

# Eradication of Mycoplasma Contaminations from Cell Cultures

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## ABSTRACT

Mycoplasma contaminations have a multitude of effects on cultured cell lines that may influence the results of experiments or pollute bioactive substances isolated from the eukaryotic cells. The elimination of mycoplasma contaminations from cell cultures with antibiotics has been proven to be a practical alternative to discarding and re-establishing important or irreplaceable cell lines. Different fluoroquinolones, tetracyclins, pleuromutilins, and macrolides shown to have strong anti-mycoplasma properties are employed for the decontamination. These antibiotics are applied as single treatments, as combination treatment of two antibiotics in parallel or successively, or in combination with a surface-active peptide to enhance the action of the antibiotic. The protocols in this unit allow eradication of mycoplasmas, prevention of the development of resistant mycoplasma strains, and potential cure of heavily contaminated and damaged cells. Consistent and permanent alterations to eukaryotic cells attributable to the treatment have not been demonstrated. *Curr. Protoc. Mol. Biol.* 106:28.5.1-28.5.12. © 2014 by John Wiley & Sons, Inc.

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## INTRODUCTION

The use of human and animal cell lines for the examination of biological functions and for the production of bioactive substances requires rigorous quality control to exclude contamination with organisms (i.e., other eukaryotic cells, bacteria or viruses). In this respect, mycoplasmas play an important but undesirable role, because a high portion of cell cultures (more than 20%) is contaminated with these wall-less bacteria (Armstrong et al., 2010).

When an infected cell culture is detected, it is commonly suggested to autoclave and discard the culture immediately and to replace it with a mycoplasma-free culture. However, the antibiotic treatment of infected cell lines is a practical option to save important cell lines. The following protocols describe the most reliable chemotherapeutic methods to eradicate mycoplasmas from contaminated cell cultures (Uphoff and Drexler, 2010).

Four classes of antibiotics have been shown to be highly effective against mycoplasmas, both in human/veterinary medicine and in cell culture technology: tetracyclines, pleuromutilins, macrolides, and fluoroquinolones (see Table 28.5.1; Uphoff and Drexler, 2002). These antibiotics can be used at relatively low concentrations, with a negligible likelihood of resistance development, and with low or no detectable effects on the eukaryotic cells. The Basic Protocol describes the use of single agents or simultaneously applied dual treatments, whereas in the alternate protocols two antibiotics with different modes of action are employed consecutively. If sufficient cell material is available, two or more independent treatments with antibiotics from different classes can be performed to minimize the possibility of treatment failure.

**Table 28.5.1** Antibiotics Active Against Mycoplasmas, Stock Solutions, Working Concentrations, and Treatment Durations

Antibiotic category	Brand name	Generic name	Stock conc. (mg/ml)	Predilution (mg/ml)	Volume per ml medium ( $\mu$ l)	Final conc. ( $\mu$ g/ml)	Treatment duration
Fluoroquinolones	Baytril	Enrofloxacin	100	1	25	25	8 days
	Ciprobay	Ciprofloxacin	2	–	5	10	2 weeks
	Mycoplasma Removal Agent (MRA)	Undisclosed	50	–	10	0.5	8 days
Fluoroquinolone + Macrolide	Plasmocin	Undisclosed	25	–	1	25	2 weeks
Pleuromutilin + Tetracycline	BM-Cyclin	Tiamulin	2.5	–	4	10	3 weeks
		Minocycline	1.25	–	4	5	
Membrane active agent + Antibiotic (undisclosed)	MycosZap	Undisclosed	Undisclosed	–	100		4 passages
		Undisclosed	Undisclosed	–	50		

**BASIC  
PROTOCOL****ELIMINATION OF MYCOPLASMAS WITH SINGLE  
FLUOROQUINOLONES OR SIMULTANEOUS DUAL ANTIBIOTIC  
TREATMENT**

This protocol describes the most convenient way to treat mycoplasma-contaminated cell cultures by adding one of the listed antibiotics (Table 28.5.1) as single regimen or the combination compound Plasmocin to the growing cell culture. The treatment time is 8 or 14 days, depending on the antibiotic employed, followed by a post-treatment period without antibiotics for at least another 2 weeks. During the treatment period the antibiotic is refreshed every second day by complete or partial medium exchange. Use the antibiotics only for the treatment of contaminated cultures and not prophylactically to prevent infections. The general use of antibiotics—especially at lower concentrations as for treatment—may lead to the selection and distribution of drug-resistant bacteria, to lapses in aseptic technique, and to delayed detection of low-level infection with bacteria (also see Background Information).

