PlasmoTest™
Mycoplasma Detection Kit

For the detection of *Mycoplasma* contamination in cell lines

Method based on the activation of Toll-Like Receptor 2

Catalog code: rep-pt1

https://www.invivogen.com/plasmotest

This package insert must be read in its entirety before using this product

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Not for diagnostic use

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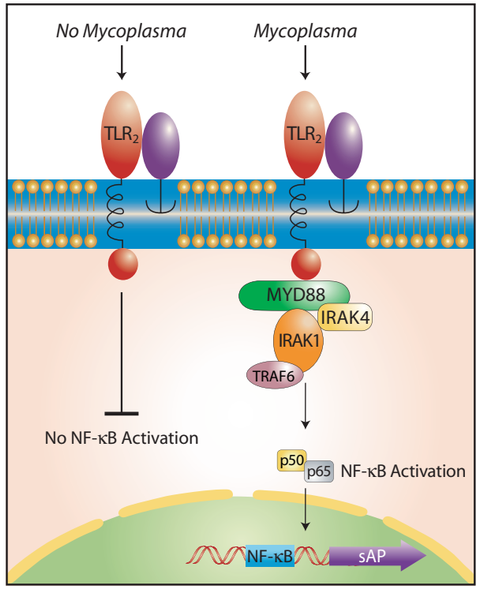
INTRODUCTION

Mycoplasma contamination of cultured cells is a major problem in both basic research and industrial production. Up to 87% of cell lines may be contaminated by *Mycoplasma*[1,2]. Unlike bacterial or fungal contaminations, mycoplasmal contaminations cannot be detected by visual inspection other than through signs of deterioration in the culture. Whereas the concentration of *Mycoplasma* in an infected cell culture range typically from 10^6 to 10^8 cfu/ml, they are invisible under an inverted microscope and may remain totally undetected for long periods[1,3]. Mycoplasmal infection can affect virtually any function and activity of eukaryotic cells leading to unreliable experimental results and potential unsafe biological products[1,4].

KIT DESCRIPTION

PlasmoTest™ is a cell-based colorimetric assay that exploits the ability of Toll-like receptor 2 to recognize mycoplasmas and to induce a signaling cascade leading to the activation of NF-κB and other transcription factors. In the presence of mycoplasmas, TLR2 expressed on the surface of **HEK-Blue™-2 cells** activates these transcription factors which in turn induce the secretion of sAP (secreted alkaline phosphatase), a reporter protein easily detectable by the purple/blue coloration of the **HEK-Blue™ Detection** medium.



Principle of PlasmoTest™, a TLR2 activation based Mycoplasma Detection Kit.

HEK-Blue™-2 Cells

HEK-Blue™-2 cells are engineered HEK293 cells stably transfected with multiple genes from the TLR2 pathway that include TLR2 and genes participating in the recognition or involved in the signaling cascade. In addition, HEK-Blue™-2 cells stably express an optimized alkaline phosphatase gene engineered to be secreted (sAP), placed under the control of a promoter inducible by several transcription factors such NF-κB and AP-1.

HEK-Blue™ Detection

HEK-Blue™ Detection is a medium specifically designed for the detection of sAP. In the presence of mycoplasma-contaminated samples, HEK-Blue™-2 cells secrete sAP in the HEK-Blue™ Detection medium resulting in a color change from pink to purple/blue.

HEK-Blue™ Detection is a powdered medium provided in individually sealed pouches. Each pouch allows the preparation of 50 ml of detection medium with HEK-Blue™ water (sterile endotoxin-free water).

HEK-Blue™ Selection Mix

HEK-Blue™ Selection is a solution that combines several selective antibiotics. These antibiotics guarantee the persistent expression of the various transgenes introduced in HEK-Blue™-2 cells.

HEK-Blue™ Water

HEK-Blue™ Water is a sterile endotoxin-free water provided in the kit to prepare HEK-Blue™ Detection medium and Positive and Negative controls.

