MycoAlert™ PLUS Mycoplasma Detection Kit

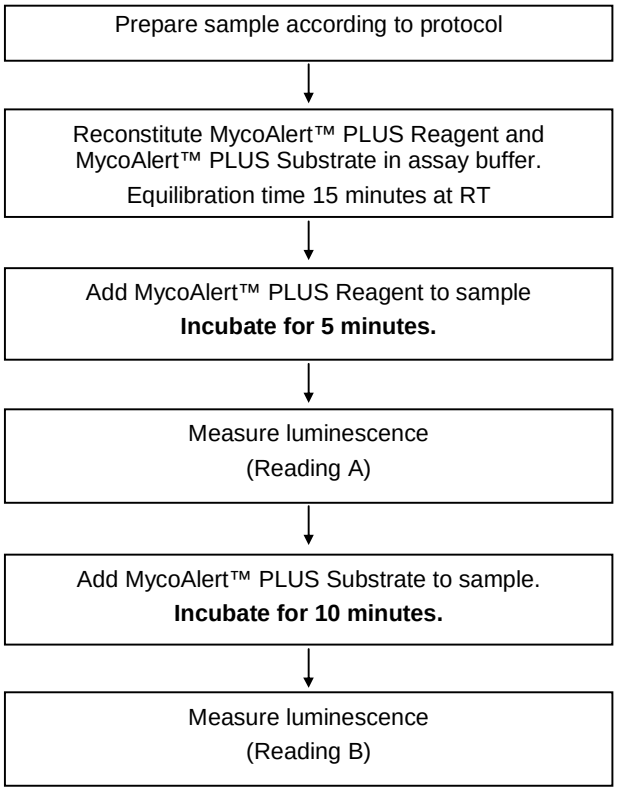
Mycoplasma detection assay - Instructions for use

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™ PLUS Assay Procedure Outline

(For detailed assay protocols see section 10)



3. Kit Contents & Ordering Information

LT07-701	**10 tests**
MycoAlert™ PLUS Reagent (lyophilized)	1 x 1.2 ml (LT27-284)
MycoAlert™ PLUS Substrate (lyophilized)	1 x 1.2 ml (LT27-287)
MycoAlert™ PLUS Assay Buffer	1 x 3 ml (LT27-290)

LT07-703	**30 tests**
MycoAlert™ PLUS Reagent (lyophilized)	3 x 1.2 ml (LT27-284)
MycoAlert™ PLUS Substrate (lyophilized)	3 x 1.2 ml (LT27-287)
MycoAlert™ PLUS Assay Buffer	3 x 3 ml (LT27-290)

LT07-705	**50 tests**
MycoAlert™ PLUS Reagent (lyophilized)	1 x 5 ml (LT27-285)
MycoAlert™ PLUS Substrate (lyophilized)	1 x 5 ml (LT27-288)
MycoAlert™ PLUS Assay Buffer	1 x 10 ml (LT27-291)

LT07-710	**100 tests**
MycoAlert™ PLUS Reagent (lyophilized)	1 x 10 ml (LT27-286)
MycoAlert™ PLUS Substrate (lyophilized)	1 x 10 ml (LT27-289)
MycoAlert™ PLUS Assay Buffer	1 x 20 ml (LT27-292)

Part codes in brackets cannot be ordered as separate item, except LT27-292 (see below).

Developed by Lonza Nottingham, Ltd.

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Related products:

MycoAlert™ Assay Control Set
LT07-518 10 Tests

MycoAlert™ Assay Buffer
LT27-292 20 ml

MycoZap™ Mycoplasma Elimination Reagent
LT07-818 1 treatment
LT07-918 5 treatments

MycoZap™ Plus-CL Antibitotic (for cell lines)
VZA-2011 10 x 1 ml
VZA-2012 1 x 20 ml

MycoZap™ Plus-PR Antibitotic (for primary cells)
VZA-2021 10 x 1 ml
VZA-2022 1 x 20 ml

MycoZap™ Prophylactic Antibitotic
VZA-2031 10 x 1 ml
VZA-2032 1 x 20 ml

4. Storage Conditions

Lyophilized 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	– For optimal assay conditions, reconstituted reagent and substrate should be used fresh within 2 hours after reconstitution. During this time they can be kept at room temperature. – If necessary, reconstituted reagent and substrate can be stored at 2-8℃ for another 5 days but should be equilibrated to room temperature* for 15-20 min before use. – For long-term storage, reconstituted reagent and substrate can be aliquoted and stored at -80℃ for up to six months. 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. They should be equilibrated to room temperature* for 15-20 min before use. – Avoid multiple cooling/freezing and heating cycles.

*Optimal assay working temperature for all components is 20-22℃. In case room temperature significantly deviat es from this optimal temperature range it is recommended to use a water bath set to 22℃.