**Materials**

Cells

Cell culture medium and supplements (e.g., fetal bovine serum and dimethyl sulfoxide) as appropriate and recommended for the particular cell line

Phosphate-buffered saline (PBS; APPENDIX 2); autoclaved

Reagent to detach adherent cells as recommended for the particular cell line (e.g., trypsin, trypsin/EDTA, TrypLE Express, TNE buffer)

0.4% Trypan blue solution (Sigma Aldrich)

Antibiotics including:

Baytril (see recipe)

Ciprobay 100 (see recipe)

MRA (see recipe)

Plasmocin (see recipe)

Liquid nitrogen

Hemocytometer, e.g., Neubauer counting chamber (Brand, Wertheim, Germany)

Centrifuge

Ampoules

Nunc freezing container *or* another freezing method**Eradication of  
Mycoplasma  
Contaminations****28.5.2**

–80°C freezer *or* liquid nitrogen  
25-cm<sup>2</sup> flasks *or* 6- or 24-well culture plates  
37°C incubator

Additional reagents and equipment for determining cell number and viability with a hemacytometer and trypan blue staining (*APPENDIX 3F*) and testing for mycoplasma contamination (*UNIT 28.4*)

### ***Pretreatment procedures***

1. If no frozen ampoules of the contaminated cell lines are available, store frozen aliquots as reserve in case of treatment failure. To do this, prepare a cell suspension. Clumps of cells in suspension cultures are broken up by pipetting the suspension up and down. Monolayer cultures are first washed with PBS (0.2 ml/cm<sup>2</sup>) and subsequently treated with 20 μl/cm<sup>2</sup> trypsin/EDTA (or another reagent to detach the cells). The culture is then incubated at 37°C until the cells are completely detached from the surface of the culture vessel (usually after 5 to 15 min; tapping the culture vessel carefully against the palm of the hand or a table edge can facilitate the detachment of the cells). Finally, add 0.2 ml/cm<sup>2</sup> fresh medium to inhibit the activity of trypsin/EDTA and transfer the cell suspension into a sterile centrifuge tube. Take an aliquot of the cell suspension to determine the cell number and viability using a hemacytometer. Centrifuge the cell suspension for 6 min at 300 × *g*, room temperature. Remove the supernatant and resuspend the cells at 0.1 × 10<sup>6</sup> to 5 × 10<sup>6</sup> cells/ml (depending on the respective cell culture) with the appropriate growth medium containing 10% to 20% FBS and 10% dimethyl sulfoxide (DMSO) as cryoprotectant. Dispense the cell suspension into labelled ampoules and apply a cooling rate of approximately 1°C/min using a Nunc freezing container or another freezing method. Transfer the frozen ampoules into a –80°C freezer or store in liquid nitrogen (see also *APPENDIX 3F*).

*Whenever possible, keep the ampoules isolated from non-infected cultures, either at –80°C for short time (during the entire treatment period of 1 to 2 months) or, preferably, in liquid nitrogen. Mark the ampoules properly as “mycoplasma-positive” to prevent misidentification/confusion of ampoules containing cured or infected cells. After successful cure, these mycoplasma-positive ampoules should be removed and the cells destroyed by autoclaving.*

2. Prepare the cell culture medium with an FBS concentration of 20%.

*Keep the higher FBS concentration before, during, and for at least 2 weeks after the treatment to ensure optimal growth conditions, even if the cells may grow well at lower concentrations.*

3. Prepare a cell suspension [detach adherent cells, break up clumps by pipetting or using other methods and determine the cell density and viability by trypan blue exclusion staining as described in step 1 (see also *APPENDIX 3F*)].

*Mycoplasma infection often impairs the growth and viability of the eukaryotic cells. The viability of the cell culture can have an impact on the selection of the best treatment option. The following order of antibiotics reflects the degree of cell culture deterioration (from excellent to poor cell culture condition): BM-Cyclin (see Alternate Protocol 1), Plasmocin, ciprofloxacin, enrofloxacin, MRA, MycoZap (see Alternate Protocol 2).*

4. Seed the cells at a medium density (see Commentary) in a 25-cm<sup>2</sup> flask or one well of a 6- or 24-well culture plate with the appropriate fresh and rich culture medium (10 ml for the flask, and 4 ml and 2 ml for the wells, respectively).