Normocin™

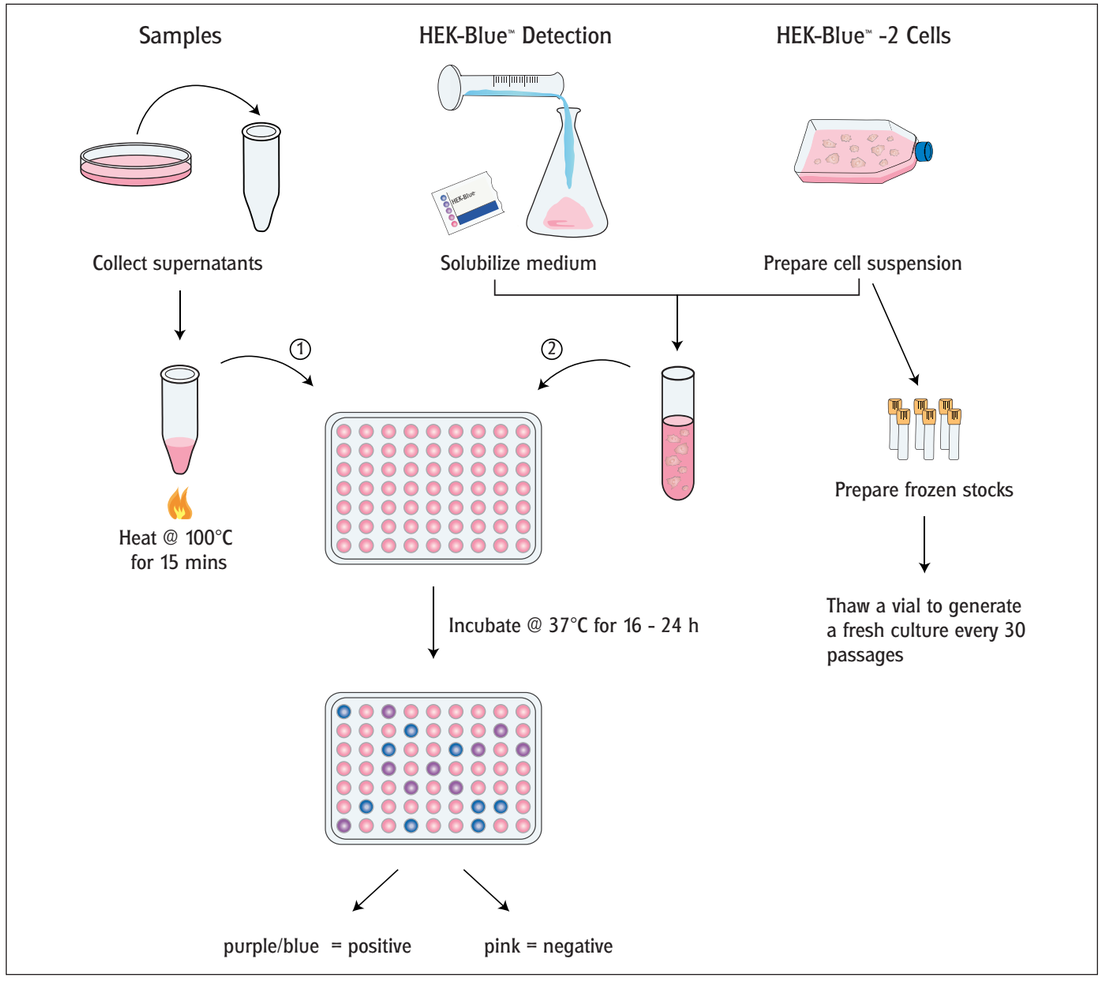
Normocin™ is a formulation of three antibiotics active against mycoplasmas, bacteria and fungi. For cell culture, Normocin™ is used at a concentration of 100 µg/ml, which represents a 1:500 dilution of stock solution.

Positive and Negative Controls

The Positive and Negative Controls are provided lyophilized. After solubilizing with 1 ml of HEK-Blue™ water (sterile endotoxin-free water), 10X solutions of Positive and Negative controls are obtained. They have to be diluted at 1/10 before use. They allow to perform 200 tests.

<u>Note:</u> The Positive Control is not a living mycoplasma.

PROCEDURES SUMMARY



1. Collect 500 µl cell culture supernatants to be checked and transfer into a microtube.
2. Heat samples at 100 °C for 15 mins.
3. Prepare HEK-Blue™ Detection by solubilizing the powder with 50 ml HEK-Blue™ water.
4. Add 50 µl of each heated sample in a well of a 96-well plate.
5. Add 50 µl of each supplied control in a well of a 96-well plate.
6. Prepare HEK-Blue™-2 cell suspension using prewarmed HEK-Blue™ Detection medium.
7. Add 200 µl (~50,000 cells) of cell suspension to each well containing the samples or controls.
8. Incubate the plate at 37 °C in a CO_2 incubator overnight (16-24 hours).
9. Detect the presence of *Mycoplasma* with the naked eye or with a spectrophotometer at 620-655 nm.

