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Document # INST-LT07-118 04/11

MycoAlert™ mycoplasma detection kit

Mycoplasma detection assay - Instructions for use

Safety

THESE PRODUCTS ARE FOR RESEARCH USE ONLY. Not approved for human or veterinary use, for application to humans or animals, or for use in clinical or *in vitro* procedures.

1. MycoAlert™ assay procedure outline

(For detailed assay protocol see section 9)

Spin 2 ml cell sample at 200 x g for 5 min.
Remove 100 µl of cleared supernatant to fresh tube/well

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Reconstitute MycoAlert™ reagent and MycoAlert™ substrate in assay buffer
Equilibration time 15 minutes.

↓

Add 100 µl MycoAlert™ reagent to sample.
Wait for 5 minutes.

↓

Measure luminescence.
(Reading A)

↓

Add 100 µl MycoAlert™ substrate to sample.
Wait for 10 minutes.

↓

Measure luminescence.
(Reading B)

2. Kit contents & ordering information

LT07-118	**10 tests**
MycoAlert™ reagent (lyophilized)	2 x 600 µl (LT27-217)
MycoAlert™ assay buffer	1 x 10 ml (LT27-218)
MycoAlert™ substrate (lyophilized)	2 x 600 µl (LT27-221)

LT07-218	**25 tests**
MycoAlert™ reagent (lyophilized)	5 x 600 µl (LT27-217)
MycoAlert™ assay buffer	1 x 10 ml (LT27-218)
MycoAlert™ substrate (lyophilized)	5 x 600 µl (LT27-221)

LT07-418	**50 tests**
MycoAlert™ reagent (lyophilized)	2 x 2.5 ml (LT27-237)
MycoAlert™ assay buffer	1 x 10 ml (LT27-218)
MycoAlert™ substrate (lyophilized)	2 x 2.5 ml (LT27-238)

LT07-318	**100 tests**
MycoAlert™ reagent (lyophilized)	1 x 10 ml (LT27-216)
MycoAlert™ assay buffer	1 x 20 ml (LT27-220)
MycoAlert™ substrate (lyophilized)	1 x 10 ml (LT27-224)

Part codes in parenthesis cannot be ordered as separate item.

Related products:

MycoAlert™ assay control set
LT07-518 10 Tests

3. Storage conditions

Kit components	Store at 2˚C-8˚C. Do not freeze. See kit label for expiry date of the whole kit. See bottle labels for expiry dates of individual components.
Reconstituted reagent and/or substrate	Only keep reconstituted components at room temperature* for the course of the experiment. Unused components can be aliquoted and stored at -20℃ up to six months. When using a frost-free freezer, be aware that temperature does not keep -20℃ constantly. For improved stability we would recommend storage at -80℃. Once thawed, reagent and/or substrate must not be refrozen and should be allowed to reach room temperature before use without the aid of artificial heat.

* If room temperature is > 30℃ it might be advisab le to use a water bath set to 20℃.

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4. Intended use

Mycoplasma are the smallest and simplest prokaryotes. Mycoplasma depend on their hosts for many nutrients due to their limited biosynthetic capabilities. They have long been recognized as common contaminants of cells in continuous culture but their presence may go undetected for months. As the mycoplasma competes with the cells for the nutrients in culture media, one of the first signs is a reduction in the rate of cell proliferation and slight changes in cellular responses including gene expression.

Mycoplasma detection in cell cultures has until now been a long, drawn out process with difficult-to-interpret results. The MycoAlert™ kit is intended for the quick and convenient detection of viable mycoplasma in cell cultures. The speed and convenience of the MycoAlert™ kit allows for the routine testing of cells in culture and commonly used constituents of complete media.

To allow for the early detection of mycoplasma contamination Lonza recommends testing at every cell passage. Frequent testing such as this will indicate when a cell line becomes infected allowing prompt remedial action to be taken. The MycoAlert™ assay can also be extended to incoming cell lines and the commonly used constituents of complete media.

5. Assay principle

The MycoAlert™ assay is a selective biochemical test that exploits the activity of certain mycoplasmal enzymes. The presence of these enzymes provides a rapid screening procedure, allowing sensitive detection of contaminating mycoplasma in a test sample (figure 1). The viable mycoplasma are lysed and the enzymes react with the MycoAlert™ substrate catalyzing the conversion of ADP to ATP.