*Keep the cell density relatively constant during the treatment and for a few weeks thereafter; a higher density of the cells demands a more frequent change of medium, which is commonly preferable to a relatively low cell density and long intervals between medium*

*changes. However, some cell lines reportedly produce their own growth factors; therefore, the medium should not be fully exchanged, depending on the cell line.*

#### **Treat infected cultures with antibiotic**

5. Depending on the antibiotic applied, prepare a predilution of the antibiotic freshly for every treatment (according to information provided in the Reagents and Solutions section and Table 28.5.1).
6. Add the predilution or the stock solution directly at the recommended concentration (according to the information provided in the Reagents and Solutions section and Table 28.5.1) to the passaged cell culture, not to the stored medium.
7. Incubate the cell culture for 2 days at 37°C in the dark.
8. Remove all cell culture medium in flasks or wells containing adherent cells or discard medium after centrifugation of suspension cells. If applicable, reduce the cell number to achieve a cell density as mentioned above (step 4). Add fresh medium and the same concentration of the respective antibiotic as used in step 6. Incubate for another 2 days.

*If the medium is not completely exchanged, add the adequate amount of antibiotic for the portion of fresh medium plus half the concentration for the residual medium.*

- 9a. If using enrofloxacin or MRA, repeat steps 6 to 8 another two times (altogether an 8-day treatment).
- 9b. If using ciprofloxacin or Plasmocin, repeat steps 6 to 8 five times (altogether a 14-day treatment).

#### **Culture cells and test post-treatment**

10. After completing the treatment, remove the culture medium and culture the cells in the same manner (enriched medium, higher cell concentration, etc.) as during the treatment period, but do not add any antibiotics. Even penicillin and streptomycin should not be added to the medium. Culture the cells for at least another 2 weeks.

*Even if initially the cells appear to be in good health during the treatment, the cells might go into a crisis after the treatment. The reason for this post-treatment crisis is not clear, but might be the result of reduced activity of the mitochondria. Thus, the cell status should be frequently examined under the inverted microscope and increased attention should be paid to the cell culture for an extended period of time. Morphological changes of the cells (e.g., shrinking, granulation and vacuolation of cells or detachment of adherent cells) and a decrease of viability indicates a potential cell culture crisis (see also Critical Parameters and Troubleshooting).*

11. Test the cultures for mycoplasma contamination (see UNIT 28.4). If the cultures are not contaminated, freeze them and store aliquots in liquid nitrogen.

*Applying the highly sensitive PCR test for the detection of mycoplasmas, we occasionally found that the treated cell cultures might show a weak false-positive signal even after a week of post-treatment passaging. This is not necessarily the result of resistance of the mycoplasmas and their regrowth, but might be caused by residual DNA in the culture medium. These cell cultures should not be discarded after being found positive, but retested after further culturing.*

12. Culture the cells at recommended conditions (where appropriate, at lower FBS concentrations, lower cell densities, and longer passage intervals), check the cells for the retention of characteristic features, and retest the culture periodically for the presence of mycoplasmas to ensure continued freedom from mycoplasma contamination.

## ELIMINATION OF MYCOPLASMAS WITH DUAL SEQUENTIAL ANTIBIOTIC TREATMENT USING BM-CYCLIN

## ALTERNATE PROTOCOL 1

This treatment employs two antibiotics usually not applied in other treatment protocols: a pleuromutilin (tiamulin) and a tetracycline (minocyclin; Schmidt and Erfle, 1984). Hence, this treatment is a good alternative if the approach with a fluoroquinolone failed and produced resistant mycoplasma clones. Due to the alternating administration of the antibiotics in three cycles, the treatment process takes 3 weeks. Although the mycoplasmas are reduced rapidly, the treatment cycles should be performed according to schedule, because both antibiotics act bacteriostatically.

### Materials

Cell culture medium and supplements as appropriate and recommended for the particular cell lines  
Fetal bovine serum (FBS)  
Trypan blue solution  
BM-Cyclin (see recipe)  
Reagents to detach adherent cells as recommended for the particular cell line (e.g., trypsin, trypsin/EDTA, TrypLE Express, TNE buffer)  
Phosphate-buffered saline (PBS; *APPENDIX 2*); autoclaved  
Liquid nitrogen

Hemocytometer, e.g., Neubauer counting chamber  
Centrifuge  
Ampoules  
Nunc freezing container *or* another freezing method  
–80°C freezer or liquid nitrogen  
37°C incubator  
25-cm<sup>2</sup> flasks *or* 6- or 24 well culture plate

Additional reagents and equipment for preparing a cell suspension (*APPENDIX 3F*) and testing the cultures for mycoplasma contamination (*UNIT 28.4*)

### Pretreatment procedures

1. If no frozen ampoules of the contaminated cell lines are available, store frozen aliquots as reserve in case of treatment failure (see Basic Protocol, step 1).
2. Prepare the cell culture medium with an FBS concentration of 20%.  
*Keep the higher FBS concentration before, during, and for at least 2 weeks after the treatment to ensure optimal growth conditions, even if the cells may grow well at lower concentrations.*
3. Prepare a cell suspension [(detach adherent cells, break up clumps by pipetting or using other methods, and determine the cell density using a hemacytometer and viability by trypan blue exclusion staining (*APPENDIX 3F*)].  
*Mycoplasma infection often impairs the growth and viability of the eukaryotic cells. The viability of the cell culture can have an impact on the selection of treatment variant. The following order of antibiotics reflects the degree of cell culture deterioration (from excellent to poor cell culture condition): BM-Cyclin, Plasmocin, ciprofloxacin, enrofloxacin, MRA, MycoZap.*
4. Seed the cells at a medium density (see Commentary) in a 25-cm<sup>2</sup> flask or one well of a 6- or 24-well culture plate with the appropriate fresh and rich culture medium (10 ml for the flask, and 4 ml or 2 ml for the wells, respectively).

