

For life science research only. Not for use in diagnostic procedures. FOR *IN VITRO* USE ONLY.

Mycoplasma Detection Kit

Enzyme immunoassay for the detection of mycoplasmas/acholeplasmas in cell culture (*M. arginini*, *M. hyorhinis*, *A. laidlawii*, *M. orale*)

Cat. No. 11 296 744 001

For 25 tests

Store at 2–8°C

Instruction Manual

Version October 2004



1. Preface

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1.2 Kit contents

Bottle/ Cap	Label	Content
1a-d blue cap	Coating antibody	<ul style="list-style-type: none"> One vial each with coating antibody: <ol style="list-style-type: none"> Anti-<i>M. arginini</i> Anti-<i>M. hyorhins</i> Anti-<i>A. laidlawii</i> Anti-<i>M. orale</i> lyophilizate; after reconstitution ready-to-use solution
2 (blue cap)	Blocking reagent	<ul style="list-style-type: none"> 120 ml ready-to-use solution <p>Note: It is possible that the solution show a slight turbidity or precipitate, respectively. This does not influence the test. Mix the content prior to use.</p>
3 (green cap)	Washing buffer	<ul style="list-style-type: none"> 2× 100 ml Washing buffer (10× conc.) after dilution ready-to-use solution
4 (green cap)	Sample buffer	<ul style="list-style-type: none"> 22 ml ready-to-use solution <p>Note: It is possible that the solution show a slight turbidity or precipitate respectively. This does not influence the test. Mix the content prior to use.</p>
5 (white cap)	Control solution	<ul style="list-style-type: none"> One vial control solution, mycoplasma-positive; inactivated lyophilizate after reconstitution ready-to-use solution
6a-d (red cap)	Detection antibody	<ul style="list-style-type: none"> One vial each with Detection antibody, biotin-conjugate: <ol style="list-style-type: none"> Anti-<i>M. arginini</i>-biotin Anti-<i>M. hyorhins</i>-biotin Anti-<i>A. laidlawii</i>-biotin Anti-<i>M. orale</i>-biotin lyophilizate after reconstitution ready-to-use solution
7 (red cap)	Streptavidin-alkaline phosphatase	<ul style="list-style-type: none"> 85 ml ready-to-use solution
8 (colorless cap)	Substrate buffer	<ul style="list-style-type: none"> 100 ml ready-to-use solution
(white cap)	Substrate tablets	One vial substrate tablets

2. Introduction

2.1 Product overview

Introduction

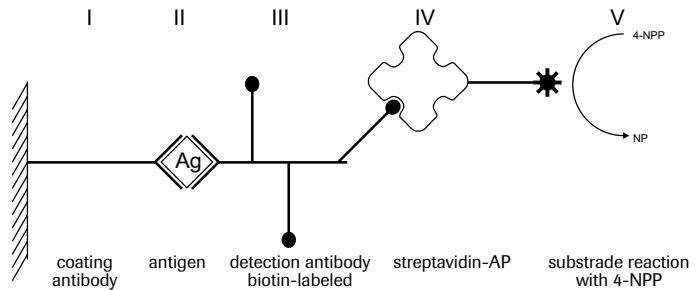
The Mycoplasma Detection Kit combines specificity and high sensitivity with short assay time and is easy to evaluate. The kit is based on the ELISA technique (14) and contains polyclonal antibodies for the detection of the most common mycoplasma/acholeplasma species contaminating mammalian cell culture (*M. arginini*, *M. hyorhinitis*, *A. laidlawii*, *M. orale*).

Test principle

Determination of each species is done separately.

Stage	Description
1	Fixation of coating antibody to the microplate wells. Blocking of unpecific binding sites.
2	<ul style="list-style-type: none">• Incubation of the test sample (e.g., cell culture supernatant, cell suspension, media)• Binding of antigen (mycoplasmas or mycoplasma fragments) to the corresponding coating antibody.
3	Detection of antigen with biotin-labeled antibody.
4	Binding of streptavidin-AP conjugate to the biotin-labeled detection antibody.
5	Visualiazion by enzymatic reaction with 4-nitrophenylphosphate as substrate.

