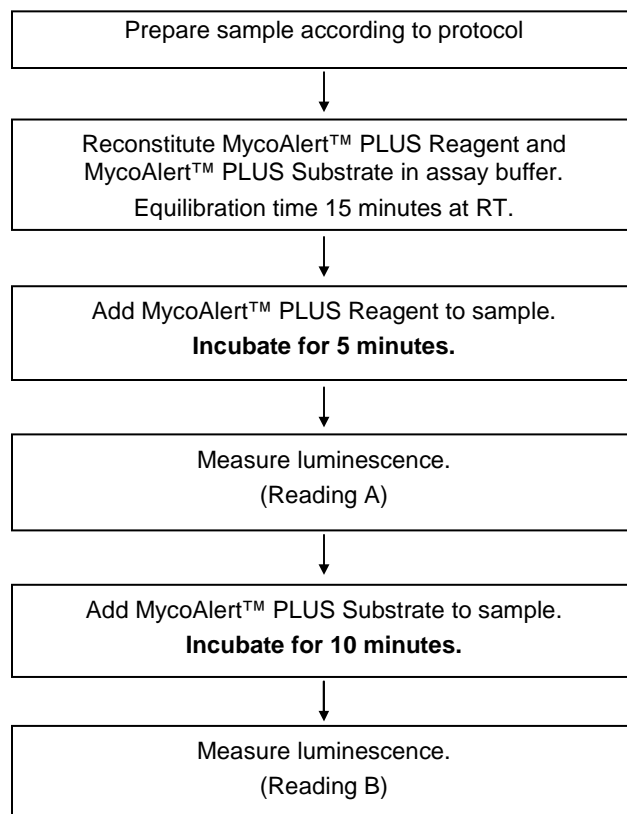


MycoAlert™ PLUS Mycoplasma Detection Kit Instructions for Use

MycoAlert™ PLUS Assay Procedure Outline

(For detailed assay protocols see Page 5)



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 items, except LT27-292 (see below).

Related Products

Mycoplasma Control Sets

Cat. No.	Product	Description
LT07-518	MycoAlert™ Assay Control Set	10 Test Kit

Mycoplasma Assay Buffer

Cat. No.	Product	Description
LT27-292	MycoAlert™ Assay Buffer	20 ml

Mycoplasma Elimination Reagents

Cat. No.	Product	Description
LT07-818	MycoZap™ Mycoplasma Elimination Reagent	1 Treatment
LT07-918	MycoZap™ Mycoplasma Elimination Reagent	5 Treatments

Mycoplasma Antibiotics

Cat. No.	Product	Description
VZA-2011	MycoZap™ Plus -CL Antibiotic (for cell lines)	10 x 1 ml
VZA-2012	MycoZap™ Plus -CL Antibiotic (for cell lines)	1 x 20 ml
VZA-2021	MycoZap™ Plus -PR Antibiotic (for primary cells)	10 x 1 ml
VZA-2022	MycoZap™ Plus -PR Antibiotic (for primary cells)	1 x 20 ml
VZA-2031	MycoZap™ Prophylactic Antibiotic	10 x 1 ml
VZA-2032	MycoZap™ Prophylactic Antibiotic	1 x 20 ml

Refer to the Lonza website or contact Scientific Support for details.

Storage Conditions

Lyophilized Components	-	Store at 2°C-8°C. Do not freeze.
	-	See kit label for expiration date of the whole kit. See bottle labels for expiration 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°C for another 5 days but should be equilibrated to room temperature* for 15-20 minutes before use.
	-	For long-term storage, reconstituted reagent and substrate can be aliquoted and stored at -80°C 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°C. In case room temperature significantly deviates from this optimal temperature range, it is recommended to use a water bath set to 22°C.

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.

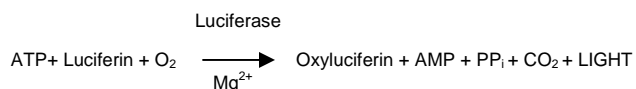
To allow for the early detection of mycoplasma contamination, Lonza recommends testing at every cell passage. Frequent testing such as this will indicate when a cell line becomes infected, allowing prompt remedial action to be taken. The MycoAlert™ PLUS Assay can also be extended to incoming cell lines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

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°C-22°C), the optimal temperature for luciferase activity.