By measuring the level of ATP in a sample both before and after the addition of the MycoAlert™ substrate, a ratio can be obtained which is indicative of the presence or absence of mycoplasma (figure 2). If these enzymes are not present, the second reading shows no increase over the first, while reaction of mycoplasmal enzymes with their specific substrates in the MycoAlert™ substrate, leads to elevated ATP levels.

This increase in ATP can be detected using the following bioluminescent reaction:

$$\text{ATP + Luciferin + } O_2 \xrightarrow[Mg^{2+}]{\text{Luciferase}} \text{Oxyluciferin + AMP + } PP_i + CO_2 + \text{LIGHT}$$

The emitted light intensity is linearly related to the ATP concentration and is measured using a luminometer. The assay is conducted at room temperature (18℃-22℃), the optimal temperature for luciferase activity.

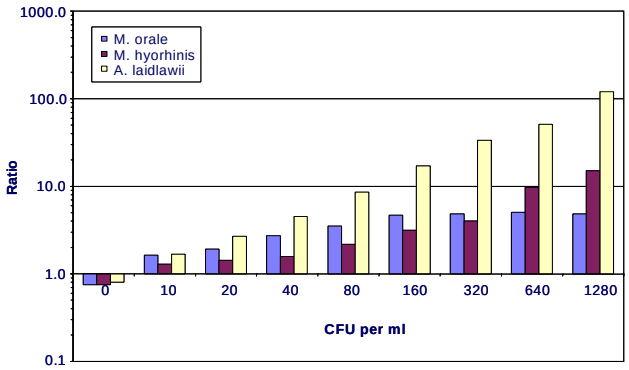

Figure 1: The graph shows a dilution series of *M. hyorhinis*, *M. orale* and *A. laidlawii* demonstrating the sensitivity of the MycoAlert™ assay.

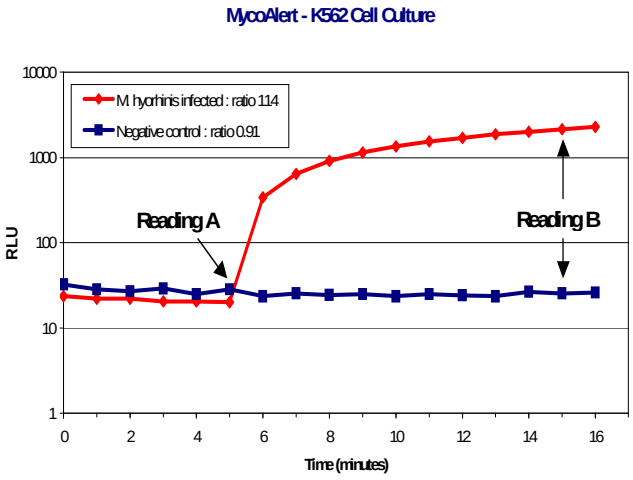

Figure 2: The kinetics of ATP generation in the presence of *M.hyorhinis* contamination.

6. Component reconstitution and storage

NOTE: Please read this section carefully to ensure optimal performance for your assay. Ensure that you follow the correct component reconstitution applicable to the kit size you have received (see table below).

The MycoAlert™ reagent and substrate are supplied as lyophilized pellets. These are reconstituted in the supplied MycoAlert™ assay buffer to produce the working solutions for use in the assay.

1. For reconstitution add MycoAlert™ buffer to the MycoAlert™ reagent and substrate, according to the table below (volumes depend on kit size).

MycoAlert™ assay buffer added	to MycoAlert™ reagent	to MycoAlert™ substrate
LT07-118 (10 test kit)	600 µl	600 µl
LT07-218 (25 test kit)	600 µl	600 µl
LT07-418 (50 test kit)	2.5 ml	2.5 ml
LT07-318 (100 test kit)	10 ml	10 ml

2. Replace screw cap and mix gently.
3. Allow equilibration to room temperature for at least 15 min.
4. Only keep reconstituted components at room temperature for the course of the experiment. Please refer to section 3 for longer term storage.

7. Additional equipment

a. Instrumentation

The MycoAlert™ kit requires the use of a luminometer. The assay has been designed for use with cuvette/tube and/or plate luminometers. Lonza provides a list of luminometers which have been tested for compatibility with the MycoAlert™ assay (**www.lonza.com/luminometers**). If your luminometer is not on that list we highly recommend assessing its sensitivity using our MycoAlert™ assay control set (LT07-518).

The parameters of the luminometer should be assessed and the conditions below used to produce the correct programming of the machine.

Cuvette/tube luminometers: Read time 1 second (integrated).

Plate luminometers: Read time 1 second (integrated). When using a microplate reader all reagents should be added manually.