*Keep the cell density relatively constant during the treatment and for a few weeks thereafter; a higher density of the cells demands a more frequent change of medium, which is commonly preferable to a relatively low cell density and long intervals between medium changes. However, some cell lines reportedly produce their own growth factors and, therefore, the medium should not be fully exchanged, depending on the cell line.*

#### **Treat cultures with antibiotics**

5. Add 4  $\mu$ l BM-Cyclin 1 (tiamulin) per milliliter cell culture directly to the passaged cell culture, not to the stored medium.
6. Incubate the cell culture for 3 days at 37°C.

*If the cell culture requires passaging or dilution after 2 days due to depleted medium or high cell density, exchange the medium and add the appropriate volume of antibiotic, detach the cells, seed out cells in a new culture vessel, and add medium and antibiotic, or dilute suspension cells and add fresh medium and antibiotic. Incubate for another day.*

7. Remove all cell culture medium in flasks or wells containing adherent cells and detach the cells with trypsin/EDTA (*APPENDIX 3F*) or another solution recommended for the cell line. Add 0.2 ml/cm<sup>2</sup> fresh culture medium to the detached cells and centrifuge the number of cells to be seeded for the next treatment step for 5 min at 300  $\times$  g, room temperature. If suspension cells are treated, directly centrifuge the cell suspension with the respective number of cells for seeding.
8. Discard the supernatant and resuspend the cells in 5 ml PBS to wash away the remaining antibiotics.
9. Centrifuge the cells for 5 min at 300  $\times$  g, room temperature, and discard the supernatant.
10. Resuspend the cells in the appropriate volume of cell culture medium, transfer them into the culture vessel, and add 4  $\mu$ l BM-Cyclin 2 (minocyclin) per milliliter cell culture directly to the passaged cell culture. Incubate for 2 days at 37°C.
11. Remove the culture medium and substitute with 0.4 ml/cm<sup>2</sup> fresh medium. Add the same concentration of BM-Cyclin 2 as used in step 10. Washing with PBS is not necessary at this step. Incubate the cell culture for 2 days at 37°C to complete one 4-day cycle of minocycline treatment.
12. Repeat steps 7 to 9.
13. Resuspend the cells in the appropriate volume of cell culture medium, transfer them into a new culture vessel, and treat the cells as described in step 5.
14. Continue the complete second treatment cycle with tiamulin and minocyclin as described in steps 6 to 12.
15. Repeat the 7-day treatment cycle once more according to steps 13, followed by steps 5 to 12.

*Alternatively, steps 4 to 12 are also possible instead of 13 followed by 5 to 12 (includes the same procedure).*

#### **Culture cells and test post-treatment**

16. After completing the treatment, resuspend the cells in fresh culture medium and culture the cells in the same manner (enriched medium, higher cell concentration, etc.) as during the treatment period, but do not add any antibiotics. Even penicillin and streptomycin should not be added to the medium. Culture the cells for at least another 2 weeks with the appropriate passaging and dilution intervals.

*Even if the cells initially appear to be in good health during the treatment, the cells might go into a crisis after the treatment. The reason for this post-treatment crisis is not clear,*

*but might be the result of reduced activity of the mitochondria. Thus, the cell status should be frequently examined under the inverted microscope and increased attention should be paid to the cell culture for an extended period of time. Morphological changes of the cells (e.g., shrinking, granulation and vacuolation of cells or detachment of adherent cells) and a decrease of viability indicate a potential cell culture crisis (see also Critical Parameters and Troubleshooting).*

17. Test the cultures for mycoplasma contamination (see *UNIT 28.4*). If the cultures are not contaminated, freeze and store aliquots in liquid nitrogen.

*Applying the highly sensitive PCR test for the detection of mycoplasmas, we occasionally found that the treated cell cultures might show a weak false-positive signal even after 2 weeks of post-treatment passaging. This is not necessarily the result of resistance of the mycoplasmas and their regrowth, but might be caused by residual DNA in the culture medium. These cell cultures should not be discarded after being found positive, but retested after further culturing.*

18. Culture the cells under recommended conditions (where appropriate at lower FBS concentrations, lower cell densities, and longer passage intervals), check the cells for the retention of characteristic features, and retest the culture periodically for the presence of mycoplasmas to ensure continued freedom from mycoplasma contamination.