KIT INFORMATION

Contents

The PlasmoTest™ contains the following components:

- 1 vial of HEK-Blue™-2 Cells (3-7 x 10^6 frozen cells)
- 2 x 1 ml HEK-Blue™ Selection (250X concentrated solution)
- 1 ml Normocin™ (50 mg/ml)
- 1 of pouch HEK-Blue™ Detection
- 60 ml bottle of HEK-Blue™ Water
- 1 tube of Positive control
- 1 tube of Negative control

Note: This kit allows to test up to 250 samples. To perform further assays, you do not need to reorder the HEK-Blue™ cells but only the reagents necessary for their maintenance (HEK-Blue™ Selection and Normocin™) and for the detection (HEK-Blue™ Detection and Controls). See "Related Products".

Storage and stability

- **PlasmoTest™ is shipped on dry ice.**
- Upon receipt HEK-Blue™-2 cells must be thawed **immediately** and grown according to handling procedures described in the next paragraph.
- Store HEK-Blue™ Selection and Normocin™ at 4 °C for 1 month and at -20 °C for >12 months.
- Store unopened pouches of HEK-Blue™ Detection pouches at 2-8 °C. Product in unopened pouches is stable for at least 6 months at 2-8 °C. Reconstituted HEK-Blue™ Detection is stable for 2 weeks at 2-8 °C and for 2 months at -20 °C. Protect from light.
- Store lyophilized Positive and Negative controls at 4 °C for 12 months. Resuspended negative control is stable at 4 °C for 6 months when properly stored. Upon resuspension, prepare aliquots of the positive control and store at 4 °C for short-term storage (1 month) or -20 °C for long-term storage (6 months).

Note: Avoid repeated freeze-thaw cycles. The stability of HEK-Blue™ Detection medium at 4°C is given only if the reagent stays at 4 °C.

ADDITIONAL MATERIALS REQUIRED

Reagents required

All reagents should be sterile and cell culture tested:

- Dulbecco's modified Eagle's medium (DMEM), high glucose (4.5 g/L)

Note: If using DMEM without glutamine, add 2 mM glutamine.

- Penicillin-Streptomycin solution
- Fetal Bovine Serum (FBS)

Note: Some lots of FBS, although sterile, contain microbial debris that may activate the HEK-Blue™-2 cells (See "Troubleshooting").

- Phosphate buffered saline (PBS) without calcium and magnesium
- Sterile endotoxin-free water or ultrapure water
- Dimethylsulfoxide (DMSO)

Supplies required

- Laminar flow hood
- Centrifuge
- Water bath (37 °C)
- Boiling water bath or heating block (100 °C)
- Inverted microscope
- CO_2 incubator
- Sterile cell culture plasticware: tubes, pipettes, 25 cm^2 and 175 cm^2 flasks, flat-bottom 96-well plates, filter tips
- Microtubes
- Cryotubes
- 250 ml sterile bottles
- 0.2 µm filters
- Counting cell (e.g. Malassez)

Optional:

- Multichannel pipettes (200 µl or 300 µl) and autoclavable reagent reservoirs
- Freezing container
- Microplate reader with 625-655 nm filter

SAFETY CONSIDERATIONS

HEK-Blue™-2 cells require **Biosafety Level 2**.

These cells were derived from HEK293 cells (transformed with adenovirus 5 DNA) that require Biosafety level 2 according to the American Center for Disease Control and Prevention (CDC) guidelines. The biosafety level may vary depending on the country. For example, in Germany HEK293 cell lines are designated Biosafety Level 1 according to the Central Committee of Biological Safety, Zentrale Kommission für die Biologische Sicherheit (ZKBS). Please check with your country's regulatory authority regarding the use of these cells. This cell line is sent with the condition that you are responsible for its safe storage, handling and use. InvivoGen is not liable for damage or injuries resulting from receipt and/or use of an InvivoGen cell culture. For more information see Laboratory Safety: Principles and Practices (Fleming *et al.*, 1995), the ATCC manual on quality control (Hay *et al.*, 1992), the Journal of Tissue Culture Methods (Caputo, 1988), and the U.S. Government Publication, Biosafety in Microbiological and Biomedical Laboratories, 4th ed. HHS publication No. (CDC) 93-8395. U.S. Department of Health and Human Services, Centers for Disease Control and Prevention. Washington DC: U.S. Government Printing Office; 1999. The entire text is available online at https://www.cdc.gov/labs/BMBL.html.

Note: InvivoGen highly recommends that protective gloves and clothing always be used and a full mask always be worn when handling frozen vials.

PREPARATION AND STORAGE OF REAGENTS

All reagents should be prepared under sterile conditions according to good laboratory practices.