Figure



2.2 Product overview, continued

Application	The kit is used for the detection of the most common mycoplasma/acholeplasma species contaminating mammalian cell culture (<i>M. arginini</i> , <i>M. hyorhinis</i> , <i>A. laidlawii</i> , <i>M. orale</i>).										
Sample material	<ul style="list-style-type: none">• Cell suspensions• supernatant from adherent cells• media• sera• detergent-treated or frozen samples										
Assay time	The assay needs one day with an overnight incubation period.										
Number of tests	The kit is designed for 25 tests.										
Kit storage/ stability	<p>The unopened kit is stable at 2–8°C until the expiration date printed on the label. The reconstituted solutions are stable at 2–8°C for 2 months. These solutions as well as Streptavidin-AP (vial 7) can also be stored in aliquots at –15 to –25°C for at least 12 month.</p> <p>Note: Repeated thawing and freezing should be avoided. Prepare the Substrate solution always fresh!</p>										
Sensitivity	<p>According to the procedure described the assay shows detection limits as given in the following table. The sensitivity and the time of the assay procedure can be adapted to the particular requirements of the user by varying the quantities used, the incubation periods and temperatures. It is recommended to perform always double determinations as described to guarantee reliability of the results. The assay has to be done always for all four organism species.</p> <p>With the method given in this pack insert the following detection limit has been reached:</p> <table border="1"><thead><tr><th>Type of mycoplasma</th><th>Detection Limit</th></tr></thead><tbody><tr><td><i>M. arginini</i></td><td>10⁶ –10⁷ CFU/ml</td></tr><tr><td><i>M. hyorhinis</i></td><td>10⁶ –10⁷ CFU/ml</td></tr><tr><td><i>A. laidlawii</i></td><td>10⁴ –10⁵ CFU/ml</td></tr><tr><td><i>M. orale</i></td><td>10⁵ –10⁶ CFU/ml</td></tr></tbody></table>	Type of mycoplasma	Detection Limit	<i>M. arginini</i>	10 ⁶ –10 ⁷ CFU/ml	<i>M. hyorhinis</i>	10 ⁶ –10 ⁷ CFU/ml	<i>A. laidlawii</i>	10 ⁴ –10 ⁵ CFU/ml	<i>M. orale</i>	10 ⁵ –10 ⁶ CFU/ml
Type of mycoplasma	Detection Limit										
<i>M. arginini</i>	10 ⁶ –10 ⁷ CFU/ml										
<i>M. hyorhinis</i>	10 ⁶ –10 ⁷ CFU/ml										
<i>A. laidlawii</i>	10 ⁴ –10 ⁵ CFU/ml										
<i>M. orale</i>	10 ⁵ –10 ⁶ CFU/ml										
Advantages	The easy handling and evaluation (macroscopic or with a microplate reader) and the high specificity and sensitivity makes the Mycoplasma Detection Kit a convenient tool for the routine examination of cell cultures and media for mycoplasma contamination.										

2.2 Background information

Preparation of antibodies	For the preparation of the polyclonal antibodies contained in the kit mycoplasma/acholeplasmas were cultivated and were purified by repeated centrifugation and washing before immunization procedures. After immunization IgG was purified from serum by precipitation and anionexchange-chromatography followed by an affinity-chromatography step to remove crossreacting antibodies. Detection antibodies were conjugated with D-biotinoyl-ε-aminocaproic acid-N-hydroxysuccinimide ester*.
Mycoplasma	Mycoplasmas are common and serious contaminants of cell cultures and this remains one of the major problems encountered in biological research using cultured cells. It has been shown that cell cultures are frequently contaminated up to 30% (1–4) with mycoplasmas, the main contaminants being the species <i>M. orale</i> , <i>A. laidlawii</i> , <i>M. arginini</i> and <i>M. hyorhinis</i> . These organisms produce a variety of effects on the infected cells in culture (e.g., changes in metabolism, growth, viability, morphology etc.) leading to experiments in cell culture with results not always reliable (1–5).
Origins of mycoplasma contamination	The origins of mycoplasmal infection of cell cultures are often bovine serum (<i>M. arginini</i> , <i>M. hyorhinis</i> , <i>A. laidlawii</i>), laboratory personnel (<i>M. orale</i>) and mycoplasma-infected cultures. Mycoplasmal infection cannot be detected with the naked eye other than by signs of deterioration in the culture. It is important to keep in mind that mycoplasmas do not always reveal their presence with macroscopic alterations of the cells or media. Many mycoplasma contaminants, particularly in continuous cell lines grow slowly and do not destroy host cells but are even though able to affect various parameters as mentioned above. Therefore there is an absolute requirement for routine, periodic assays for possible covert contamination of all cell cultures, particularly continuous or established cell lines.
Detection techniques	A variety of techniques have been developed for the detection of cell culture mycoplasmas, e.g., DNA staining with dyes like 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI)* or bisbenzimidazole (H 33258), biochemical detection methods, e.g., with 6-methylpurine deoxyriboside (6-MPDR), mycoplasma culture method, electron microscopy (2, 3, 6–13). However, each method shows certain disadvantages. Detection via DNA staining requires a fluorescence microscope and much experience in evaluating the results. The 6-MPDR assay is dependent on a indicator cell line and time-consuming, which is also true for electron microscopy. The culture method being also time-consuming, does not detect all relevant mycoplasma species, e.g., <i>M. hyorhinis</i> , which is not easy to cultivate <i>in vitro</i> .

