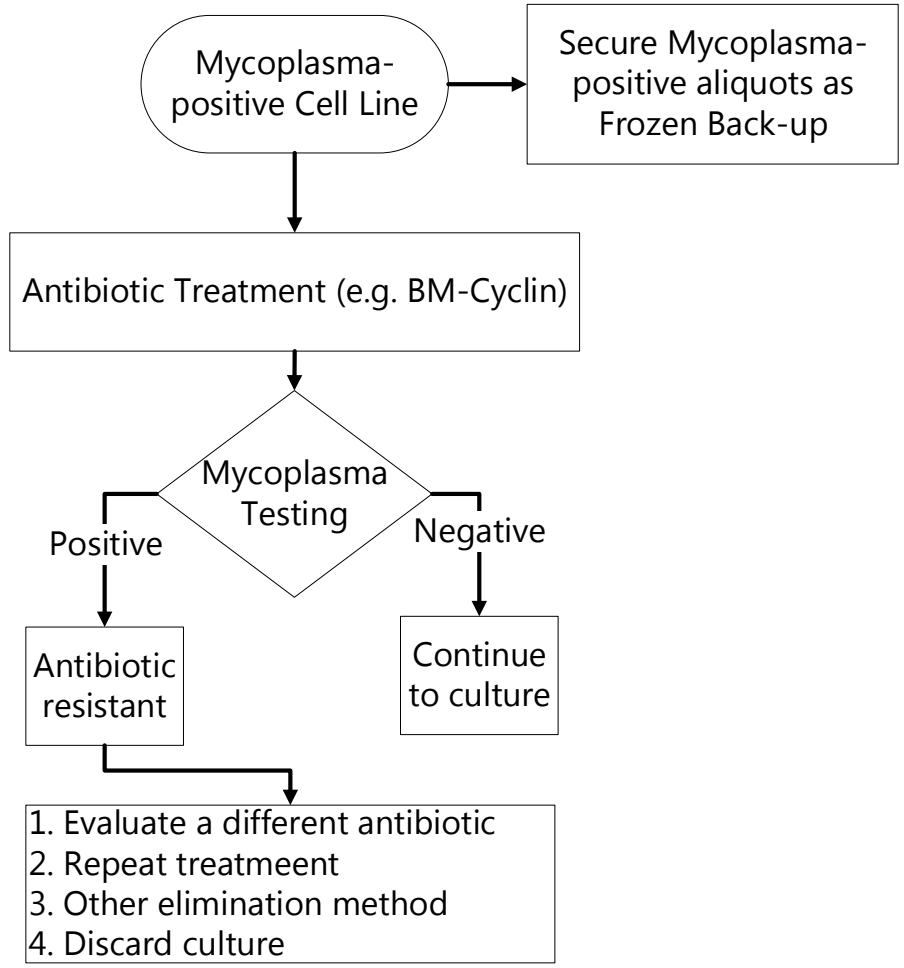
- Disinfect all laboratory surfaces and equipment (*e.g.,* biosafety cabinets, incubator, water bath, laboratory bench)

Antibiotics and mycoplasma



When cell cultures are too valuable or difficult to replace, there are chemical agents that can be used to eliminate mycoplasma infection.

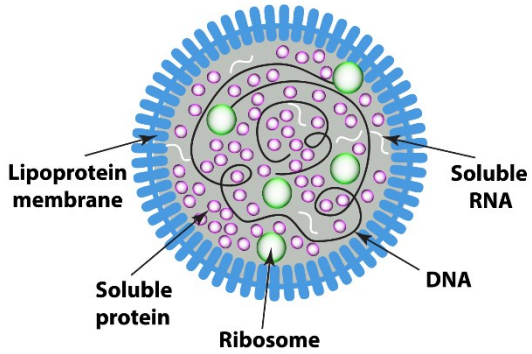
- Tetracycline and macrolide antibiotics can remove mycoplasma from cell culture
  - BM-Cyclin, a combination of tiamulin and minocycline, inhibits bacterial protein synthesis
  - Plasmocin™ contains bactericidal components that affect protein synthesis & DNA replication
- If you must use antibiotics, a recommended workflow* is illustrated to the right

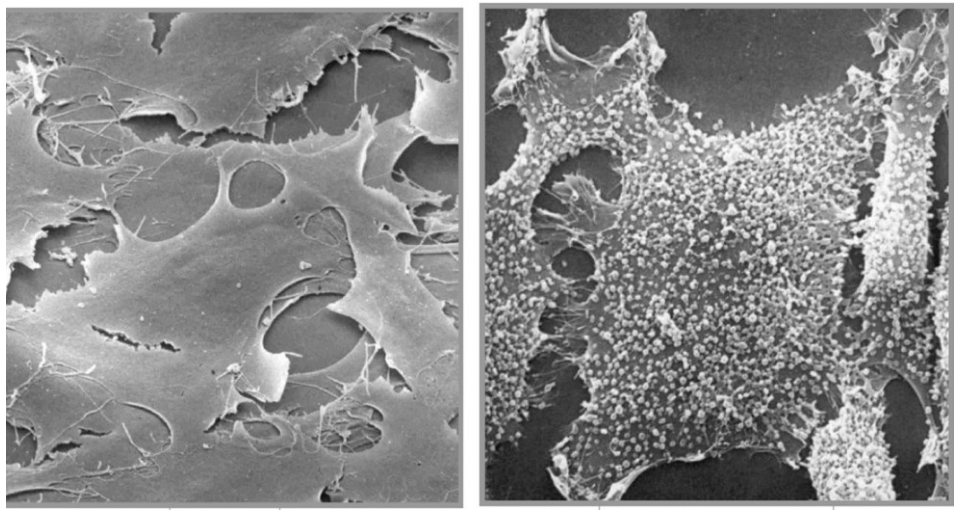


* Derived from: Cytotechnology 39(2):75-90, 2002.

