How To Use

Product 1: Mycoplasma Detection Broth Media (Liquid Form)

Catalog Numbers: M-HAYFLICK-Y; M-FREY-Y; M-FRIIS-Y
Specification: 100 mL per bottle

Product 2: Mycoplasma Detection Agar Media (Plates)

Catalog Numbers: M-HAYFLICK-X; M-FREY-X; M-FRIIS-X
Specification: 10 plates per pack, 10 packs per case; 60 mm diameter plates

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Product Description

These media are designed for use in *direct culture-based mycoplasma detection methods* as described in the **USP** and **EP** regulations. They are suitable for both *laboratory-scale mycoplasma cultivation* and *industrial-scale mycoplasma fermentation*.

> **HAYFLICK medium**: General-purpose medium for culturing most *Mycoplasma* species.
>
> **FREY medium**: Optimized for *Mycoplasma hyorhinis* and other synovial-type mycoplasmas.
>
> **FRIIS medium**: Recommended for *Mycoplasma hyopneumoniae* and lipid-dependent species such as *M. hyosynoviae*.

The liquid medium is used to amplify Mycoplasma from test samples or cultures suspected of contamination. Before the medium reaches the species-specific pH tolerance limit, transfer should be made to fresh liquid medium or corresponding agar plates.

Note: Color change in liquid broth alone does not confirm Mycoplasma growth; *both liquid enrichment and plate subculture* are required for definitive detection.
Except for *M. orale* and *M. fermentans* (which turn deep red due to alkaline metabolism), other Mycoplasma species exhibit a color change from **red → orange → yellow** during acid production and growth.

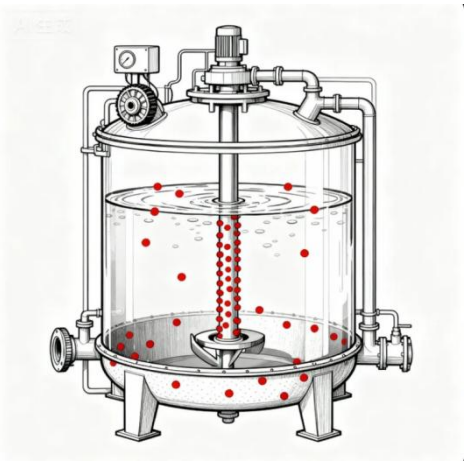
Protocol 1: Detection of Mycoplasma in Test Samples

Storage Conditions

Liquid media: Store at 2–8 °C for up to 6 months, or at −20 °C/−80 °C for up to 1 year. Avoid repeated freeze–thaw cycles.

Agar plates: Store at 2–8 °C for up to 6 months.

Sample Requirements
Prior to Mycoplasma testing, samples must be confirmed free of bacterial contamination and antibiotic residues.



Presence of bacteria or antibiotics may cause *false negatives*, especially in previously contaminated samples.

Ensure sample pH is within the optimal Mycoplasma growth range (7.0–7.8). Deviations may also result in false negatives.

Sampling Method
For large-volume or vessel samples, perform *multi-point sampling*, including from tank corners and bottom sediments, regardless of agitation. Uneven sampling may lead to false negatives or variable Mycoplasma loads within the same batch.

Sample Preservation

Test samples as soon as possible.

If delay is unavoidable, store at 2–8 °C using CDC-recommended *virus transport media* that suppress bacteria and fungi but not Mycoplasma.

Samples must be tested within 3 days of collection.

Inoculation
Mix the sample thoroughly and inoculate into liquid media at a ratio of approximately **1:10 (sample : medium)** into **M-HAYFLICK-Y**, **M-FREY-Y**, and **M-FRIIS-Y**.

Use sterile screw-cap tubes or cell culture flasks.

Incubate at **35–37 °C** in a **20% CO_2 atmosphere**.

Avoid cross-contamination between samples and media.

Negative Control
Include 10–20 mL of uninoculated medium as a negative control and incubate under identical conditions.

Observation of Liquid Media
Color change time varies widely by *species* and *inoculum size*.

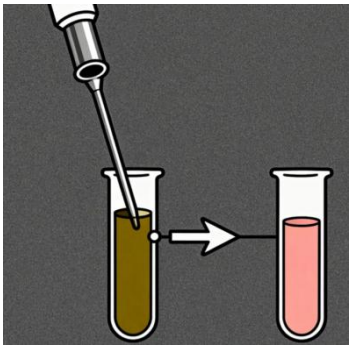
M. pneumoniae typically requires **4–5 days** before color shift.

M. hyorhinis may change color within **24 hours**.
Generally, the more nutritionally dependent the species, the faster the color change.

Observe every **12–24 hours**; **do not** wait 2–3 days between checks.

When the color turns **orange (acid-producing Mycoplasma)** or **deep red (alkaline-producing Mycoplasma)**, proceed with **medium change**, **agar plating**, or **sample preservation**.

Avoid waiting until complete yellowing or dark purple-red, as this indicates extreme pH changes that may lead to widespread Mycoplasma death.



Agar Plate Inoculation
Mix the color-changed broth well, then inoculate the corresponding agar plate (e.g., Hayflick broth → Hayflick agar).

Apply **200 µL** of liquid sample per plate and gently tilt to spread evenly.

To prevent contamination during long incubation (10–14 days), place plates in sterile sampling bags before incubating.