### **ELIMINATION OF MYCOPLASMAS WITH A MEMBRANE-ACTIVE PEPTIDE AND AN ANTIBIOTIC (Myc Zap)**

### **ALTERNATE PROTOCOL 2**

Myc Zap is a combination treatment consisting of a membrane-active peptide, such as alamethicin, gramicidin S, or surfactin, and a conventional antibiotic (described in Nir-Paz et al., 2002). During the first part of the treatment most of the mycoplasmas are lysed and washed away by the surface-active agent. In the second part, the remaining mycoplasmas are killed by the antibiotic. This combination treatment shows almost no effect on the growth parameters of the eukaryotic cells during the eradication procedure. This treatment is recommended when the cells are already in very poor condition prior to treatment and the number of available cells would suffice only for a single type of treatment. Sometimes, the cells recover rapidly after starting the treatment due to the immediate reduction/elimination of the mycoplasmas.

#### **Materials**

Cell culture medium for the particular cell lines  
Fetal bovine serum (FBS)  
Dimethyl sulfoxide (DMSO)  
Trypan blue  
Reagents to detach adherent cells as recommended for the particular cell line (e.g., trypsin, trypsin/EDTA, TrypLE Express, TNE buffer)  
Myc Zap reagent 1 and Myc Zap reagent 2 (see recipe)

Hemocytometer, e.g., Neubauer counting chamber  
Labeled ampoules  
Nunc freezing containers, optional  
–80°C freezer *or* liquid nitrogen  
25-cm<sup>2</sup> flasks

Additional reagents and equipment for preparing a cell suspension (*APPENDIX 3F*) and testing the cultures for mycoplasma contamination (*UNIT 28.4*)

#### **Pretreatment procedures**

1. If no frozen ampoules of the contaminated cell lines are available, store frozen aliquots as reserve in case of treatment failure. To do this, prepare a cell suspension.

Clumps of cells in suspension cultures are broken up by pipetting the suspension up and down. Monolayer cultures are first washed with PBS (0.2 ml/cm<sup>2</sup>) and subsequently treated with 20 μl/cm<sup>2</sup> trypsin/EDTA (or another reagent to detach the cells). The culture is then incubated at 37°C until the cells are completely detached from the surface of the culture vessel (usually after 5 to 15 min; tapping the culture vessel carefully to the heel of the hand or a table edge can facilitate the detachment of the cells). Finally add 0.2 ml/cm<sup>2</sup> fresh medium to inhibit the activity of trypsin/EDTA and transfer the cell suspension to a sterile centrifuge tube. Take an aliquot of the cell suspension to determine the cell number and viability using a hemacytometer. Centrifuge the cell suspension for 5 min at 300 × g, room temperature. Resuspend the cells at 0.1 × 10<sup>6</sup> to 5 × 10<sup>6</sup> cells/ml (depending on the respective cell culture) with the appropriate growth medium containing 10% to 20% FBS and 10% dimethyl sulfoxide (DMSO) as cryoprotectant.

2. Dispense the cell suspension into labeled ampoules and apply a cooling rate of ~1°C/min using a Nunc freezing container or another freezing method. Transfer the frozen ampoules to a –80°C freezer or store in liquid nitrogen.

*Whenever possible, keep the ampoules isolated from non-infected cultures either at –80°C for short time (during the entire treatment period of 1 to 2 months) or, preferably, in liquid nitrogen. Mark the ampoules properly as “mycoplasma-positive” to prevent misidentification/confusion of ampoules containing cured or infected cells. After successful cure, these mycoplasma-positive ampoules should be removed and the cells destroyed by autoclaving.*

3. Prepare a cell suspension [(detach adherent cells, break up clumps by pipetting or using other methods and determine the cell density and viability by trypan blue exclusion staining (APPENDIX 3F)].

#### ***Treat cultures with antibiotics***

4. Add 500 μl MycoZap reagent 1 to 4.5 ml cell culture medium supplemented with a maximum of 5% FBS.

*The activity of the membrane-active peptide is influenced by the concentration of the FBS. Thus, the FBS concentration of the cell culture medium should not exceed 5% during the treatment with MycoZap reagent 1.*

5. Seed 5 × 10<sup>5</sup> cells in 4.5 ml cell culture medium supplemented with maximally 5% FBS in a 25-cm<sup>2</sup> flask. Add 5 ml medium containing MycoZap reagent 1 as prepared in step 4 and incubate the cells at 37°C until the culture reaches a medium density, but at least for 2 days.
6. Remove the complete cell culture medium in the flask containing adherent cells or after centrifugation of suspension cells. If applicable, dilute the cell cultures to the medium cell density.
7. Add 9.5 ml fresh medium (containing the FBS concentration appropriate for the cell culture or an increased FBS concentration of 20%) and 0.5 ml of MycoZap reagent 2. Incubate for 2 days.
8. Repeat step 7 another two times to complete a 6-day antibiotic treatment.