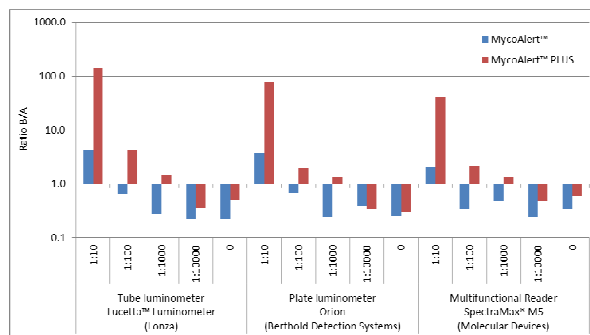


Figure 1: The graph shows a dilution series of MycoAlert™ Assay Control demonstrating the increased sensitivity of various luminometer models when using MycoAlert™ PLUS Assay, compared to the first generation MycoAlert™ Assay.

Component Reconstitution

NOTE: Please read this section carefully to ensure optimal performance for your assay. Please be sure to 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 minutes.
4. If not used immediately for the assay, please refer to Page 2 for long-term storage.

Additional Equipment

a. Instrumentation

The MycoAlert™ PLUS kit is compatible for use with tube or plate luminometers and multi-functional 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.lonza.com/FAQ for availability of any detailed recommendations for specific reader models.

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 µl; 50-200 µl; 200-1000 µl
- Bench centrifuge

Assay Samples & Controls

The 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°C for testing same day
- Supernatant can be frozen and stored at -80°C 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 minutes 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. No. CC-5002)
- Gentamicin/Amphotericin B

NOTE: For testing these types of samples, please refer to the respective protocol for testing fresh media and supplements on Page 5. For other sample types, please refer to our FAQ database www.lonza.com/FAQ or contact Lonza Scientific Support.

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.

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.

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 on Page 5.
- Before starting the experiment, program the luminometer or multi-functional plate reader according to the recommendation on Page 4 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.

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.
3. Transfer 100 µl* of sample into a luminometer tube or plate.
 4. Add 100 µl* of MycoAlert™ PLUS Reagent to each sample and **wait 5 minutes**.
 5. Place tube/plate in the luminometer or plate reader and initiate the program (Reading A).
 6. Add 100 µl* of MycoAlert™ PLUS Substrate to each sample and **wait 10 minutes**.
 7. Place cuvette/tube/plate in luminometer or plate reader and initiate the program (Reading B).
 8. Calculate ratio = Reading B/Reading A.

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

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 hours quarantine before re-testing.

NOTE: For this protocol you will need 90 µl extra MycoAlert™ PLUS Assay Buffer per sample that 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.

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.
3. Transfer 100 µl* of sample into a luminometer cuvette / tube/ plate well.
 4. Add 100 µl* of MycoAlert™ PLUS Reagent to each sample and **wait 20 minutes**.
 5. Place tube/plate in luminometer or plate reader and initiate the program (Reading A).
 6. Add 100 µl* of MycoAlert™ PLUS Substrate to each sample and **wait 30 minutes**.
 7. 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.

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: <ul style="list-style-type: none"> - Quarantine cells & retest in 24-48 hours - Alternatively, you may repeat the test with the dilution protocol
> 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).

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

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

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

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°C. 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 course of time 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.

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

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
 - o If gloves are contaminated by touching anything outside the cabinet, re-sanitize as above
 - o 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
 - o Direct verbal communication away from the cabinet
 - o 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
 - o 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
- Clean up 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

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.

The MycoAlert™ Assay and kits are protected by a granted patent in Australia [AU2004233290(B2)], Brazil [BRPI0407750(A)], Canada [CA2512636(A1)], China [CN1756848(B)], Europe [EP1613763(A1) and EP2264181(A1)], the UK [GB2400659B], Japan [JP2006523450], South Korea [KR20060005997(A)], Norway [NO20053276(A)], the US [US7585644], and the World Intellectual Property Organization [WO2004094656(A1)].

All trademarks herein are marks of Lonza Group or its affiliates.

Lonza strongly recommends that cell cultures with mycoplasma contamination be discarded and fresh stocks obtained. When that is 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.