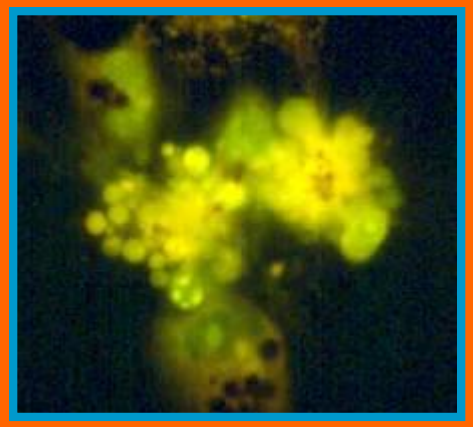
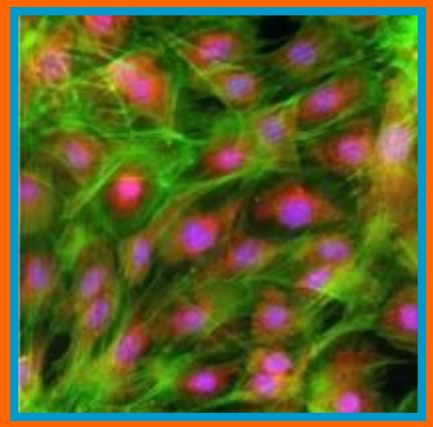
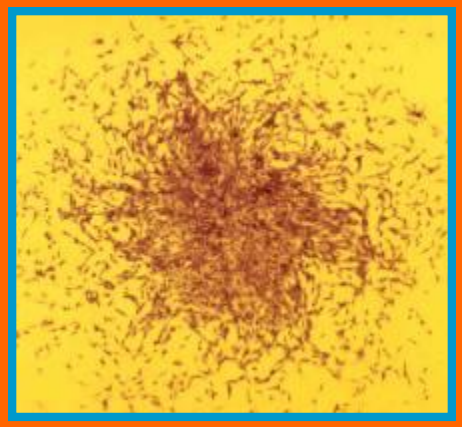






CORNING

Life Sciences

Understanding and Managing Cell Culture Contamination

Presented by John So
Field Applications Scientist

Talk objectives

Two parts:

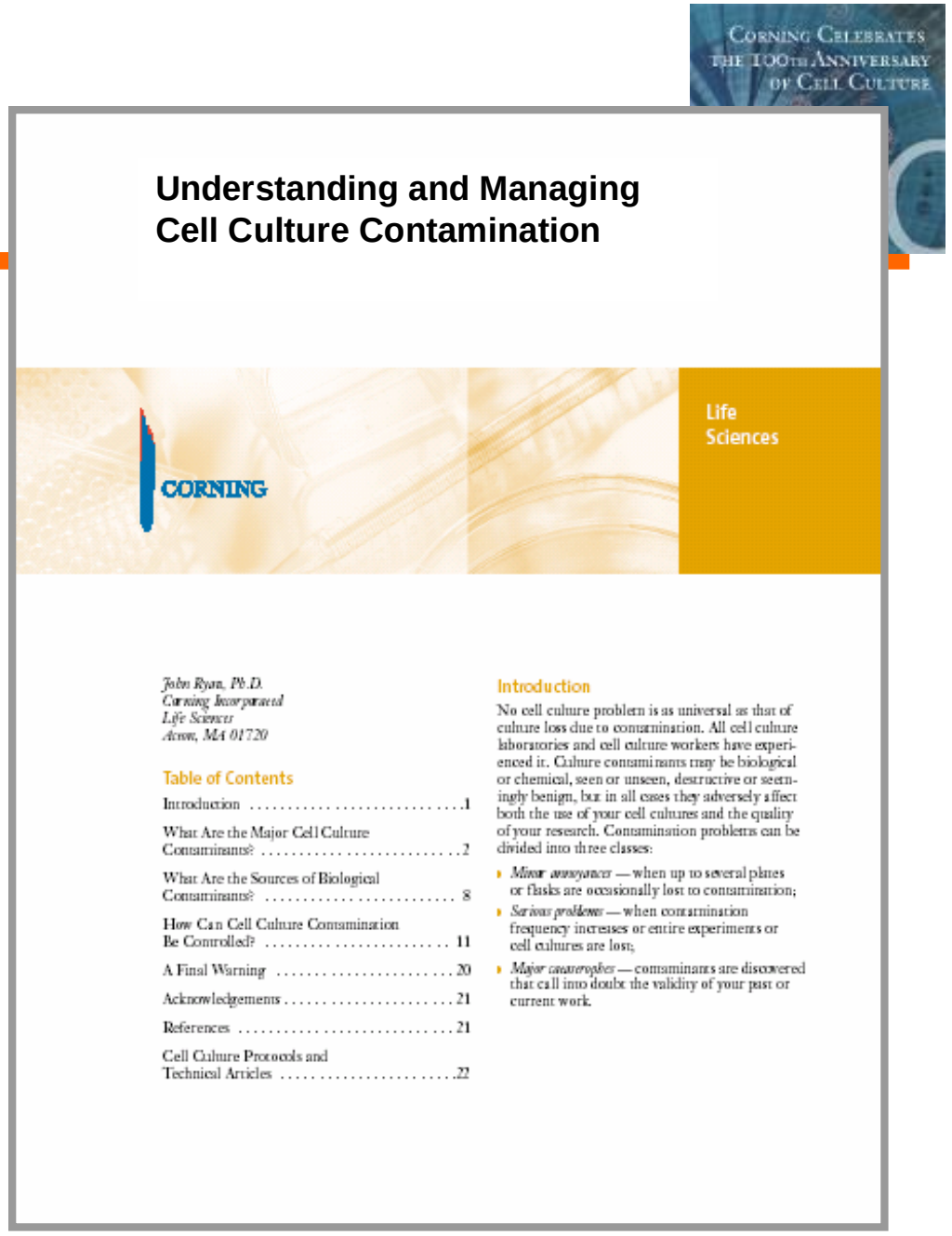
1) Understanding the types, nature and sources of contamination

2) Explore some key concepts and strategies for preventing culture loss from contamination

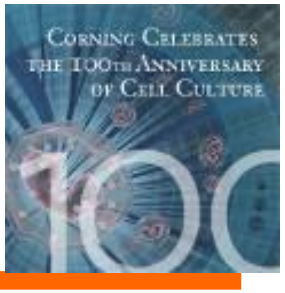
My goal is to give you a new perspective on the problem of contamination and at least one new strategy for managing these problems

For more information...

Please check
Corning's web site:
www.corning.com/lifesciences



Consequences of contamination



- Personal embarrassment
- Loss of time, money and effort
- Adverse effects on the cultures
- Loss of valuable cells and/or products
- Bad data/erroneous publications

Key concept

Contamination cannot be eliminated, but it can be managed to reduce both the frequency of occurrence and the seriousness of its consequences

1st step is understanding the types of contaminants

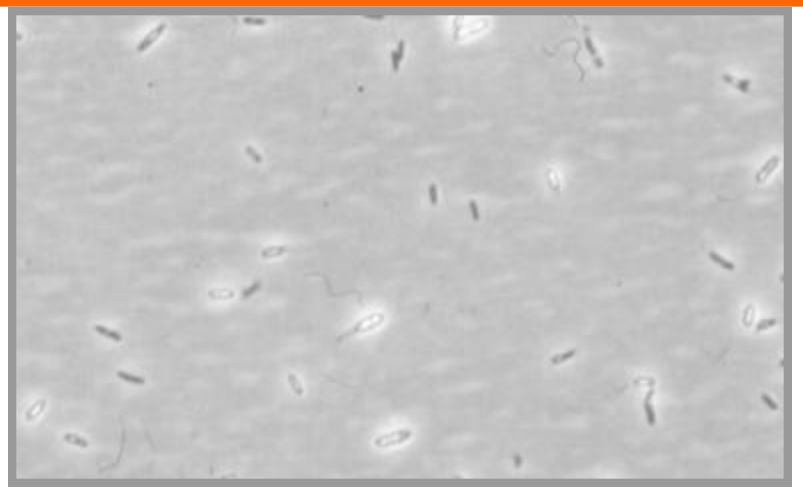
Types of culture contaminants

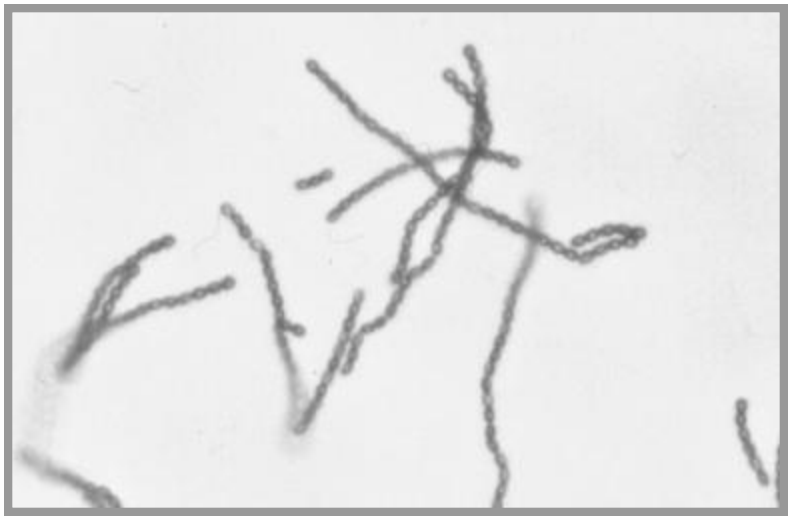
Most culture contaminants fall into two groups:

- Those that are usually easy to detect (if no antibiotics are used) - bacteria, yeast and fungi
- Those that are difficult to detect - toxic chemicals, viruses, parasites, insects, mycoplasma & other cell lines. These last two cause the most serious (and potentially embarrassing) problems

Bacterial contaminants

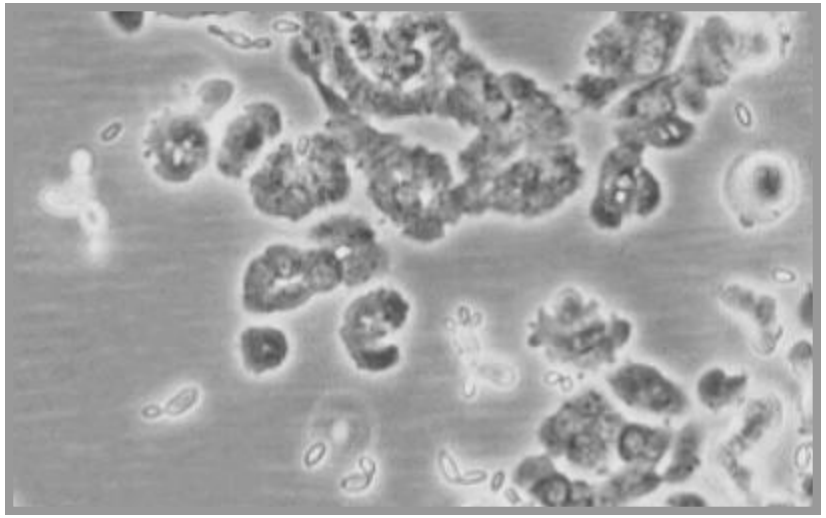
- Common
- Easy to mistake for cellular debris, especially at low levels
- Look for
  - signs of motility
  - size and shape uniformity
  - Acidic pH

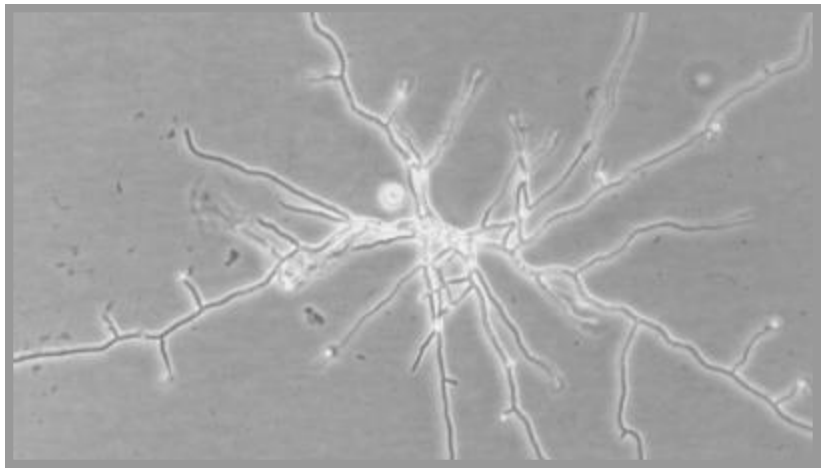




Fungal and yeast contaminants

- Also common
- May form clumps or mats
- Larger size makes them easier to identify
  - yeast will usually show signs of budding
  - fungi may initially form small colonies on medium surface





Chemical contaminants

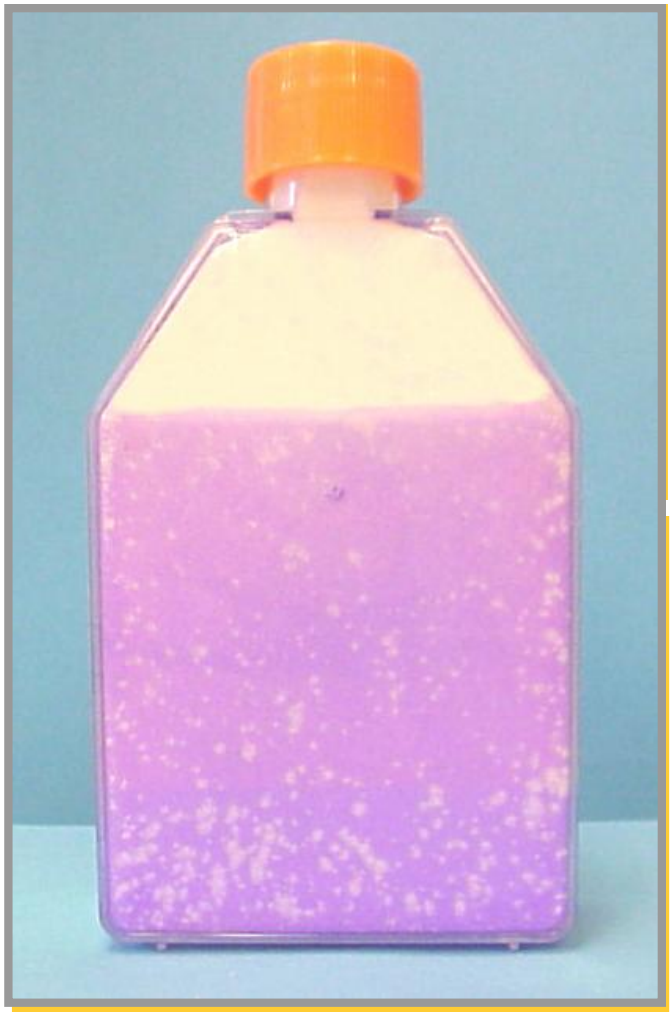
Not very common but difficult to trace:

- Toxic by-products from photoactivation of riboflavin, tryptophan and HEPES buffer in media by fluorescent light exposure
- Metal ions, endotoxins and other contaminants in water, media and sera
- Disinfectant or pesticide residues in incubators
- Impurities in gases used in incubators

Viral contaminants

Not very common:

- Detected by their adverse affects on cultures
- Many unknown problems are wrongly blamed on viruses
- Fetal bovine sera contains bovine viruses

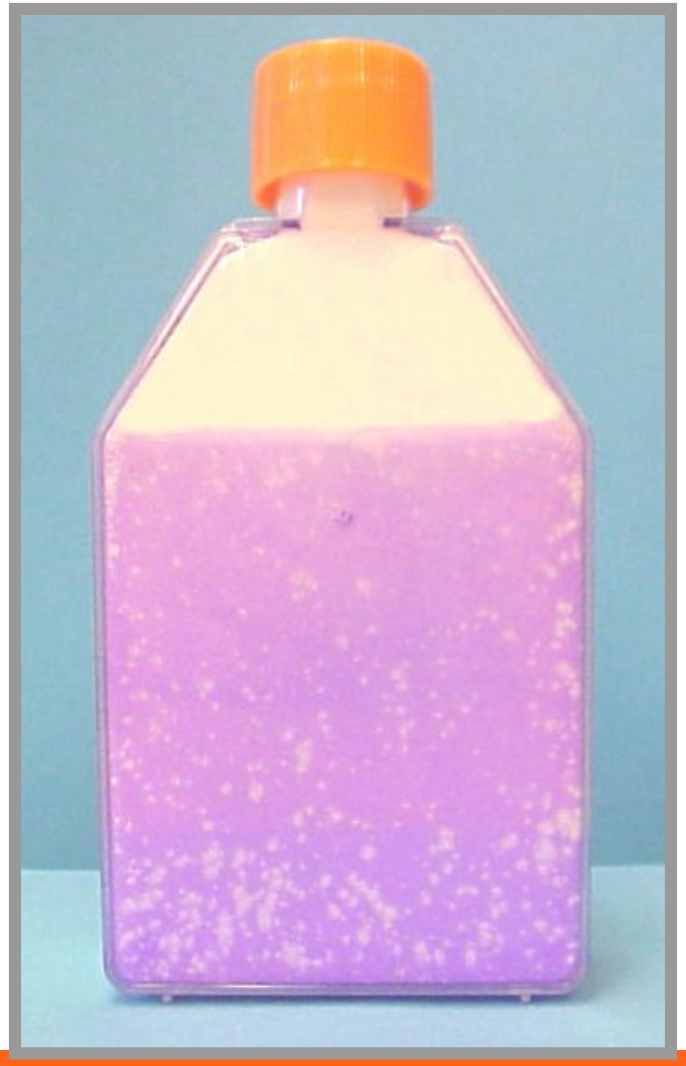


Viral contaminants

Detection:

- Immunostaining
- ELISA
- PCR
- Commercial: BioReliance

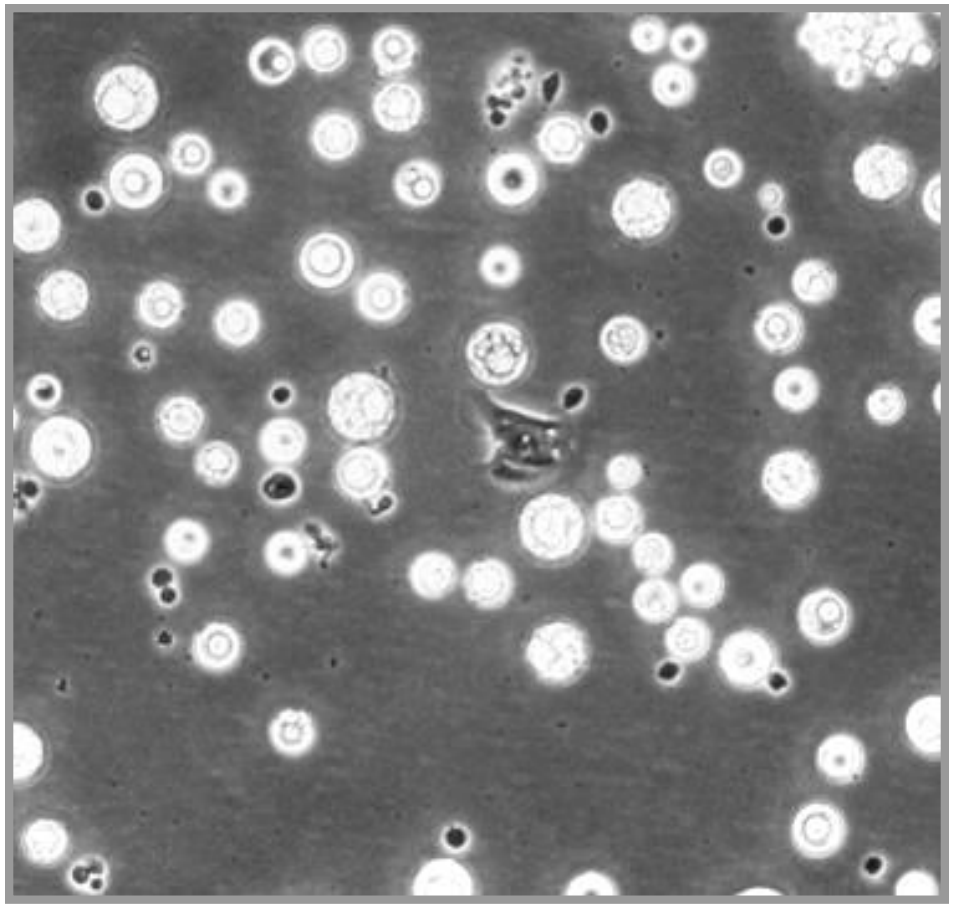
No reliable way to completely eliminate viral contamination



Insects and parasites

Fortunately, these are very rare:

- Insects & spider mites can contaminate both cultures and sterile supplies
- Amebas, protozoa, & other parasites have been found in cultures

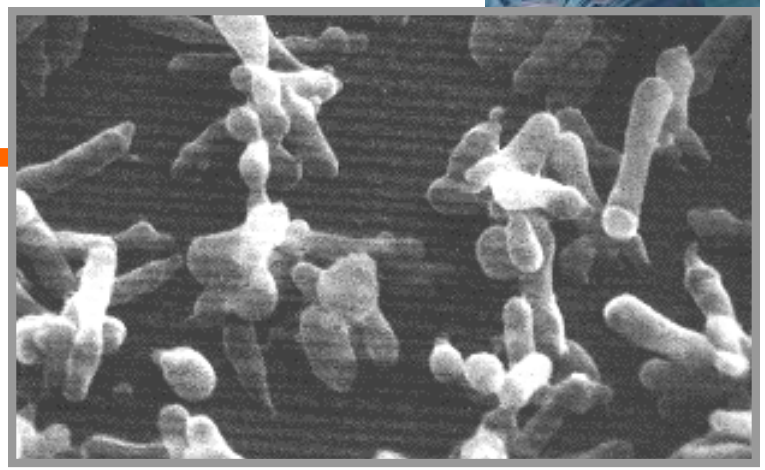


Sporozoa in a flounder culture

Mycoplasma

Very common (15+% in US)

- Smallest free living organisms (0.2 - 0.3µm)
- No cell wall; can not be seen under phase contrast microscopy
- Effect virtually every aspect of cell behavior & growth, even microarrays
- Up to 10^8 mycoplasma/mL medium without turbidity



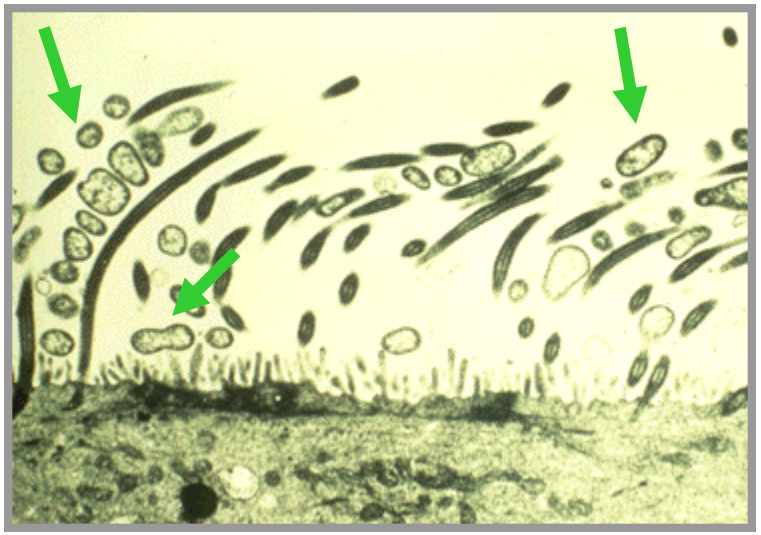


Photo courtesy of Richard F. Ross and F. Chris Minion, Iowa State University

Mycoplasma

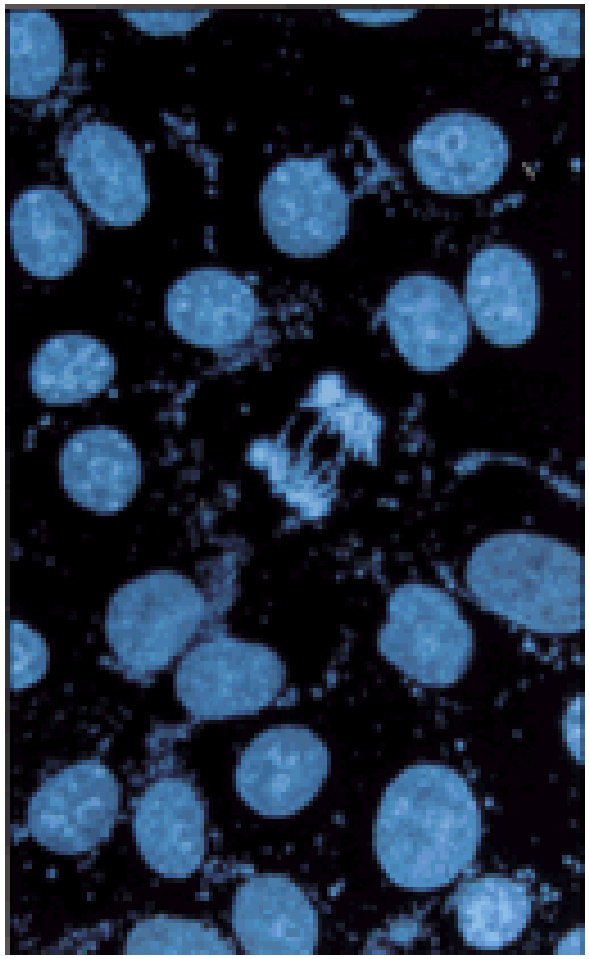
- Most common form of contaminate in cell culture today!
- Approximately 180 different species exist
- The most common method of transmission is from other contaminated cultures

Types found in mammalian culture
M. orale (human 50%)
M. arginini (bovine)
M. Hyorhinis (pig)
M, fermentans
M. Salvarium (human)
A. Laidlawii (environment)