#### ***Culture cells and test post-treatment***

9. After completing the treatment, remove the culture medium and culture the cells in the same manner (enriched medium, higher cell concentration, etc.) as during the treatment period, but do not add any antibiotics. Even penicillin and



streptomycin should not be added to the medium. Culture the cells for at least another 2 weeks.

*Even if the cells initially appear to be in good health during the treatment, the cells might go into a crisis after the treatment. The reason for this post-treatment crisis is not clear, but might be the result of reduced activity of the mitochondria. Thus, the cell status should be frequently examined under the inverted microscope and increased attention should be paid to the cell culture for an extended period of time. Morphological changes of the cells (e.g., shrinking, granulation and vacuolation of cells or detachment of adherent cells) and a decrease of viability indicates a potential cell culture crisis (see Critical Parameters and Troubleshooting).*

10. Test the cultures for mycoplasma contamination (see UNIT 28.4). If the cells are clean, freeze and store aliquots in liquid nitrogen.

*Applying the highly sensitive PCR test for the detection of mycoplasmas, we occasionally find that the treated cell cultures might show a weak false positive signal even after 2 weeks of post-treatment passaging. This is not necessarily the result of a resistance of the mycoplasmas and their regrowth, but might be caused by residual DNA in the culture medium. These cell cultures should not be discarded after being found positive, but retested after further culturing.*

11. Culture the cells at recommended conditions (where appropriate at lower FBS concentrations, lower cell densities, and longer passage intervals), check the cells for the retention of characteristic features, and retest the culture periodically for the presence of mycoplasmas to ensure continued freedom from mycoplasma contamination.

## REAGENTS AND SOLUTIONS

*Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2; for suppliers, see APPENDIX 4.*

*Store the antibiotics at the recommended concentrations, temperatures, and usually in the dark, and do not use them after the expiration date. Upon formation of precipitates, completely dissolve the crystals at room temperature in the dark before use.*

### **Baytril**

Baytril (Bayer) contains 100 mg/ml of enrofloxacin. Dilute 1:100 with RPMI 1640 medium immediately prior to use. The dilution should be prepared freshly for every anti-mycoplasma treatment. This solution is used as 1:40 final dilution in cell culture (at 25  $\mu$ g/ml final concentration).

### **BM-Cyclin**

BM-cyclin (Roche) contains the pleuromutilin tiamulin (BM-Cyclin 1) and the tetracycline minocycline (BM-Cyclin 2), both in lyophilized states. Dissolve the antibiotics in 10 ml sterile distilled water (dH<sub>2</sub>O), divide into 1-ml aliquots, and store up to 2 years at  $-20^{\circ}\text{C}$ . These stock solutions have concentrations of 2.5 mg/ml and 1.25 mg/ml, respectively.

*Repeated freezing and thawing of the solutions is not detrimental to the activity of the antibiotics. The dissolved solutions can be used at 1:250 dilutions in cell culture (at 10  $\mu$ g/ml and 5  $\mu$ g/ml final concentration, respectively).*

### **Ciprobay**

Ciprobay (Bayer) is a ready-to-use solution containing 2 mg/ml ciprofloxacin. It can be used 1:200 in cell culture (at 10  $\mu$ g/ml final concentration). Aliquots (1-ml) should be taken sterilely from the bottle and stored up to 2 years at  $4^{\circ}\text{C}$ . Crystals form at  $4^{\circ}\text{C}$  and can be dissolved at room temperature.

## **MRA**

MRA (Mycoplasma Removal Agent, ICN) is a ready-to-use dilution containing 50 µg/ml of a 4-oxo-quinolone-3-carboxylic acid derivative (specific type of reagent not disclosed). It is used in the treatment of cell cultures in 1:100 dilutions (at 0.5 µg/ml final concentration).

## **MycoZap**

MycoZap (Lonza) is the combination of an antimicrobial peptide (MycoZap reagent 1) and an antibiotic (MycoZap reagent 2) (specific types of reagents not disclosed) that are employed consecutively. The solutions are ready-to-use.