3. Procedures and required materials

3.1 Before you begin

Sample material	<p>Cell suspensions, supernatant from adherent cells, media, sera and also detergent-treated or frozen samples can be used.</p> <p>The presence of antibiotics within the sample does not interfere with the assay results. However, since the number of mycoplasmas can be reduced the cells may be cultivated free of antibiotics for at least two passages to increase the sensitivity of the assay.</p> <p>So far it is not necessary to spin samples with cells in suspension down before performing the assay.</p> <p>To concentrate mycoplasmas a centrifugation step can be done though a number of mycoplasmas will be lysed due to the centrifugation.</p> <p>The assay results will not be influenced by contamination from bacteria or yeast.</p>
Sample preparation	<p>Dilute 2 ml of a sample with 0.5 ml Sample buffer (vial 4).</p>
Positive mycoplasma control	<p>Positive mycoplasma control should be tested in parallel using the Control solution, mycoplasma-positive (vial 5).</p> <p>Mycoplasmas in this solution have been inactivated by W-propiolactone treatment.</p>
Negative mycoplasma control	<p>Sterile media analogous to the sample should be used.</p> <p>If there is none available Washing buffer (vial 3) can be used.</p> <p>Negative controls are also diluted with Sample buffer:</p> <p>0.25 ml Sample buffer (vial 4) is added to 1 ml media or Washing buffer.</p> <p>Note: If a medium containing serum is used, we recommend to use sera of the same lot in the medium used as a negative control.</p>
Microplates	<p>It is recommended to use only commercially available microplates (96 wells each of approx. 300 µl) with high homogeneity and high binding capacity for peptides/proteins, even in the presence of detergents (e.g., “immunoplate” microplates from Nunc GmbH, Denmark).</p>
Assay procedure	<p>Determination of each species has to be performed separately, i.e. coating antibody and detection antibody have to correspond. For each sample double determinations should be done for each species.</p> <p>Neither coating antibodies nor detection antibodies should be mixed. Avoid cross-contamination of the wells!</p>

3.1 Before you begin, continued

Assay capacity

To perform a successful assay it is not necessary that the mycoplasmas/acholeplasmas are present as viable organisms. Therefore the handling of the samples, *e.g.*, sample collection, storage, transport or the use of antibiotics in routine biochemical work is not critical.

The Mycoplasma Detection Kit has been developed for routine testing of cell cultures and media for mycoplasma contamination. Since mycoplasmas can show a great variety in subspecies the assay could not have been tested for all variants. Therefore detection of all variants of the four species to be tested in the assay can not be guaranteed. It has been shown that 94% of all tested contaminated routine cell cultures were detected with the Mycoplasma Detection Kit is, based on the ELISA principle. These results are based on a blind study with about 200 examined cell cultures of which more than 30% were found to be mycoplasma-positive.

In this study the Mycoplasma Detection Kit has been compared with the DAPI method and the culture method. Correspondence between enzyme immunoassay and culture method is approx. 80%, but especially some *M. hyorhinis*-contaminated cell cultures were not detected with the culture method.

Correspondence between enzyme immunoassay and the DAPI method is approx. 75%, but with the DAPI method the detection of contaminated cell cultures was significantly lower than with the enzyme immunoassay.