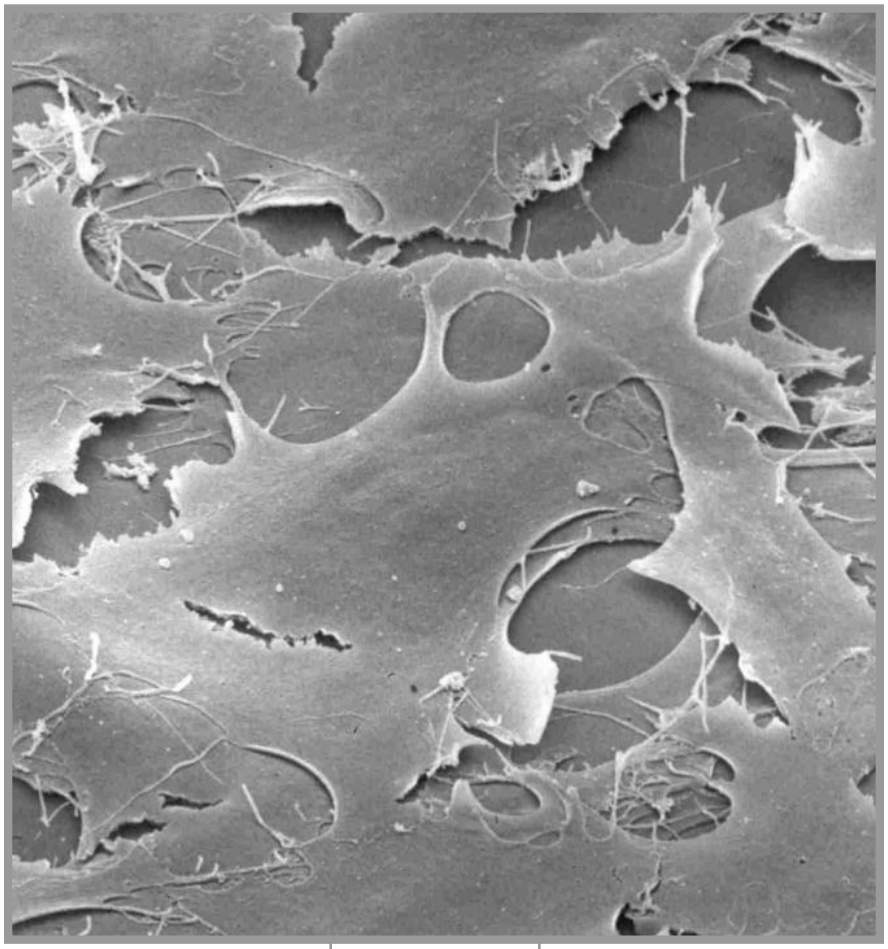
Mycoplasma

Mycoplasma contamination

- Effects cellular metabolism, growth, viability, DNA/RNA, protein synthesis, and morphology
- Interfere with screening assays
- Cause chromosomal breakage
- Leads to unreliable or erroneous data

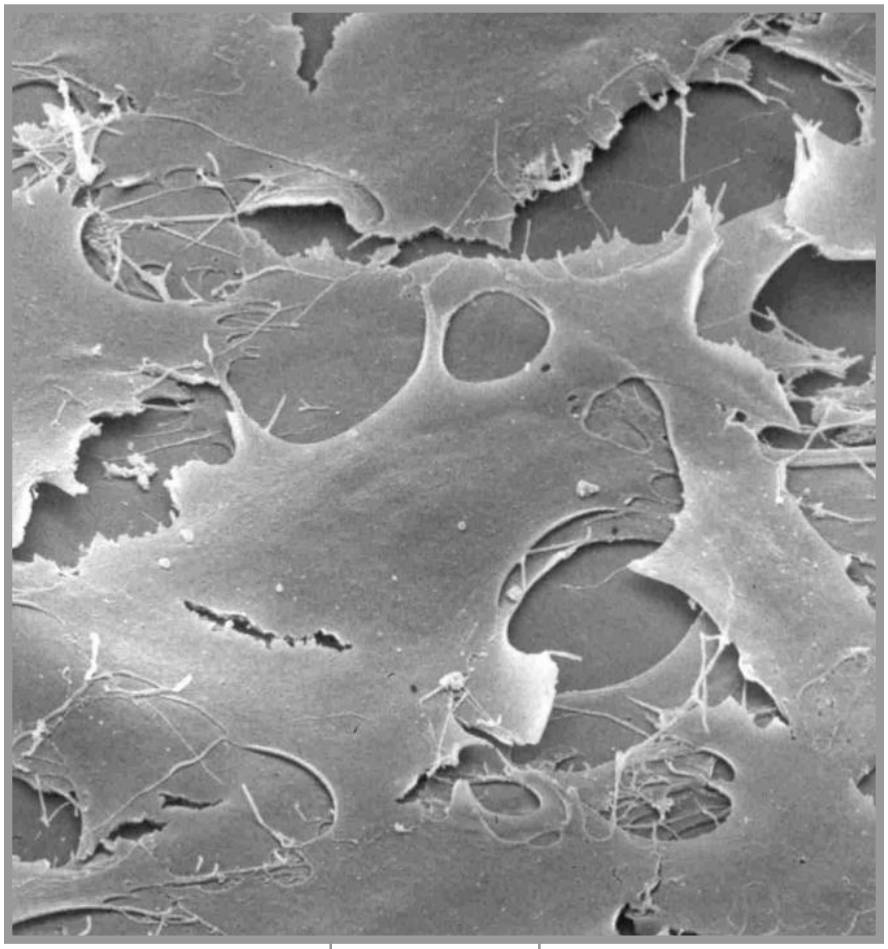


Mycoplasma in 3T6 cultures

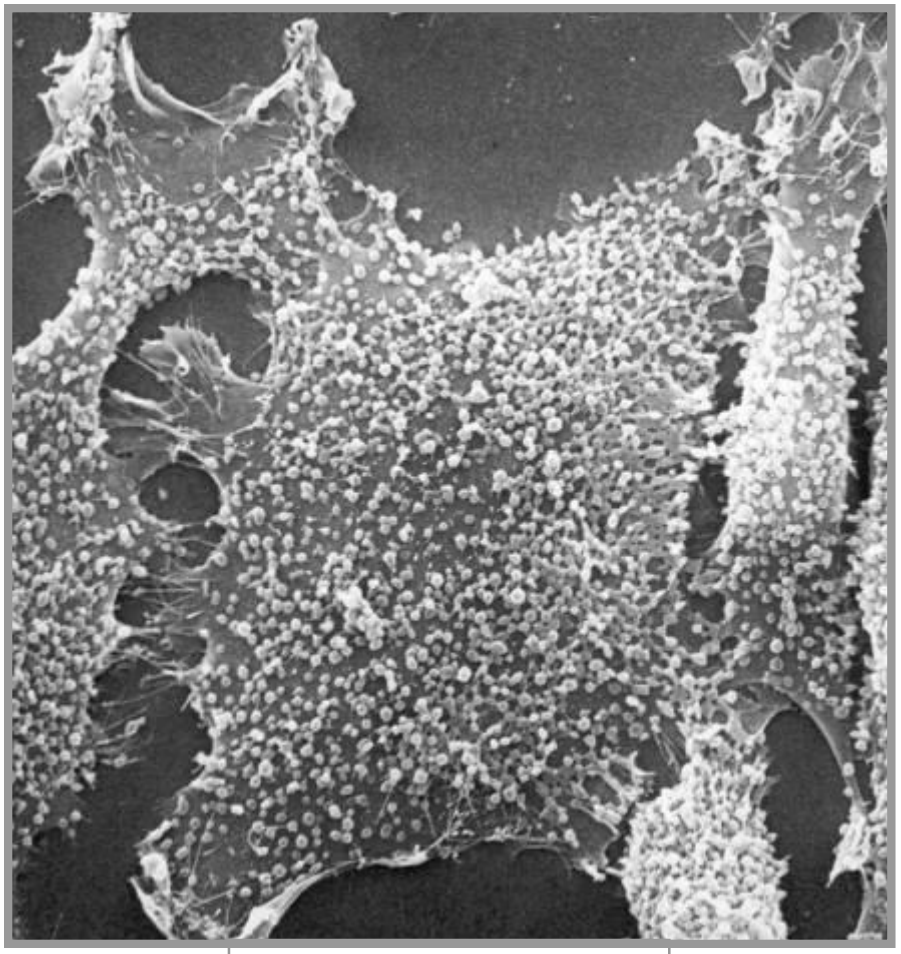


Clean

Mycoplasma in 3T6 cultures



Clean



Contaminated

Mycoplasma - How big is the problem?

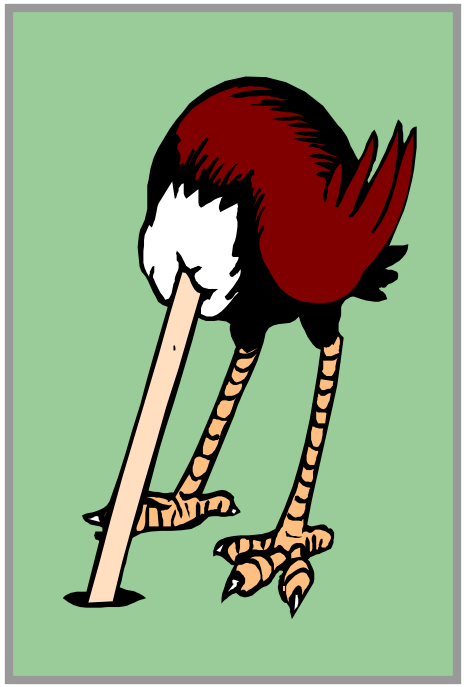
# Cultures Tested	# Positive
• FDA **20,000**	**3,000+ (15%)**
• Bionique Testing Labs **11,000**	**1,218 (11.1%)**
• Microbiological Associates **2,863**	**370 (12.9%)**
• ATCC **5,362**	**752 (14%)**

This problem is worldwide!

# Cultures Tested	# Positive
• Netherlands 1,949	488 (25%)
• Czechoslovakia 327	121 (37%)
• Argentina	65%
• Japan	80+%
• Israel	32%

Why is mycoplasma so widespread?