Beta counters: Mode - out of coincidence or luminescence; read time 1 second (integrated).

b. Additional equipment and consumables

- 10 ml sterile pipettes
- Luminometer cuvettes or white walled microplates (ideally with an opaque bottom)
- Micropipettes: 50-200 µl; 200-1000 µl
- Bench centrifuge

8. Assay samples & controls

Following sample types are suitable for use with MycoAlert™ assay. Cell supernatant must be spun at 1500 rpm (200 x g) for 5 minutes to remove any remaining cells. Cells present in the sample will increase the background, resulting in the loss of sensitivity and resolution. In essence it reduces the chance of seeing a low-lying infection.

a. Fresh supernatant (optimal sample)

1. Cell supernatant **during** passage of suspension cell culture.
2. Supernatant from adherent cells **prior** to trypsinisation.
3. Cell supernatant from cells brought “up from frozen” – cells out of liquid nitrogen with uptake into media. Leave minimum of 1-2hr before testing under normal culture conditions.

NOTE: Cells diluted into fresh media after passaging or trypsinization result in a much lower signal - leave minimum 24hr under normal culture conditions before testing.

Supernatant can be stored at room temperature or 4℃ for testing the same day.

b. Refrigerated supernatant

Supernatants might be stored at 4℃ for ≤ 5 days. Bring to room temperature without the aid of artificial heat before testing. Do not freeze supernatant.

c. Other samples suitable for MycoAlert™:

- Cell free media
- FCS
- BPE
- Other tissue culture reagents

d. Positive control

A MycoAlert™ positive control is available as separate item (LT07-518). This control does not contain mycoplasma, i.e. it is not a source of

mycoplasma. We recommend to include a positive control sample in every experiment.

e. Negative control

Use 100 µl of MycoAlert™ buffer or HPLC grade water as a negative control. We recommend to include a negative control sample in every experiment.

9. Assay protocol

NOTE: To ensure that the optimal performance of the assay is achieved for your experiment please make certain that you have carefully read the component reconstitution and storage procedure.

1. Bring all reagents up to room temperature before use.
2. Reconstitute the MycoAlert™ reagent and MycoAlert™ substrate in MycoAlert™ assay buffer. Leave for 15 minutes at room temperature to ensure complete rehydration.
3. Transfer 2 ml of cell culture or culture supernatant into a centrifuge tube and pellet any cells at 1500 rpm (200 x *g*) for 5 minutes.
4. Transfer 100 µl of cleared supernatant into a luminometer cuvette/tube or well.
5. Program the luminometer to take a 1 second integrated reading (this is usually the default setting on most cuvette luminometers).
6. Add 100 µl of MycoAlert™ reagent to each sample and wait 5 minutes.
7. Place cuvette or plate in luminometer and initiate the program (Reading A).
8. Add 100 µl of MycoAlert™ substrate to each sample and wait 10 minutes.
9. Place cuvette or plate in luminometer and initiate the program (Reading B).
10. Calculate ratio = Reading B/Reading A.

10. Interpretation of results

The ratio of Reading B to Reading A is used to determine whether a cell culture is contaminated by mycoplasma.

Ratio	Interpretation
< 0.9	Negative for mycoplasma
0.9 - 1.2	Borderline: quarantine cells & retest in 24 h
> 1.2	Mycoplasma contamination

The interpretation of the different ratios obtained, within each experimental situation, may vary according to the cell types and conditions used.

However, the test has been designed to give ratios of less than 0.9 with uninfected cultures. Cells which are infected with mycoplasma will routinely produce ratios greater than 1.

Table 1. Interpretation of MycoAlert™ assay results illustrating examples of healthy and infected cell lines.

Cell Line	MycoAlert™ ratio	Conclusions
Infected cells		
K562	123.26	Positive
A549	4.10	Positive
U937	8.26	Positive
HepG2	1.17*	Borderline,quarantine and retest in 24 hours
Healthy cells		
HL60	0.72	Negative
COS-7	0.46	Negative

** see Troubleshooting section 11*

11. Troubleshooting

High background levels?

Take great care when handling any of the reagents. Skin has high levels of ATP on its surface that can contaminate the reagents leading to falsely high readings. Wear latex gloves or equivalent.

Ensuring optimal performance

The optimal working temperature for all reagents is 22℃. If reagents have been refrigerated always allow time for them to reach room temperature before use.

Pipettes

As with all assays involving manual pipetting in order to gain maximal accuracy and to reduce variability pipettes should be calibrated regularly.