## **Plasmocin**

Plasmocin (InvivoGen) contains two antibiotics; one is a macrolide active against protein synthesis of the bacteria by inhibiting the 50S ribosomal subunit, and one is a fluoroquinolone inhibiting the DNA replication (gyrase inhibitor) (specific reagents not disclosed). The mixture is a ready-to-use solution and applied 1:1000 in the cell culture (at 25 µg/ml final concentration).

## **COMMENTARY**

### **Background Information**

Mycoplasmas can have a multitude of effects on eukaryotic cells and can alter almost every cellular parameter, from proliferation via signaling pathways to virus susceptibility and production. Most striking are the effects resulting from the competition for nutrients that leads to the depletion of a number of essential nutrients. As a result, many downstream effects, such as altered levels of protein, DNA, and RNA synthesis, and alterations of cellular metabolism and cell morphology, can occur.

Mycoplasmas do not gain energy by oxidative phosphorylation, but from the fermentative metabolism of diverse nutrients. This can lead to an alteration of the pH in the medium and to the production of metabolites that are toxic to eukaryotic cells (e.g., NH<sub>3</sub>). The dependence of many mycoplasmas on cholesterol, sterols, and lipids can result in an alteration of the membrane composition. Other activation and suppression processes have also been described (e.g., lymphocyte activation, cytokine expression, induction of chromosomal aberrations). It has also been noted that many experimentally analyzed parameters that were at first attributed to the eukaryotic cells later had to be ascribed to the contaminating mycoplasmas or were at least influenced by them (Rottem and Barile, 1993).

Due to the unpredictable impact of mycoplasmas on the eukaryotic cells, contaminated cell cultures should not be used for experimentation.

A number of methods have been suggested to eradicate mycoplasmas from cell cultures. They comprise physical, chemical, immunological, and chemotherapeutic treatment. Some treatments are restricted to surfaces only (e.g., exposure to detergents), to eukaryotic-cell-free solutions, such as FBS (e.g., filtration through microfilters,  $\gamma$ -irradiation treatment), to specific mycoplasma species only (e.g., culture with anti-mycoplasma antisera); others appear not to be practicable in a standard cell culture laboratory (e.g., in vivo passage of continuous cell lines through nude mice cell cloning), or are ineffective in eliminating the mycoplasmas quantitatively (e.g., heat treatment, exposure to complement) (Drexler and Uphoff, 2002). The fact that some mycoplasma species have the ability to invade the eukaryotic cell should also be taken into consideration. *Mycoplasma fermentans* is one of the main infecting mycoplasma species that has the ability to enter eukaryotic cells. Thus, eliminating procedures must also be active intracytoplasmically (Yavlovich and Rottem, 2007).

Chemotherapeutic treatment can be efficiently employed using specific antibiotics. Because mycoplasmas possess no rigid cell walls and have a greatly reduced metabolism, many of the commonly used antibiotics exhibit no effect on the viability of the mycoplasmas. They are naturally resistant to antibiotics targeting cell wall biosynthesis (e.g., penicillin) and have an acquired resistance against other antibiotics that are often prophylactically used

in cell culture (e.g., streptomycin), or the antibiotics are effective only at concentrations that are also detrimental to the eukaryotic cells. As the cell culture technology is based on use of sterile techniques, the general and prophylactic application of antibiotics is no longer necessary. Only special circumstances require prophylactic use, e.g., for primary cell cultures, the selection of transfected cells, when cells are handled under semi-sterile conditions, and for the specific situation to eliminate contaminations. The indiscriminate and general use of antibiotics could lead to the selection of drug-resistant organisms, to lapses in aseptic technique, and to delayed detection of low-level infection with either mycoplasmas or other bacteria (Uphoff and Drexler, 2001).

### Critical Parameters and Troubleshooting

Tetracyclines, pleuromutilins, and macrolides act bacteriostatically by inhibiting protein synthesis: tetracyclines bind to the 30S subunits of the bacterial 70S ribosomes, whereas the pleuromutilins and macrolides bind to the 50S subunits. Fluoroquinolones are bactericides that inhibit the bacterial DNA gyrase and topoisomerase IV, which are essential for mycoplasmal DNA replication and lead to DNA fragmentation. The risk of development of resistant mycoplasma clones and their dissemination to other cell cultures and laboratories is always a critical aspect for the use of antibiotics. These untoward outcomes can be avoided or at least minimized by the application of antibiotics with different mechanisms of action, by sufficient treatment durations, and by constant concentrations of the antibiotics in the medium (Uphoff et al., 2012).