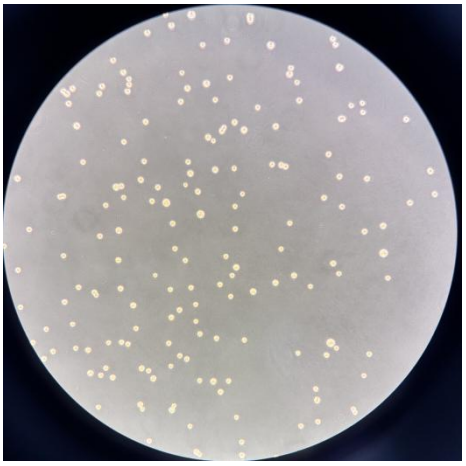
Colonies typically appear only when the CFU count reaches a detectable level; hence, liquid enrichment is essential.

Microscopic Observation

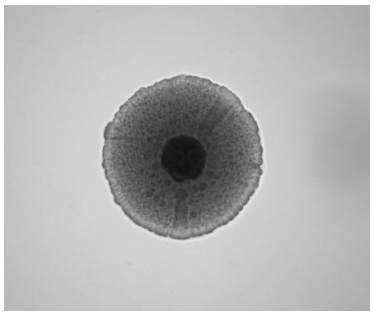
Examine plates under an **inverted microscope (4× objective, 10× eyepiece)**.

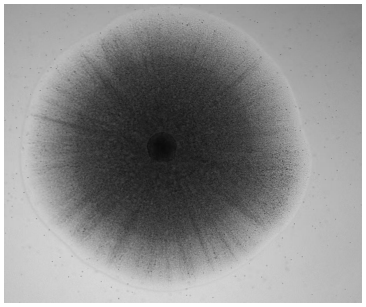
For colony counting, digital imaging and CFU-counting software can be used.

Colonies appear as **“fried-egg”** morphology typical of Mycoplasma.



For morphological identification at species level, use a **100× objective**, as colony edge and central zone vary among species.





If cellular debris complicates visualization, apply a selective stain that colors Mycoplasma light blue (periphery) and dark blue (center). *Note: staining kills Mycoplasma.*

Subculture in Liquid Medium

To expand culture, transfer inoculum at a **1:10 ratio** from color-changed broth into fresh medium of the same formulation (e.g., Hayflick → Hayflick).

Sample Pretreatment for Contaminated or Antibiotic-Containing Samples

Filter through **0.45 µm** to remove bacteria.

Then filter through **0.1 µm** to capture Mycoplasma on the membrane while allowing antibiotics and antibacterial peptides to pass.

Place the membrane into the broth medium for culture.

CFU Enumeration Considerations
Mycoplasma colonies grow slowly, minimizing counting errors. Plates can be refrigerated before observation. CFU counts from plates provide reliable quantification of Mycoplasma in the sample.

Protocol 2: Mycoplasma Cultivation and ATCC Strain Revival

Storage
Same as described in Protocol 1.

Preparation of Lyophilized ATCC Strains
Some ATCC Mycoplasma lyophilized preparations contain *cryoprotectants* that inhibit recovery.

Perform serial 10-fold dilutions in fresh medium to dilute inhibitory components.

Refer to ATCC strain-specific datasheets for details.

Medium Selection
Due to extensive metabolic pathway loss, Mycoplasma species differ in nutritional requirements.

Autotrophic or easily cultivated species such as *M. pneumoniae* (ATCC 15531) and *M. orale* (ATCC 23714): use Hayflick (Frey or Friis also acceptable, with potentially better yield).

More fastidious species such as *M. hyorhinis* (ATCC 25204): use Frey medium (Friis also possible; Hayflick less suitable).

Highly lipid-dependent species such as *M. hyopneumoniae*: use Friis exclusively (Hayflick/Frey not recommended).
Typical growth rate:

M. hyopneumoniae ≥ *M. hyorhinis* > *M. orale* >> *M. pneumoniae* (under appropriate medium conditions).

Inoculation Ratios

Adjust inoculum-to-medium ratio based on growth rate and laboratory schedule:

For slow growers (*M. pneumoniae*, ATCC 15531): use higher inoculum (1:5–1:7) to allow observable color change by Day 3–4.

For fast growers (*M. hyorhinis*, ATCC 25204): use larger medium volume to prevent overgrowth during weekends.

It is recommended to establish a growth curve for each strain.

Agar Plate Observation

Follow the same procedure as described in Protocol 1.

Medium Renewal

Same as described in Protocol 1.

Fermentation Cultivation

For fermentation-scale culture, consult the company's technical department to determine nutrient supplementation strategies.

Add supplements *slowly* to avoid abrupt environmental shifts that may cause large-scale Mycoplasma death.

Preservation of Mycoplasma Cultures

Preserve cultures during the *logarithmic growth phase*, not at CFU maximum.

When the broth color is **orange-red to light red**, add **20% sterile glycerol** and store at **2–8 °C**.

Storage stability: ~1 year. Revive every 6–12 months to maintain viability.

Strain Recovery (Rejuvenation)
If culture pH approaches the species' growth limit, metabolism slows or halts.

Transfer into fresh, suitable medium before color reaches the critical point (deep yellow or purple-red).

After **2–3 serial passages**, growth rate typically recovers.