Borderline ratios around 1

The sensitivity of the assay does allow for detection of covert contamination, and if the ratio is marginally above 1 (for example up to 1.2), it is recommended that the sample be retested. Any cultures maintained in quarantine can be tested after a further 24-48 hours in culture to see if the ratios have increased.

A ratio of less than 1 is produced by the ongoing consumption of ATP over the time course of the assay. Consistent ratios of around 1 demonstrate that this consumption and subsequent drop in RLUs is not being seen and indicates an instrument sensitivity issue.

To try to overcome this, increase the integration time from 1 second up to a max of 10 seconds; check to make sure that filters (not even plain glass) are not present between the sample and detector, and ensure the instrument is in luminescence or "out of coincidence" mode.

Negative RLUs or ratios

If automatic background subtraction is enabled on the instrument it will cause negative RLUs for the B reading and consequently negative ratios. This option MUST be disabled for the instrument to work correctly with the MycoAlert™ assay.

If technical support is required please contact Lonza Scientific Support teams.

12. MycoAlert™ tested species

The MycoAlert™ mycoplasma detection kit is a generic biochemical test for mycoplasma and other mollicutes (e.g. *acholeplasmas, mesoplasmas, spiroplasmas*) and will detect mollicutes of mammalian, avian, insect and plant origin.

The following 44 mollicute species were tested using the MycoAlert™ assay. Species were obtained from the National Collection of Type Cultures UK. The six most common species in cell culture are in bold.

Species	Origin/Source	Result
Acholeplasma laidlawii	**Mammalian/Avian**	**Positive**
Acholeplasma modicum	Bovine	Positive
Acholeplasma morum	Mammalian	Positive
Mesoplasma entomophilum	Insect	Positive
Mesoplasma florum	Plant/insect	Positive
Mycoplasma agussizii	Tortoise	Positive
Mycoplasma alkalescens	Bovine	Positive
Mycoplasma alligatoris	Alligator	Positive
Mycoplasma arginini	**Bovine/Porcine**	**Positive**
Mycoplasma arthriditis	Human	Positive
Mycoplasma bovirhinis	Bovine	Positive
Mycoplasma bovis	Bovine	Positive
Mycoplasma bovoculi	Bovine	Positive
Mycoplasma buccale	Human	Positive
Mycoplasma californicum	Bovine	Positive
Mycoplasma canadense	Bovine	Positive
Mycoplasma cloacale	Avian	Positive
Mycoplasma conjunctivae	Ovine & Caprine	Positive
Mycoplasma crocodyli	Crocodile	Positive
Mycoplasma equirhinis	Equine	Positive
Mycoplasma faucium	Human	Positive
Mycoplasma fermentans	**Human**	**Positive**
Mycoplasma gallinaceum	Mammalian/Avian	Positive
Mycoplasma gallisepticum	Avian	Positive
Mycoplasma genitalium	Human	Positive
Mycoplasma hominis	**Human**	**Positive**
Mycoplasma hyopneumoniae	Human	Positive
Mycoplasma hyorhinis	**Porcine**	**Positive**
Mycoplasma hyosynoviae	Porcine	Positive
Mycoplasma iguanae	Iguana	Positive
Mycoplasma lipophilum	Human	Positive
Mycoplasma muris	Murine	Positive
Mycoplasma neurolyticum	Murine	Positive
Mycoplasma opalescens	Canine	Positive
Mycoplasma orale	**Human**	**Positive**
Mycoplasma pirum	Human	Positive
Mycoplasma pneumoniae	Human	Positive
Mycoplasma primatum	Mammalian	Positive
Mycoplasma pulmonis	Human	Positive
Mycoplasma pulmonis	Rat	Positive
Mycoplasma salivarium	Human	Positive
Mycoplasma spermatophilum	Human	Positive
Mycoplasma synoviae	Avian	Positive
Spiroplasma citri	Plant/Insect	Positive

13. Preventing mycoplasma contamination

Mycoplasmas, the smallest and simplest form of bacteria, are common contaminants of cells grown in culture. Studies indicate that between 15% - 35% of all continuous culture cells are contaminated with mycoplasma (Rottem and Barile, 2003). Infections can seriously impact the reliability, reproducibility and consistency of results obtained with these cultures, and can be easily spread within the culture environment. To that end we recommend aseptic techniques to prevent mycoplasma contamination and cross contamination with other cell lines.