Generally, there are four different approaches to the treatment of infected cell cultures with antibiotics: (1) the use of a single antibiotic compound (e.g., the fluoroquinolones), where basically the same procedure is employed for each antibiotic of that group; (2) the simultaneous application of two different antibiotics (e.g., in the case of Plasmocin); (3) the use of a combination therapy applying two antimycoplasma agents subsequently in alternating cycles (e.g., BM-Cyclin); and (4) the use of a membrane-active peptide to get rid of the majority of the mycoplasmas present in the medium and on the cells, followed by the application of an antibiotic to eliminate the remaining mycoplasmas inside and outside the cells (e.g., MycoZap).

The simultaneous application of antibiotics with different modes of action is generally not recommended, because the action of the bactericidal fluoroquinolones depends on the proliferation of the cells, which is compromised by bacteriostatic agents. However, the employment of Plasmocin showed a similar efficiency as the antibiotic treatment with a single fluoroquinolone (Uphoff et al., 2012). Whenever possible, we recommend using two different treatment procedures separately in parallel approaches to increase the chance of cure should one method fail.

After addition of the antibiotic, heavily infected cells might recover immediately and the viability of the culture might increase rapidly. However, in other cases, the delicate health of the cells is further aggravated by exposure to the antibiotic(s). One reason might be the partial inhibition of mitochondrial respiration and/or proliferation by the antibiotic(s). Even though the optimal concentrations of the antibiotics were determined in many trials, different cell types or cells under different infection conditions might behave differently upon treatment. Thus, in some instances, the cultures might be killed by the treatment (Uphoff and Drexler, 2002). In these events, the treatment must be repeated with an aliquot that was stored frozen prior to the treatment. To counteract the treatment-associated harm, a few general suggestions should be followed to improve the culture conditions and to reduce the stress of infection and treatment on the eukaryotic cells (these rules are suitable for most cell lines, but some cell lines require special care, which must be determined by the user):

- Keep the concentration of the antibiotic constant during the treatment period; degradation of the antibiotic can be avoided by frequent complete exchange of the medium noting the following caveats.
- Observe the culture daily under the inverted microscope for alteration in general appearance, growth, or morphology, decrease in cell viability, detachment of cells, formation of granules, vacuoles, and so forth.
- In the case of rapid deterioration of the cell culture, interrupt the treatment for a few days and let the cells recover (but this should only be the last resort); culture conditions should be changed immediately after recognition of the alterations, because if the cells are already beyond a certain degree of damage, it is usually difficult to reverse the progression of apoptosis.
- If possible, frequently detach slowly growing adherent cells to facilitate the

exposure of all mycoplasmas to the antibiotic; the contaminants should not have the opportunity to survive in sanctuaries, such as cell membrane pockets. It is similarly helpful to break up clumps of suspension cells by vigorous pipetting or using other reagents (e.g., trypsin, trypsin/EDTA, TrypLE Express, or Accutase).

- Fluoroquinolones exhibit phototoxic potential. Therefore, protect antibiotics and cultures from light, as much as possible.

Some cell lines are sensitive to a complete exchange of the medium. If the medium can only be exchanged partially, an additional 50% of the antibiotic concentration should be added to the remaining conditioned medium, whereas 100% of the antibiotic concentration is added to the fresh medium.

In the rare event of resistance, cells of the untreated frozen back-up aliquots can be thawed and treated again with another protocol. As MRA, ciprofloxacin, enrofloxacin, and one component of Plasmocin all belong to the group of fluoroquinolones, it is likely that the use of an alternative compound from the same group will produce the same end result (cure, resistance, or culture death).

Adherent cells are detached by methods appropriate for the cell line being treated. It is important to break up all clumps and clusters and to detach cells from the surface of the culture vessels. Fluoroquinolones were shown to be active and even accumulate inside the eukaryotic cells. However, the membranes might be barriers that cannot be passed by other antibiotics. Intracellular mycoplasmas or mycoplasmas trapped within clumps of eukaryotic cells or even in cavities formed by the cell membrane of a single cell might be protected from the antibiotic. This is also the reason for the advice to keep the concentration of the antibiotic constantly high by frequently exchanging the medium.

### Anticipated Results

The procedures described in this unit should result in complete eradication of mycoplasma contamination. The success rate of a single treatment is between 75% and 85%. However, the employment of a double or even a triple treatment approach, in parallel or sequentially, increases the success rate to almost 100%, provided that the cell cultures survive the infection as well as the treatment (Uphoff et al., 2012).

Whenever resistant mycoplasma clones are recognized, the cell culture should immediately be destroyed by autoclaving to avoid the distribution of the resistant clones in the laboratory or between laboratories.

### Time Considerations

Depending on the antibiotics used, the complete decontamination procedure including pre-treatment, treatment, and post-treatment periods requires 4 and 6 weeks. The cured cell cultures should be cultured further for 1 or 2 months and be tested regularly for mycoplasma contamination to ensure consistent freedom from mycoplasma contamination.

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