- Highly Contagious
  - Other contaminated cultures in the lab
  - From ourselves – bad aseptic technique
- Overuse of antibiotics (+65%)
- Obtaining cells from untested sources
- Denial - 50% do not test for mycoplasma

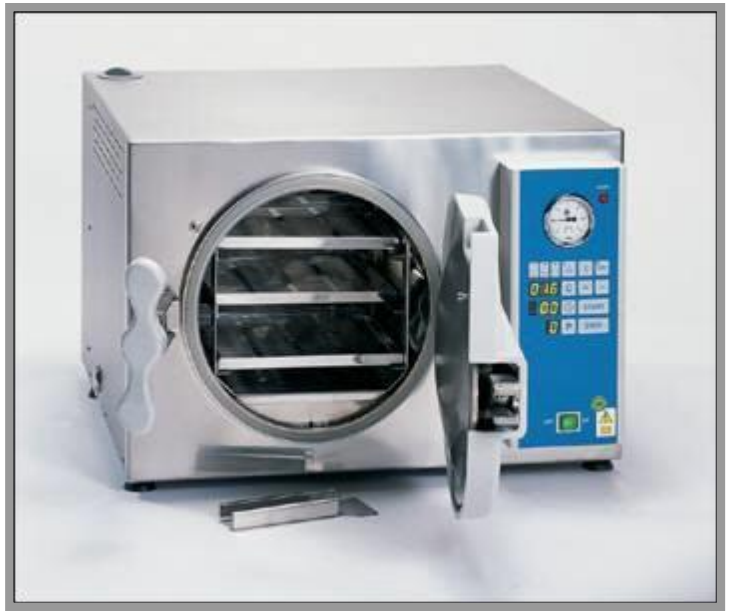


4 steps for controlling mycoplasma

1. Avoid continuous use of antibiotics – they do not prevent contamination
2. Be careful of “free” cell lines – they are often worth what you pay for them
3. Test cultures for mycoplasma – it is not that difficult (It is worth your peace of mind and reputation)
4. Rely on a tested cell repository as the source of all of your cells (ATCC, DSMZ, etc)

How can cultures be cured?

- Autoclaving – it always works
- Commercial clean up kits
  - may only temporarily reduce contamination below detectable levels
  - may change characteristics or kill cells
- Have a pro do it - $2000+



If you detect mycoplasma?

- Autoclaving – it always works

LAST RESORT

- Tylosin (MP Biomedicals)
- MRA; mycoplasma removal agent (MP Biomedicals)
- Ciprofloxacin (Bayer)
- BM Cycline (Roche)

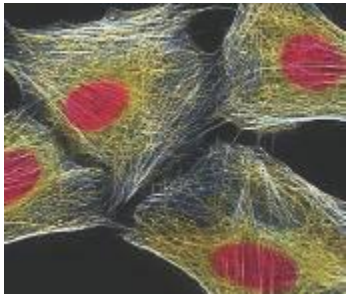
<u>Non- Antibiotic</u>

- Mynox Mycoplasma Elimination Reagent (Sigma)

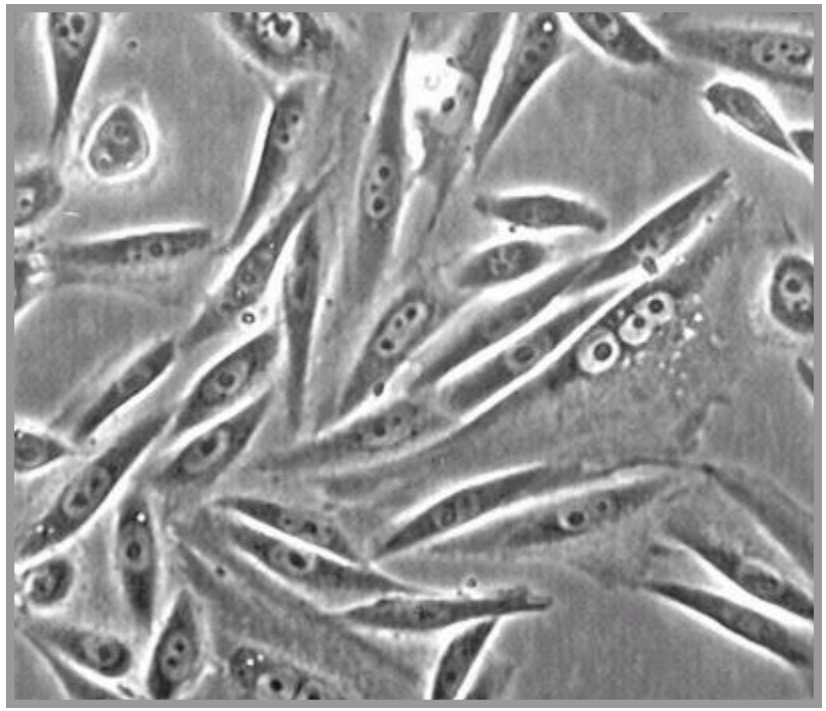
Caution: May just reduce below detectable levels.

Cell culture mix-ups

- Contamination of one cell line by another was first reported in the late1950's
- HeLa cells are the most "famous" cell culture contaminant



Henrietta Lacks



Can you tell what cell line this is?

History of cross-contaminated cell lines

- **1963:** 28 animal cell lines are contaminated; 25 are actually human.
- **1966:** Gartler showed that 18 of the most widely used human cell lines were actually HeLa cells
- **1976:** Stulberg and Peterson tested 246 cell lines:14% were the wrong species, 15% were intraspecies contaminated
- **1980:** Nelson-Rees showed over 100 cancer cell lines were actually HeLa cells
- **1999:** DNA fingerprinting showed 18% of 252 cell lines sent to the German repository contained other cells (PNAS 98:7656-7658 (1999)

Cross-contaminated cell lines

All below are HeLa cells

- Chang liver (CCL-13)
- KB (CCL-17)
- L132 (CCL-5)
- Detroit 98 (CCL-18)
- WISH (CCL-25) (amnion)
- Intestine 407 (CCL-6)
- Hep-2 (CCL-23)
- AV3 (CCL-21) (amnion)
- MA-160
- Plus many, many more...

Other recently uncovered mix ups

- ECV-304 endothelial cells with T-24 bladder tumor cells
- SNB-19 with U-373 with U-251, all glioblastomas
- OV-1063 female derived but has a Y-chromosome

Visit the ATCC web site, www.atcc.org for the latest updates

Cross-contaminated cell lines

Internet survey of 485 researchers from over 48 countries

- 32% use HeLa cells
- 9% use HeLa contaminated cell lines
- 35% of the cells lines were obtained from other researchers
- Only 33% test their cell lines for cell identity
- 1969-2004 (publications 10X, total 2.7X)

*** From a talk, *Lack of Awareness and Prevention of cell line Cross-contamination among mammalian cell culturists.* Presented by G.C. buehring et al., at the 2004 SIVB annual meeting**

NCI-60 panel

- Group of 60 cancer cell lines that are maintained by the National Cancer Institute and widely used in basic research and drug discovery
- In 2001 it was discovered that the breast cancer cell line MCF-7 and its drug resistant daughter cell line MCF-7/ AdrR (Adriamycin resistant) were unrelated. The drug resistant cell line was actually identical to a ovarian cancer (OVCAR-8) cell line in the panel.
- SNB19 and U251, once thought to be distinct CNS lines are actually the same.
- MDA MD 231 (metastatic breast cancer) is actually the same as M14 (melanoma line)

Excerpt from Science Vol315, Feb 2007

How does it happen?

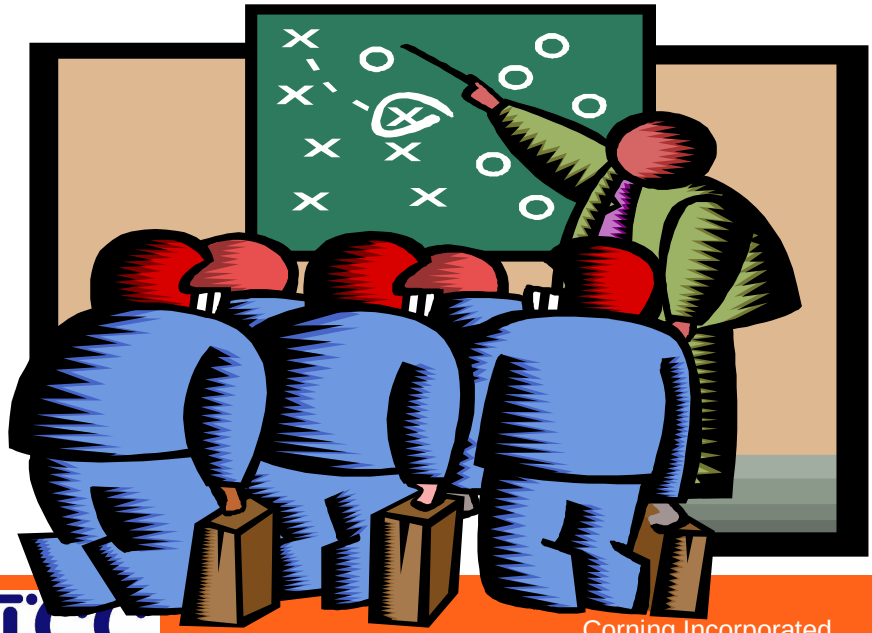
Some real life examples:

- Bad technique and accidents
  - using the same media bottle for multiple cell lines
  - using unfiltered pipet tips
- Mistaken ID (flasks and cells look alike)
- Mix-ups (dangerous acronyms)
- Obtaining cells from untested sources

Key concept

- **The seriousness of a contaminant is usually directly proportional to the difficulty of detecting it; those that go undetected the longest have the most serious consequences and are the most embarrassing**

2nd step is to have strategies to detect, reduce and control contamination problems



How do we reduce and control contamination problems?