Pipetting

In case of varying the described pipetting procedure take care that the volume of the detection antibody and the conjugate solution applied is not larger than the volume of the Blocking solution in order to avoid contact with uncoated surfaces.

3.2 Preparation of kit working solutions

Additional equipment and reagents required

Sodium azide

Preparation of kit working solutions

The following table shows the preparation of kit working solutions.

Solution	Composition/ preparation	Storage and Stability	Use
Coating anti-body solution	Reconstitute coating antibody (vials 1a-d) with 30 ml double dist. water at 15-25°C for 20 min and mix.	At 2-8°C for 2 month or in aliquots at -15 to -25°C for at least 12 months	Protocol step 1
Control solution, mycoplasma-positive	Reconstitute Control solution (vial 5) with 25 ml Washing buffer solution at 15-25°C for 20 min and mix.	At 2-8°C for 2 month or in aliquots at -15 to -25°C for at least 12 months	Protocol step 4
Detection anti-body solution	Reconstitute Detection antibody (vials 6a-d) with 25 ml Washing buffer solution at 15-25°C for 20 min and mix.	At 2-8°C for 2 month or in aliquots at -15 to -25°C for at least 12 months	Protocol step 6
Washing buffer	To reconstitute one bottle, fill 100 ml 10× concentrate in an autoclaved 1 liter bottle and add 900 ml double distilled water. Add 1 g/l sodium azide to avoid microbial contamination, if not used instantly. Stirr with an auto-claved magnetic stirrer.	Recommended to consume soon, reconstitute only as much as needed, take routine measures to avoid microbial contamination.	Protocol step 3, step 5, step 7, step 9
Substrate solution	Dissolve one substrate tablet (vial 9) in 4 ml Substrate buffer (vial 8) (for one determination).	Always prepare fresh!	Protocol step 10

3.3 Protocol

Before you begin

- Before performing the assay all reagents should have reached 15–25°C. The assay should be performed at the temperatures indicated below.
- For checking one cell culture sample 16 wells are required:
- 2 estimations for each of four mycoplasma species = 8 wells
- 4 negative controls
- 4 positive controls

Procedure

Please refer to the following table.

Step	Action																				
1	<p>Coating with coating antibody</p> <ul style="list-style-type: none"> • Pipette 0.25 ml of Coating antibody (vials 1a–d) each into 4 wells of a microplate (see pipetting scheme). <table border="1" style="margin-left: 20px;"> <tr> <td>x</td> <td>x</td> <td>x</td> <td>x</td> <td>anti-<i>M. arginini</i> coating antibody (x) (vial 1a)</td> </tr> <tr> <td>#</td> <td>#</td> <td>#</td> <td>#</td> <td>anti-<i>M. hyorhinae</i> coating antibody (#) (vial 1b)</td> </tr> <tr> <td>=</td> <td>=</td> <td>=</td> <td>=</td> <td>anti-<i>A. laidlawii</i> coating antibody (=) (vial 1c)</td> </tr> <tr> <td>⊗</td> <td>⊗</td> <td>⊗</td> <td>⊗</td> <td>anti-<i>M. orale</i> coating antibody (⊗) (vial 1d)</td> </tr> </table> <ul style="list-style-type: none"> • Cover microplate tightly and incubate 2 h at 37°C. 	x	x	x	x	anti- <i>M. arginini</i> coating antibody (x) (vial 1a)	#	#	#	#	anti- <i>M. hyorhinae</i> coating antibody (#) (vial 1b)	=	=	=	=	anti- <i>A. laidlawii</i> coating antibody (=) (vial 1c)	⊗	⊗	⊗	⊗	anti- <i>M. orale</i> coating antibody (⊗) (vial 1d)
x	x	x	x	anti- <i>M. arginini</i> coating antibody (x) (vial 1a)																	
#	#	#	#	anti- <i>M. hyorhinae</i> coating antibody (#) (vial 1b)																	
=	=	=	=	anti- <i>A. laidlawii</i> coating antibody (=) (vial 1c)																	
⊗	⊗	⊗	⊗	anti- <i>M. orale</i> coating antibody (⊗) (vial 1d)																	
2	<p>Blocking</p> <ul style="list-style-type: none"> • Remove the solutions from the wells by suction and tapping on a clean and dry cloth (e.g., cellulose) • Pipette 0.25 ml Blocking solution (vial 2) into all antibody-coated wells. • Incubate 30 min at 37°C. 																				
3	<p>Washing</p> <ul style="list-style-type: none"> • Remove the solutions from the wells by suction and then by tapping on a clean and dry cloth. • Wash wells 3 × with Washing buffer solution and finally remove Washing buffer thoroughly. 																				
4	<p>Application of sample and controls</p> <ul style="list-style-type: none"> • Pipette 0.2 ml of the prepared sample into each of the sample wells (s), pipette 0.2 ml of the negative control (medium with Sample buffer, vial 4) and positive control – Control solution into each of the control wells (+/-) (see pipetting scheme); <table border="1" style="margin-left: 20px;"> <tr> <td>S</td> <td>S</td> <td>n.c.</td> <td>p.c.</td> <td rowspan="4">S : = sample n.c.: = negative control p.c.: = positive control</td> </tr> <tr> <td>S</td> <td>S</td> <td>n.c.</td> <td>p.c.</td> </tr> <tr> <td>S</td> <td>S</td> <td>n.c.</td> <td>p.c.</td> </tr> <tr> <td>S</td> <td>S</td> <td>n.c.</td> <td>p.c.</td> </tr> </table> <ul style="list-style-type: none"> • Cover microplate tightly and incubate overnight at 2–8°C. 	S	S	n.c.	p.c.	S : = sample n.c.: = negative control p.c.: = positive control	S	S	n.c.	p.c.	S	S	n.c.	p.c.	S	S	n.c.	p.c.			
S	S	n.c.	p.c.	S : = sample n.c.: = negative control p.c.: = positive control																	
S	S	n.c.	p.c.																		
S	S	n.c.	p.c.																		
S	S	n.c.	p.c.																		
5	<p>Washing</p> <p>As described under step 3</p>																				