5. 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™ PLUS kit is intended for the quick and convenient detection of viable mycoplasma in cell cultures. The speed and convenience of the MycoAlert™ PLUS 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™ PLUS assay can also be extended to incoming cell lines and the commonly used constituents of complete media.

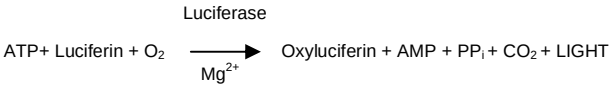
6. Assay Principle

The MycoAlert™ PLUS 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. The viable mycoplasma are lysed and the enzymes react with the MycoAlert™ PLUS 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™ PLUS Substrate, a ratio can be obtained which is indicative of the presence or absence of mycoplasma. If mycoplasma enzymes are not present, Read B shows no increase over Read A. If mycoplasma are present, reaction of mycoplasmal enzymes with the MycoAlert™ PLUS Substrate leads to elevated ATP levels and an increase in

Read B.

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



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.

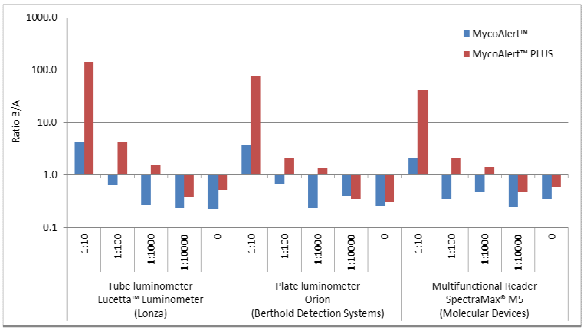


Figure 1: The graph shows a dilution series of MycoAlert™ Assay Control demonstrating the increased sensitivity of various luminometer models when using MycoAlert™ PLUS Assay compared to the first generation MycoAlert™ Assay.

7. Component Reconstitution

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™ PLUS Reagent and substrate are supplied as lyophilized pellets. These are reconstituted in the supplied MycoAlert™ PLUS Assay Buffer to produce the working solutions for use in the assay.

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

MycoAlert™ Assay Buffer added	to MycoAlert™ Reagent	to MycoAlert™ Substrate
LT07-701 (10 test kit)	1.2 ml	1.2 ml
LT07-703 (30 test kit)	1.2 ml	1.2 ml
LT07-705 (50 test kit)	5 ml	5 ml
LT07-710 (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. If not used immediately for the assay, please refer to section 4 for long-term storage

8. Additional Equipment

a. Instrumentation

The MycoAlert™ PLUS kit is compatible for use with tube or plate luminometers and multifunctional plate readers capable of detecting luminescence. We highly recommend assessing the sensitivity of your instrument using our MycoAlert™ Assay Control Set (LT07-518) according to the control set instructions. The parameters of the instrument should be assessed and the conditions below used to produce the correct programming of the machine.

Type	Read time
Tube luminometers	1 second (integrated)
Plate luminometers	1 second (integrated).
Multifunctional plate readers	Default settings*

* Please view our FAQ database **www.lonzabio.com/FAQ** for availability of any detailed recommendations for specific reader models.

b. Additional equipment and consumables

- 5 ml and 10 ml sterile pipettes
- Luminometer cuvettes or white walled microplates (ideally with an opaque bottom)
- Micropipettes: 1- 10 ul; 50-200 µl; 200-1000 µl
- Bench centrifuge

9. Assay Samples & Controls

Following sample types are suitable for use with MycoAlert™ PLUS Assay.

a. Cell supernatant

- Cell supernatant during passage of suspension cell culture.
- Supernatant from adherent cells prior to trypsinisation.

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

- 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 loss of sensitivity and possibly interfering with detection of low-level infections.
- For optimal assay performance, supernatant should be tested as soon as possible after collection.
- Supernatant can be kept at room temperature or 4℃ for testing same day
- Supernatant can be frozen and stored at -80℃ for 6 months. For this purpose, freeze supernatant immediately after spinning out cells. Thaw without the aid of artificial heat and equilibrate to room temperature for at least 15 min before testing

b. Other samples suitable for MycoAlert™ PLUS:

- Unused media
- 10-20% FCS or FBS
- L-glutamine
- Penicillin-Streptomycin mixture
- Neutralized Trypsin (diluted 1:2, i.e. neutralized with 2x volume of TNS solution, Lonza, Cat# CC-5002)
- Gentamicin/Amphotericin B

Note: For testing these types of samples, please refer to the respective protocol for testing fresh media and supplements in section 11. For other sample types, please refer to our FAQ database www.lonzabio.com/FAQ or contact Lonza Scientific Support.

c. 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 including a positive control sample in every experiment.

d. Negative control

Use 100 µl of MycoAlert™ PLUS Assay Buffer or deionized water as a negative control. We recommend including a negative control sample in every experiment.

10. Standard Assay Protocol (20 min) - For Cell Supernatant

Notes:

- 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.
- For testing unused media or supplements please refer to the respective protocol described in section 11.
- Before starting the experiment program the luminometer or multifunctional plate reader according to the recommendation in section 8a or the settings you have established for MycoAlert™ PLUS Assay.