Basics:

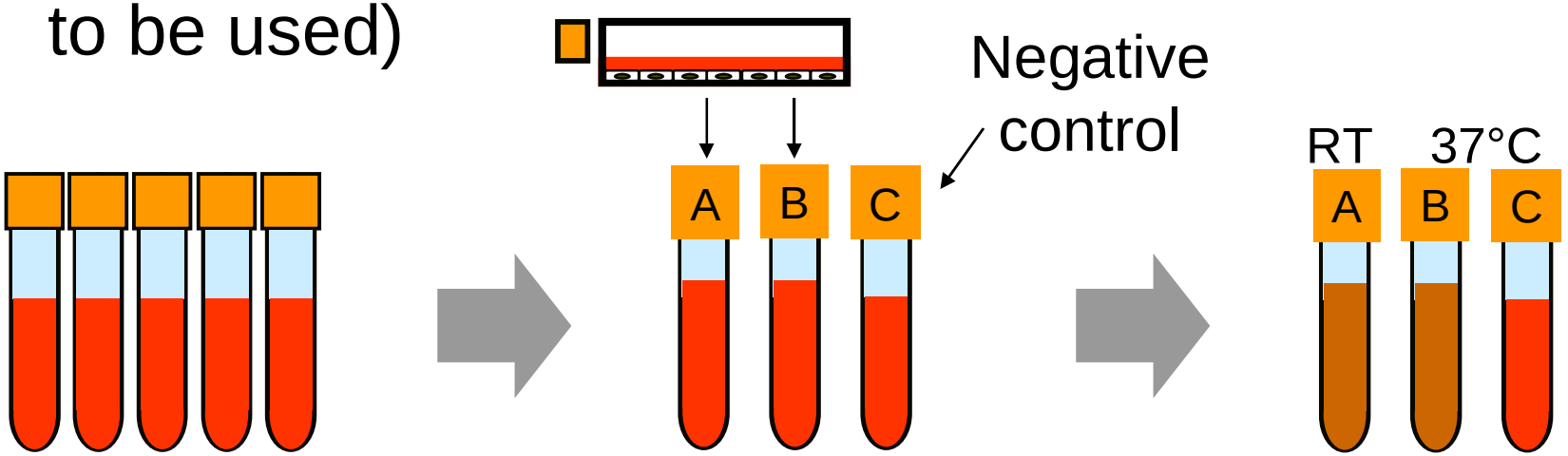
- Keep the lab clean
- Use good aseptic techniques
- Educate lab personnel

Better:

- Quarantine incoming cultures
- Monitor cultures for contamination
- Use antibiotics strategically
- Use a frozen cell repository strategically
- Reduce opportunities for accidents

Testing for bacteria, yeast and fungi

The simple approach (and, as a result, more likely to be used)



Make a rack of antibiotic-free medium + sera in 15mL tubes & store at 4°C

Add 1mL samples from suspicious culture or untested medium to each of 2 tubes

Incubate and examine tubes periodically by eye & at 400x

Testing methods for mycoplasma detection

- **Direct culture in special media**
  - not a good do-it-yourself technique
- **PCR and ELISA kits** –16S and/or 23S rRNA genes

  VenorGeM Mycoplasma PCR detection Kit (Sigma)

  MycoSensor QPCR Assay (Stratagene)

  MycoProbe Mycoplasma Detection Kit (R & D system)
  Based on labeled oligos probes to 16rRNA of 8 forms
- **Fluorescent DNA stains** - DAPI & Hoechst 33258
  - Good do-it-yourself approach if fluorescent microscope is available

Testing methods for Mycoplasma detection

- **Contract testing** - ATCC & Bionique Labs
  - CellShipper from Bionique is $140 for 4 cultures
  - ATCC has a direct culture/DNA stain for $125

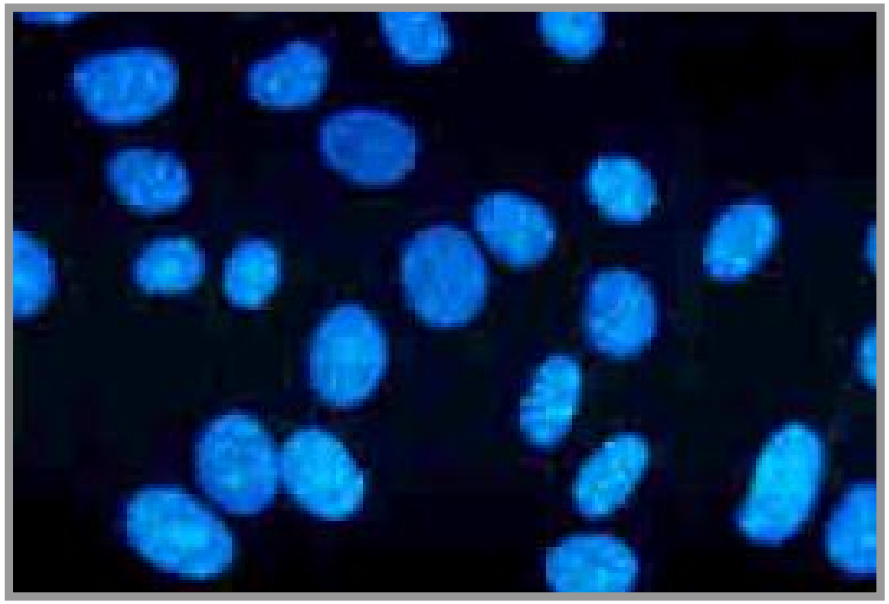
  Baseclear (Netherlands)

  Cell Essentials (MA)

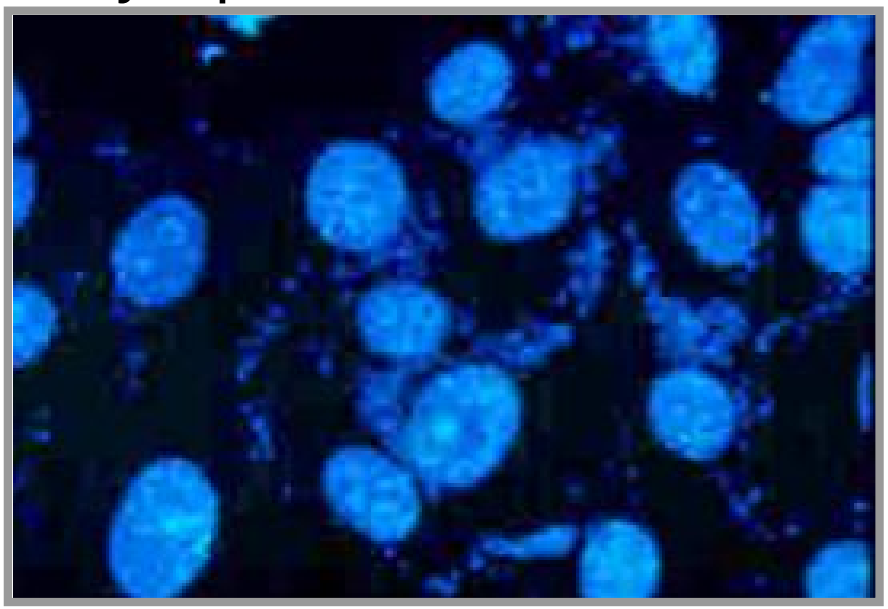
Fluorescent DNA mycoplasma staining

- Relatively simple and inexpensive technique

Clean VERO cells



Mycoplasma contaminated

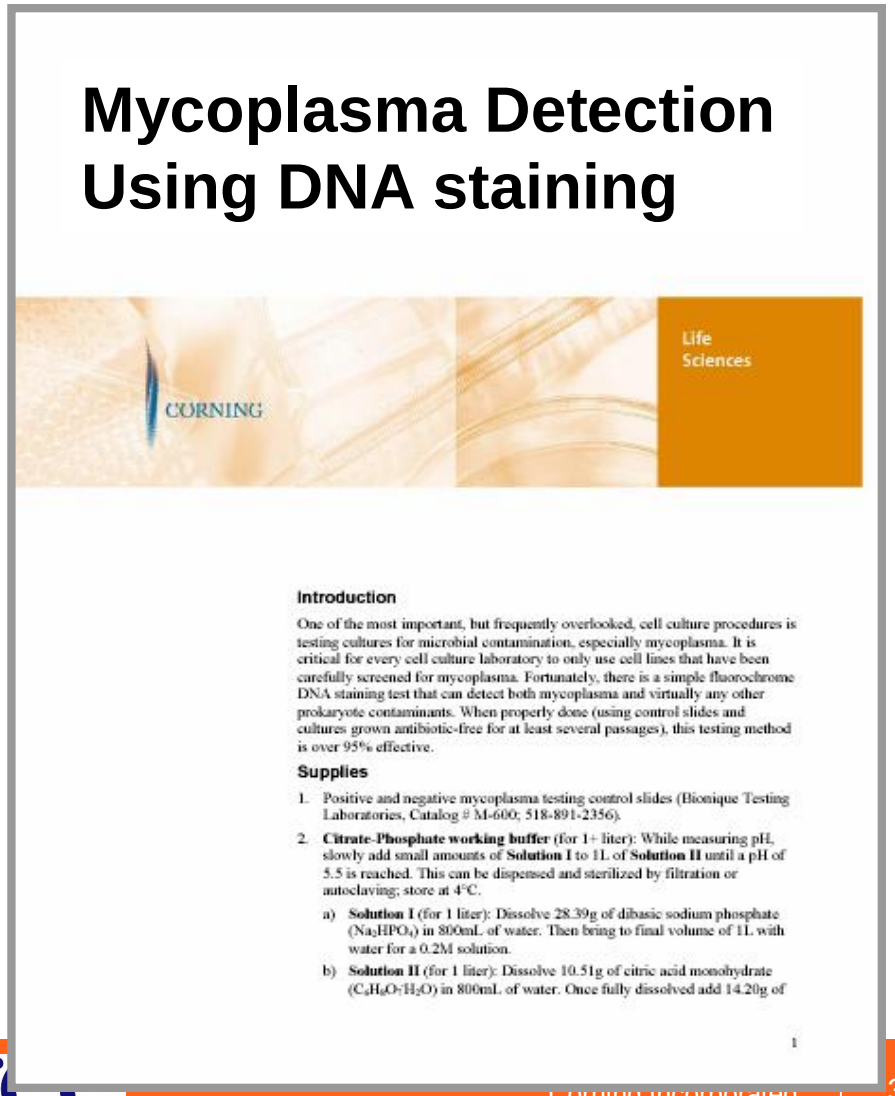


Photos courtesy of Bionique Testing Labs: www.bionique.com

For more information on DNA mycoplasma staining…

Protocol on Corning web site:

- www.Corning.com/lifesciences
- Important to use control slides – See Bionique Labs or Fisher Scientific



Mycoplasma Detection Using DNA staining

CORNING Life Sciences

Introduction

One of the most important, but frequently overlooked, cell culture procedures is testing cultures for microbial contamination, especially mycoplasma. It is critical for every cell culture laboratory to only use cell lines that have been carefully screened for mycoplasma. Fortunately, there is a simple fluorochrome DNA staining test that can detect both mycoplasma and virtually any other prokaryote contaminants. When properly done (using control slides and cultures grown antibiotic-free for at least several passages), this testing method is over 95% effective.

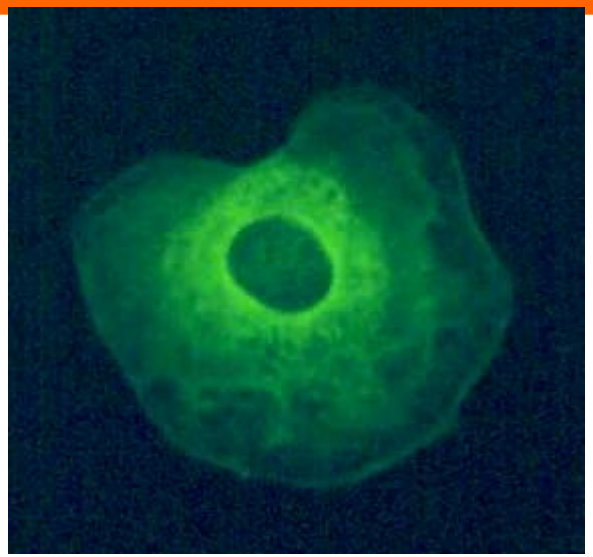
Supplies

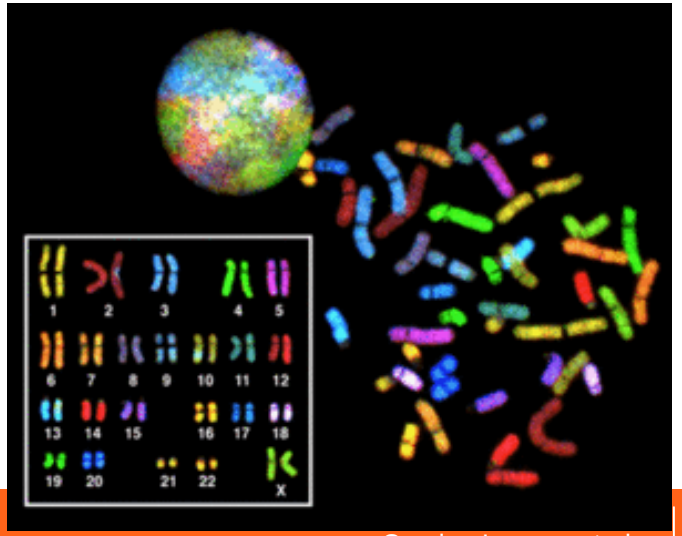
1. Positive and negative mycoplasma testing control slides (Bionique Testing Laboratories, Catalog # M-600; 518-891-2356).
2. **Citrate-Phosphate working buffer** (for 1+ liter): While measuring pH, slowly add small amounts of **Solution I** to 1L of **Solution II** until a pH of 5.5 is reached. This can be dispensed and sterilized by filtration or autoclaving; store at 4°C.
   a) **Solution I** (for 1 liter): Dissolve 28.39g of dibasic sodium phosphate (Na_2HPO_4) in 800mL of water. Then bring to final volume of 1L with water for a 0.2M solution.
   b) **Solution II** (for 1 liter): Dissolve 10.51g of citric acid monohydrate ($C_6H_8O_7 \cdot H_2O$) in 800mL of water. Once fully dissolved add 14.20g of

1

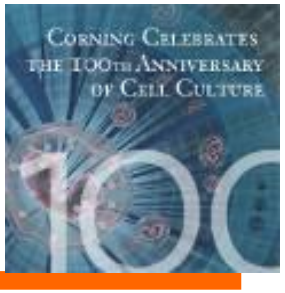
Testing for cross contamination of cells

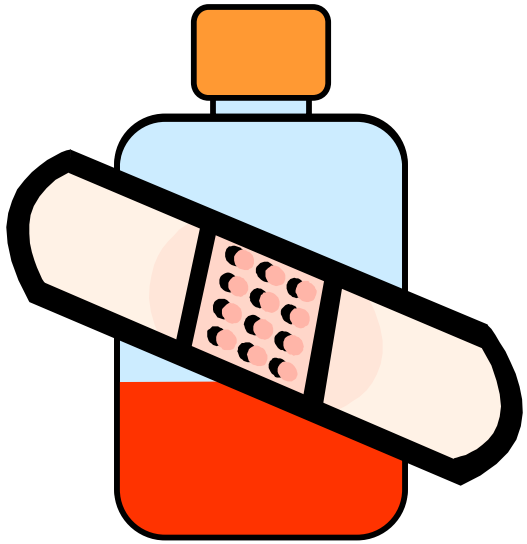
- Common methods
  - Karyotyping
  - Isoenzyme analysis
  - Species specific antisera
  - DNA fingerprinting
  - RFLP
  - STR (short tandem repeats)





Using antibiotics strategically





Antibiotics do not stop mycoplasma, they help it by hiding bad technique!

Using antibiotics strategically

- **Long-term, continuous use of antibiotics:**
  - **Does not prevent contamination**
  - **Is indirectly responsible for many mycoplasma problems**
  - **Allows contamination to spread further before detection**
  - **Makes it difficult to trace source of contamination.**
- **Short-term use of antibiotics is very useful:**
  - **For primary cultures (1-2 weeks)**
  - **For experiments where the cells will not be reused**

Accidents

“an unfortunate event resulting from carelessness, unawareness, ignorance or a combination of causes...”

1981 Webster’s Dictionary

Cell culture accidents can be devastating!

Cell culture accidents

Possible causes

- Mislabeling
- Poor communications
- Overwork
- Human nature
  - Friday afternoons
  - Let someone else do it
- Equipment failures or supplies shortages

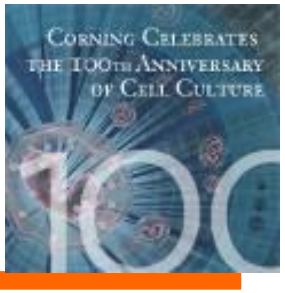
Possible solutions

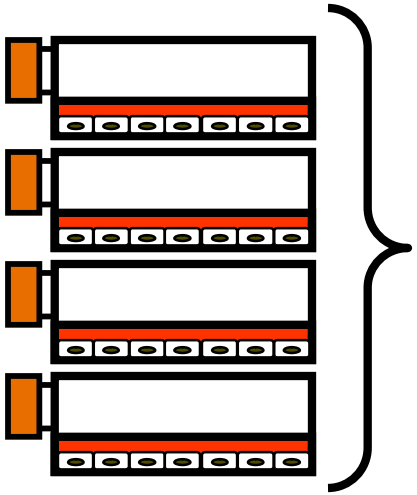
- Assigned label colors
- Written procedures
- Good supervision
- Assigned tasks, especially for LN_2 freezers and incubators
- Careful planning & backups

Using frozen cells strategically

- Convert long-term cultures into a series of discreet, short-term cultures taken from your LN_2 freezer's validated working stock
- This approach eliminates serious problem of culture evolution and eliminates periodic testing for mycoplasma and other contaminants
- This works if you:
  - Test all frozen cell stocks for contamination and viability
  - Store cultures below -130°C

How I do it - Step 1

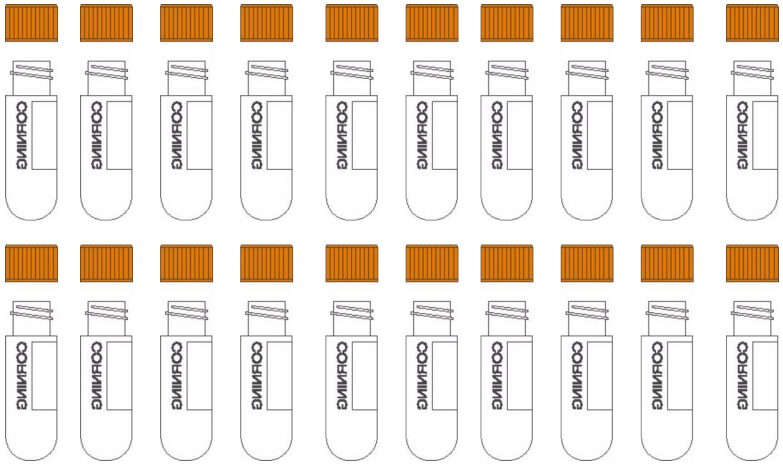




No antibiotics!