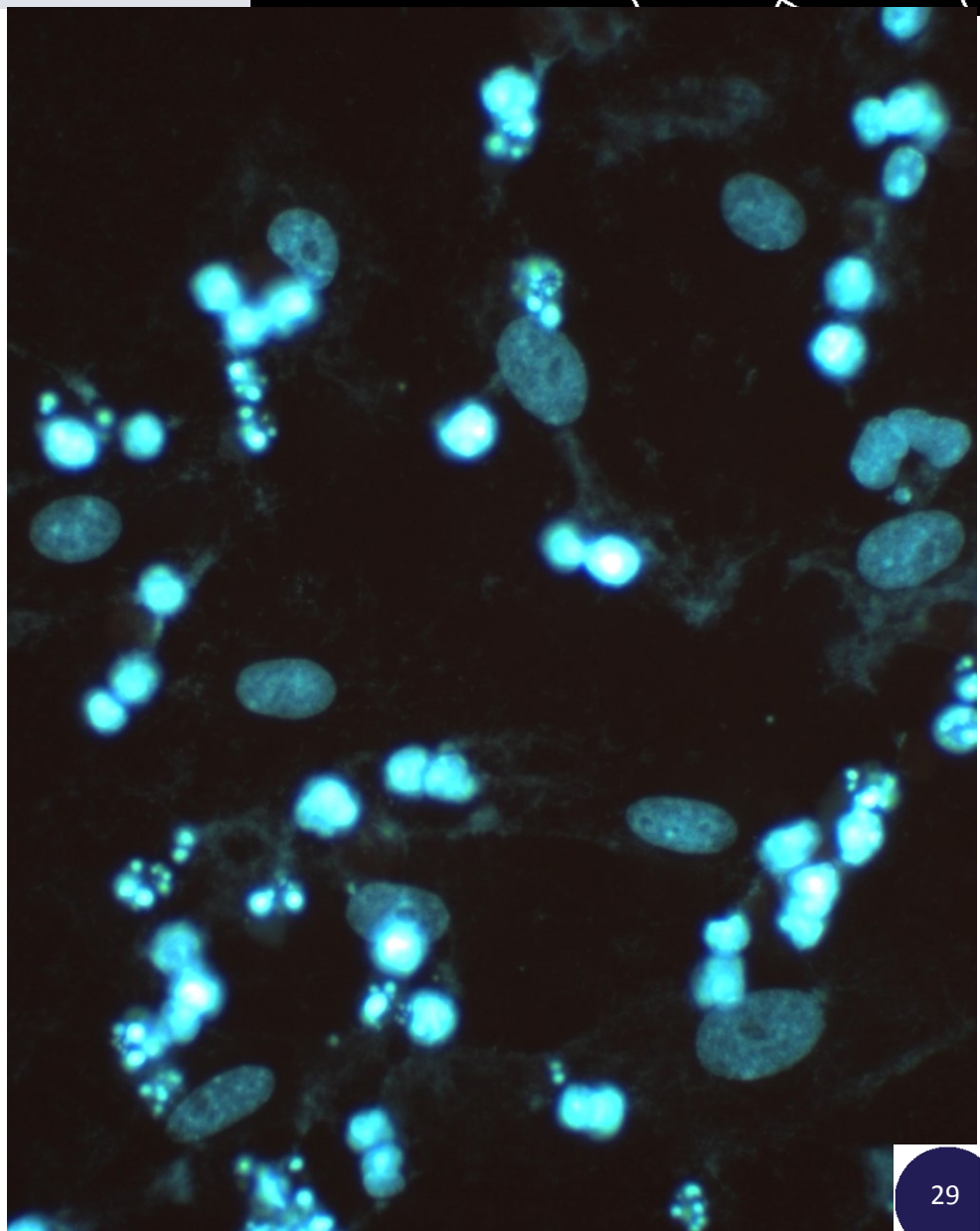
Conclusions

- Mycoplasma contamination affects roughly 15-35% of continuous cell cultures, resulting in deleterious effects
- The best practices for avoiding contamination and preventing the spread of existing contamination include:
  - keeping a documented history of your cell line
  - following cell culture best practices
  - routine testing
- Eliminating mycoplasma-infected cultures in the lab should occur quickly and is the best way to prevent contamination
- Direct, indirect, and PCR-based mycoplasma testing methods are available to ensure your cultures are mycoplasma free
- ATCC offers a variety of mycoplasma testing services, including a new rapid PCR-based test using FTA paper!





Disclaimer



Questions & Answers

Q&A