continued on next page

3.3 Protocol, continued

Step	Action																				
6	<p>Application of detection antibody</p> <ul style="list-style-type: none"> Pipette 0.2 ml Detection antibody-solution into the 4 corresponding wells of each antibody (see pipetting scheme). <table border="1"> <tr> <td>x</td> <td>x</td> <td>x</td> <td>x</td> <td>anti-<i>M. arginini</i> detection antibody (x) (vial 6a)</td> </tr> <tr> <td>#</td> <td>#</td> <td>#</td> <td>#</td> <td>anti-<i>M. hyorhinae</i> detection antibody (#) (vial 6b)</td> </tr> <tr> <td>=</td> <td>=</td> <td>=</td> <td>=</td> <td>anti-<i>A. laidlawii</i> detection antibody (=) (vial 6c)</td> </tr> <tr> <td>⊗</td> <td>⊗</td> <td>⊗</td> <td>⊗</td> <td>anti-<i>M. orale</i> detection antibody (⊗) (vial 6d)</td> </tr> </table> <ul style="list-style-type: none"> Cover microplate tightly and incubate 2 h at 37°C. 	x	x	x	x	anti- <i>M. arginini</i> detection antibody (x) (vial 6a)	#	#	#	#	anti- <i>M. hyorhinae</i> detection antibody (#) (vial 6b)	=	=	=	=	anti- <i>A. laidlawii</i> detection antibody (=) (vial 6c)	⊗	⊗	⊗	⊗	anti- <i>M. orale</i> detection antibody (⊗) (vial 6d)
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⊗	⊗	⊗	⊗	anti- <i>M. orale</i> detection antibody (⊗) (vial 6d)																	
7	<p>Washing</p> <ul style="list-style-type: none"> Remove the solutions from the wells by suction and then by tapping on a clean and dry cloth. Wash wells 4× with Washing buffer solution and finally remove Washing buffer thoroughly. 																				
8	<p>Application of Streptavidin-AP</p> <ul style="list-style-type: none"> Pipette 0.2 ml Streptavidin-AP-solution (vial 7) into each of the 16 wells (see pipetting scheme). <table border="1"> <tr> <td>Δ</td> <td>Δ</td> <td>Δ</td> <td>Δ</td> <td rowspan="4">Δ := Streptavidin-AP-solution</td> </tr> <tr> <td>Δ</td> <td>Δ</td> <td>Δ</td> <td>Δ</td> </tr> <tr> <td>Δ</td> <td>Δ</td> <td>Δ</td> <td>Δ</td> </tr> <tr> <td>Δ</td> <td>Δ</td> <td>Δ</td> <td>Δ</td> </tr> </table> <ul style="list-style-type: none"> Cover microplate tightly and incubate 1 h at 37°C. 	Δ	Δ	Δ	Δ	Δ := Streptavidin-AP-solution	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ			
Δ	Δ	Δ	Δ	Δ := Streptavidin-AP-solution																	
Δ	Δ	Δ	Δ																		
Δ	Δ	Δ	Δ																		
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9	<p>Washing</p> <ul style="list-style-type: none"> Remove the solutions from the wells by suction and tapping on a clean and dry cloth. Wash wells 3 × with Washing buffer solution and finally remove Washing buffer thoroughly. 																				
10	<p>Substrate reaction</p> <ul style="list-style-type: none"> Pipette 0.2 ml Substrate solution (freshly prepared) into each of the 16 wells (see pipetting scheme). <table border="1"> <tr> <td>□</td> <td>□</td> <td>□</td> <td>□</td> <td rowspan="4">□ = Substrate solution (freshly prepared)</td> </tr> <tr> <td>□</td> <td>□</td> <td>□</td> <td>□</td> </tr> <tr> <td>□</td> <td>□</td> <td>□</td> <td>□</td> </tr> <tr> <td>□</td> <td>□</td> <td>□</td> <td>□</td> </tr> </table> <ul style="list-style-type: none"> Incubate 30 min at 18–25°C. 	□	□	□	□	□ = Substrate solution (freshly prepared)	□	□	□	□	□	□	□	□	□	□	□	□			
□	□	□	□	□ = Substrate solution (freshly prepared)																	
□	□	□	□																		
□	□	□	□																		
□	□	□	□																		
11	<p>Evaluation</p> <p>Evaluate visually or measure samples against Substrate solution in a micro-plate reader at 405 nm.</p>																				