- Wear appropriate personal protective equipment (sterile gloves, lab coat, safety glasses).
- Perform all tissue culture work in a biosafety cabinet at appropriate containment level.
- Sanitize the biosafety cabinet with 70% ethanol before commencing work.
- Wash gloved hands with 70% ethanol and allow to air dry for 30 seconds.

    - If gloves are contaminated by touching anything outside the cabinet, re-sanitize as above.
    - Discard gloves after handling contaminated cultures and at the end of all culture procedures.
- Use 70% ethanol to disinfect exterior surfaces of all materials and equipment required for experiment before placing in to the biosafety cabinet.
- Ensure air flow in the biosafety cabinet circulates properly.
    - Direct verbal communication away from the cabinet.
    - Minimize rapid movement within and immediately outside the cabinet.
- Use sterile flasks, plates, bottles and dishes for all cell cultures and media.
- Dedicate separate media for each cell line.
- Minimize exposure of sterile media, cell cultures, and equipment to the environment.
    - Uncover sterile culture vessels immediately before use; re-cover as soon as work is finished.
    - Keep sterile lab equipment (pipettes, reservoirs, plates, etc) wrapped until ready to use.
    - Return cultures to incubator as soon as work is complete.
- Avoid splashes, spills, and aerosols.
- Do not transfer liquid by pouring; use a new, sterile pipette for each transfer to or from a different bottle.
- Cleanup after tissue culture work is complete.
    - Disinfect all equipment and material with 70% ethanol before removing from cabinet.
    - Disinfect work surfaces inside of biosafety cabinet with 70% ethanol.
- Use the **MycoAlert™ mycoplasma detection kit** to routinely screen cell cultures.

Lonza strongly recommends that cell cultures with mycoplasma contamination be discarded and fresh stocks obtained. When that's not possible, **MycoZap™ elimination reagent** provides a reliable method of mycoplasma elimination.

References

Mycoplasma:

Denecke,J., Becker,K., Jurgens,H., Reinhold,G., and Wolff,J. (1999) Falsification of Tetrazolium Dye (MTT) Based Cytotoxicity Assay Results due to Mycoplasma Contamination of Cell Cultures. *Anticancer Reseach*, **19**: 1245-1248.

Miller,J., Kassem,S., Pepper,S.D., Hey,Y., Ward, T. H., and Margison,G.P. (2003) Mycoplasma infection significantly alters microarray gene expression profiles. *Biotechniques*, **35(4)**: 812-814.

Razin,S., Yogeu,D, and Naot,Y: (Dec 1998) Moleculer Biology and Pathogenicity of Mycoplasmas. *Microbiol and Molecular Biology Reviews*, 1094-1156.

Rowe,J.A., Scragg,I., Kwiatkiwski,D., Ferguson,D., Carucci,D., and Newbold,C. (1998) Implications of mycoplasma contamination in Plasmodium falciparum cultures and methods for its detection and eradication. *Molecular and Biochemical Parasitology,* **92**: 177-180.

Rottem, S. and Barile, M.F. (1993). Beware of Mycoplasma. *Trends in Biotechnology*, **11**(4): 143-151.

MycoAlert™ citations:

Jian-Zhong Qin, Lawrence Stennett, Patricia Bacon, Barbara Bodner, Mary J.C. Hendrix, Richard E.B. Seftor, Elisabeth A. Seftor, Naira V. Margaryan, Pamela M. Pollock, Amy Curtis, Jeffrey M. Trent, Frank Bennett, Lucio Miele, and Brian J. Nickoloff. (2004). p53-independent NOXA induction overcomes apoptotic resistance of malignant melanomas. *Molecular Cancer Therapy*, **3**: 895-902.

Yong Zhou, James S. Hagood and Joanne E. Murphy-Ullrich. (2004). Thy-1 Expression Regulates the Ability of Rat Lung Fibroblasts to Activate Transforming Growth Factor-ß in Response to Fibrogenic Stimuli *American Journal of Pathology,* **165**: 659-669.

Product warranty

When used according to the preceding protocol Lonza's MycoAlert™ assay will provide a sensitive measure of mycoplasma infection in cell cultures. It is intended as a presumptive screening tool, and any positives should be re-tested by a second confirmatory method.

Lonza warrants that this product will perform according to established product specifications. It is sold with the understanding that the purchaser will determine if the product is suitable for his or her application. Lonza shall not be liable for any damages or injury to persons or property arising from the purchase or use of the product.

THESE PRODUCTS ARE FOR RESEARCH USE ONLY.

Not approved for human or veterinary use, for application to humans or animals, or for use in clinical or *in vitro* procedures.

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