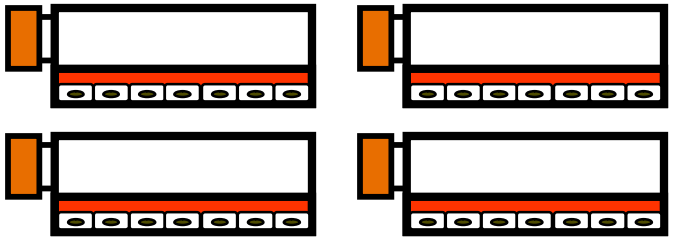
Harvest, pool and freeze down 20 vials

Seed stock



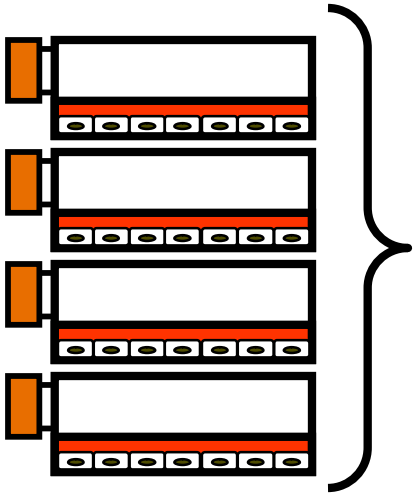
Thaw 1 vial and check recovery

Expand



No antibiotics!

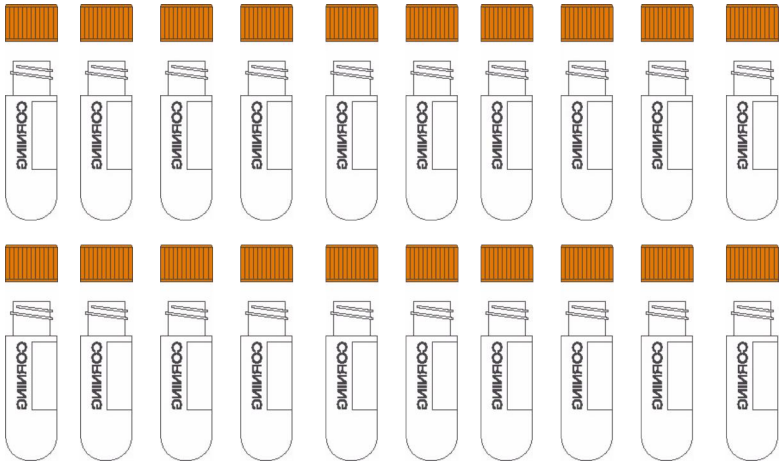
How I do it - Step 2



Cells from seed stock

Harvest, pool and freeze down 20 more vials

Certified working stock



Thaw I vial, check viability and test for contamination

Once tested, a new vial is used every 3 months

For more information on freezing cells……

- Check the Corning technical web site: www.corning.com/lifesciences

General Guide for Cryogenically Storing Cell Cultures

CORNING

Life Sciences

John Ryan, Ph.D.
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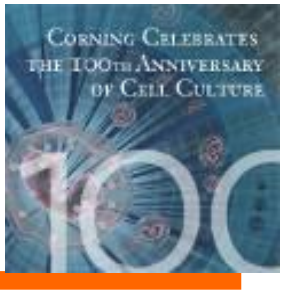
Table of Contents

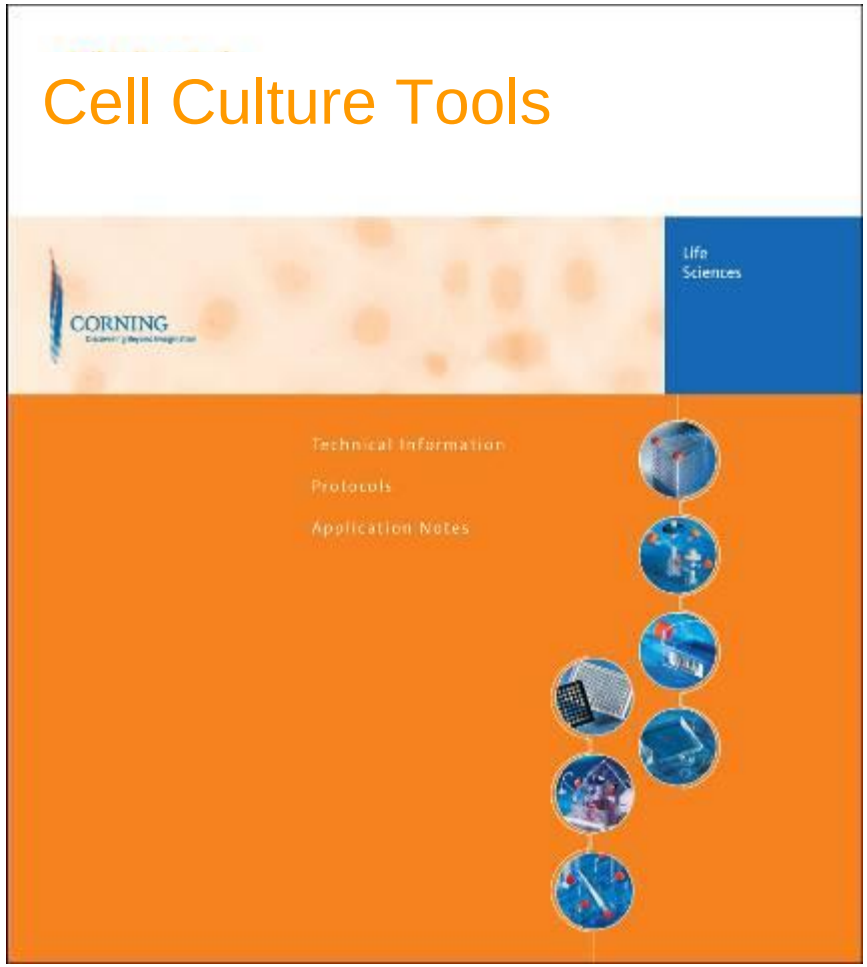
Introduction

Maintaining healthy, growing cell cultures is a demanding task made more difficult by the ever-present risk of their loss through accidents or contamination. In addition, actively growing cell cultures are not static but, like all populations of microorganisms, subject to age-related or environmentally-induced changes which can result in their ongoing evolution and potential loss.

These problems are reduced by using cryogenic preservation to stop biological time for cell cultures, effectively putting them into true suspended animation. This concept, long a favorite ploy of science fiction writers and movie producers, has been a reality since the important discovery by Polge, Smith and Parkes (11) in 1949 that glycerol prevents injury to cells caused by freezing. Many cook book-style protocols are now available for freezing cells and these procedures usually perform well (3,6,13-16). It is essential, however, when problems arise or protocol adaptations and improvements must be made, that the underlying concepts on which they are based are well understood. This guide examines both the basic theoretical concepts and practical aspects necessary for successfully freezing animal cells and managing a cell repository.

For more information on cell culture...





- Corning® Cell Culture Selection Guide
- Introduction to Animal Cell Culture
- Understanding And Managing Cell Culture Contamination
- General Guide For Cryogenically Storing Animal Cell Cultures
- Corning® Guide For Identifying and Correcting Common Cell Growth Problems
- Mycoplasma Detection using DNA Staining
- Corning® Roller Bottles Selection And Use Guide
- Cell Culture Scale Up Utilizing Disposble Spinner Flasks

Keys to success

Monitor cultures for contamination

Use antibiotics strategically

Use frozen cells strategically

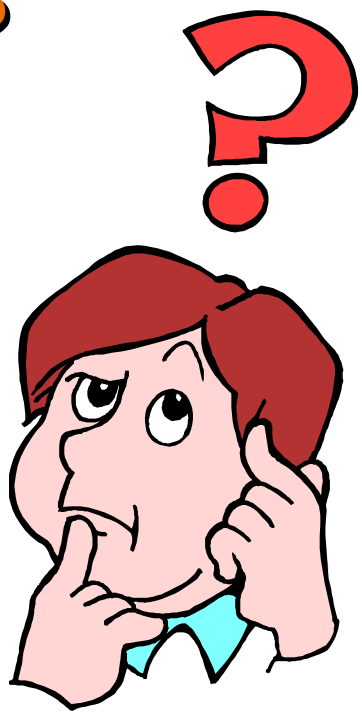
Good aseptic technique

Understand nature of contamination

Good housekeeping

Thanks for participating in this seminar

Any questions?



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