4. Results

Evaluation

Evaluation is carried out visually ("positive" = yellow, "negative" = practically colorless) or with the aid of a microplate reader at a wavelength of $\lambda = 405$ nm against substrate solution. The cut off limit "mycoplasma-positive, -negative" must be determined by the user. Following exactly the protocol, no color development can be observed in the absence of mycoplasmas in the sample. In the presence of mycoplasmas above the sensitivity limit an OD > 0.2 is measured. Wells containing only medium and Sample buffer (see step 3 in the assay procedure or step II in the pipetting scheme) are used as a negative control.

False positive signals

Using media containing serum from swine increase the probability that the assay will show false positive signals. In this case the determination of mycoplasma-contamination cannot be performed.

In extremely rare cases the presence of an unspecific cross reactivity was observed in horse sera, causing false positive results. In that case the test should be redone after culturing the cells in sera of a different serum lot. Usually horse sera do not produce an elevated background level in the assay.

5. Appendix

5.1 Trouble shooting

Trouble shooting table Please refer to the following table.

Problem	Possible cause	Recommendation
Some of the negative control values are too high	Negative control solution has been contaminated with positive control.	Repeat assay without positive control.
All values of the negative controls and samples are similar high	The blocking solution has not been pipetted. Possible interference of the medium.	<ul style="list-style-type: none">• Repeat the assay as described.• Repeat the assay using a different medium. (eventually use a different serum lot).
No or low signal in the positive control	Incorrect pipetting.	Repeat the assay as described.
	The reagents have not reached the correct temperature.	Make sure that all reagents have reached 15–25°C before performing the assay.

5.2. References

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5.3 Related products

Roche Applied Science offers a large selection of reagents and systems for life science research. For a complete overview of related products and manuals, please visit and bookmark our homepage <http://www.roche-applied-science.com>.

Product	Pack Size	Cat. No
BM-Cyclin Antibiotic combination for the elimination of mycoplasma from cell cultures	275 mg	10 799 050 001
4',6-Diamidine-2- phenylindole dihydrochloride (DAPI)	10 mg	10 236 276 001
Uracil-DNA glycosylase (UNG)	25 U (25 µl) 100 U (100 µl)	11 269 062 001 11 444 646 001
Mycoplasma PCR ELISA	1 kit (96 reactions)	11 663 925 910

*available from Roche Applied Science

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