1. Bring all reagents up to room temperature before use.
2. Reconstitute the MycoAlert™ PLUS Reagent and MycoAlert™ PLUS Substrate in MycoAlert™ PLUS Assay Buffer. Leave for 15 minutes at room temperature to ensure complete rehydration.
3. **Sample preparation:**
   - Transfer at least 2 ml of cell culture or culture supernatant into a centrifuge tube and pellet any cells at 200 x g for 5 minutes.
4. Transfer 100 µl* of sample into a luminometer tube or plate.
5. Add 100 µl* of MycoAlert™ PLUS Reagent to each sample and **wait 5 minutes**.
6. Place tube/plate in the luminometer or plate reader and initiate the program (Reading A).
7. Add 100 µl* of MycoAlert™ PLUS Substrate to each sample and **wait 10 minutes**.
8. Place cuvette/tube/plate in luminometer or plate reader and initiate the program (Reading B).
9. Calculate ratio = Reading B/Reading A.

*Volumes can be reduced to 90 µl if total well volume is < 300 µl.

11. Dilution Protocol (55 min) - For Fresh Media or Supplements

This alternative protocol is recommended for testing unused media or supplements. It involves a sample dilution step (1:10) that helps eliminating any inhibitory influence of the culture media components to the MycoAlert™ PLUS Assay Reactions.

This protocol might be also recommended if your sample shows a borderline ratio and you would like to circumvent 24-48 h quarantine before re-testing.

Note: For this protocol you need 90 µl extra MycoAlert™ PLUS Assay Buffer per sample, which can be ordered separately (LT27-292).

1. Bring all reagents up to room temperature before use.
2. Reconstitute the MycoAlert™ PLUS Reagent and MycoAlert™ PLUS Substrate in MycoAlert™ PLUS Assay Buffer. Leave for 15 minutes at room temperature to ensure complete rehydration.
3. **Sample preparation:**
   - Transfer at least 2 ml of sample into a centrifuge tube and pellet any cells at 200 x g for 5 minutes.
   - Transfer 90 µl of MycoAlert™ PLUS Assay Buffer into a new luminometer tube or plate and add 10 µl of cleared supernatant. Mix by pipetting.
4. Transfer 100 µl* of sample into a luminometer cuvette / tube/ plate well.
5. Add 100 µl* of MycoAlert™ PLUS Reagent to each sample and **wait 20 minutes**.
6. Place tube/plate in luminometer or plate reader and initiate the program (Reading A).
7. Add 100 µl* of MycoAlert™ PLUS Substrate to each sample and **wait 30 minutes**.
8. Place cuvette/tube/plate in luminometer or plate reader and initiate the program (Reading B).
9. Calculate ratio = Reading B/Reading A.

*Volumes can be reduced to 90 µl if total well volume is < 300 µl.

12. 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
< 1	Negative for mycoplasma
1 - 1.2	Borderline: – Quarantine cells & retest in 24-48 h – Alternatively, you may repeat the test with the dilution protocol (see section 11a)
> 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 1 with uninfected samples and routinely produce ratios greater than 1 for samples infected with mycoplasma.

Note: For correct interpretation of ratios for unused media or supplements, make sure that samples had been diluted 1:10 (see protocol 10a). Use of neat samples may lead to positive results due to inhibitory media components (see table 1, example RPMI).

Table 1. Interpretation of MycoAlert™ PLUS Assay Results - Examples illustrating ratios obtained with healthy and infected cells or clean and infected media.

Sample	MycoAlert™ PLUS Ratio		Conclusion
	Neat	1:10 Dilution	
Infected Cells			
K562	3.69	1.86	Positive
H9 (hESCs)	299.50	174.00	Positive
Infected Media			
IMDM/20% FBS	900.50	1943.50	Positive
Water	188.00	174.00	Positive
Healthy Cells			
HepG2	0.97	0.62	Negative
K562	0.89	0.45	Negative
Clean Media			
DMEM	1.00	0.48	Negative
RPMI	1.34*	0.45	Negative

* Use of neat samples may lead to positive results due to inhibitory media components.

13. Troubleshooting

High background levels (Read A)

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.

Cells remaining in supernatant can increase ATP background. When removing supernatant from pelleted cells, take care to minimize carry-over of cells into the supernatant.

Ensuring optimal performance

The optimal working temperature for all reagents is 20-22℃. 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 may indicate 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™ PLUS assay.

For additional troubleshooting guidelines please refer to the separate troubleshooting document at **www.lonza.com/mycoalert**. If technical support is required please contact Lonza Scientific Support teams.

14. 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 first generation MycoAlert™ Assay, some of them were re-tested with MycoAlert™ Plus Assay (see asterisks). 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 pneumonia**	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

15. 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™ PLUS 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

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

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.

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