Cell culture medium for HEK-Blue™-2 Cells

- **for thawing and recovery of the frozen cell line:**

Growth medium = DMEM high glucose supplemented with 10% FBS, Penicillin-Streptomycin and 100 µg/ml Normocin™. Warm at 37 °C before use and store at 4 °C.

- **for cell culture maintenance:**

Growth medium supplemented with 1X HEK-Blue™ Selection. Warm at 37 °C before use and store at 4 °C.

- **for freezing**

Growth medium supplemented with 10% sterile DMSO. Prepare extemporaneously, no storage.

HEK-Blue™ Detection Medium

1. Pour the contents of one pouch of HEK-Blue™ Detection into a sterile vial/bottle.
2. Solubilize the powder with 50 ml of endotoxin-free water.
3. Swirl gently until powder is completely dissolved.
4. Warm reconstituted HEK-Blue™ Detection to 37 °C for 30 minutes to 1 hour.
5. Filter the medium through a 0.2 µm membrane into a sterile vial/bottle.

Note: We recommend using filter units providing a large filter area to facilitate filtration.

6. Keep the HEK-Blue™ Detection medium at 37 °C before use or store at 2-8°C for up to 2 weeks.

Preparation of Positive and Negative controls (lyophilized powder)

- Solubilize the Positive and Negative controls by adding 1 ml of HEK-Blue™ water (sterile endotoxin-free water) in each tube. 10X solutions of Positive and Negative controls are obtained.
- Mix vigorously by vortexing as the 10X Positive and Negative controls may stick to the tube wall.
- Prepare a 1/10 dilution of 10X Positive and Negative controls with HEK-Blue™ water.

10X and 1X solutions of Positive and Negative controls are stable for 6 months at 4 °C when properly stored.

HANDLING PROCEDURES OF HEK-BLUE™ -2 CELLS

All procedures should be performed under sterile conditions (see "Troubleshooting").

HEK-Blue™-2 cells are shipped on dry ice. Upon receipt the cells must be thawed immediately and grown according to the procedure described below.

Note: Do not freeze the cells upon receipt as it may result in irreversible damage to the cell line.

Thawing of frozen HEK-Blue™-2 cells

1. Thaw the HEK-Blue™-2 cells vial by gentle agitation in a 37 °C water bath. To reduce the possibility of contamination, keep the O-ring and cap out of the water. Thawing should be rapid (~2 minutes).
2. Remove the vial from the water bath as soon as the contents are thawed, and decontaminate by dipping in or spraying with 70% ethanol.

All of the operations from this point should be carried out under strict aseptic conditions.

3. Gently transfer the content of the vial in a sterile tube containing 15 ml of growth medium and spin at 300 x g (RCF) for 5 minutes.
4. Remove the supernatant containing the cryoprotective agent and resuspend the cells with 1 ml of growth medium.
5. Transfer the content of the vial to a 25 cm^2 tissue culture flask containing 5 ml of growth medium.

Note: To avoid excessive alkalinity of the medium during recovery of the cells, place the tissue culture flask containing the growth medium into a CO_2 incubator for at least 15 minutes prior to the addition of the cells.

6. Place the flask at 37 °C in a CO_2 incubator overnight.
7. Follow the growth of the cells by daily observation of the culture with an inverted microscope. When 80-90% confluency is reached, detach the cells in presence of PBS by tapping the flask and grow the cells in growth medium supplemented with 1X HEK-Blue™ Selection.

Note: HEK-Blue™-2 cells functions can be altered by the action of trypsin. Do not use trypsin to detach HEK-Blue™-2 cells .

Cell maintenance

- Maintain and subculture the cells in growth medium supplemented with 1X HEK-Blue™ Selection.
- Renew growth medium twice a week.
- Cells should be passaged when a 70-80% confluency is reached. Do not let the cell grow to 100% confluency.

Note: Do not forget to supplement growth medium with HEK-Blue™ Selection, a solution of selective antibiotics that guarantee the persistent expression of the transgenes introduced in HEK-Blue™-2 Cells.

The HEK-Blue™-2 cell line should not be passaged more than 30 times to remain fully efficient.

Storage of cells

After the recovery of the frozen cells we strongly recommend to expand the HEK-Blue™-2 cells in 175 cm^2 tissue culture flasks containing growth medium supplemented with 1X HEK-Blue™ Selection. These cells can be frozen according to the following procedure to make your own frozen stock.

1. Harvest the cells in presence of PBS by tapping the flask when the culture has reached 80% confluency.
2. Resuspend the cells in growth medium and estimate the cell concentration by using a counting cell.
3. Centrifuge the cells 5 min at 300 x g (RCF).

- Resuspend the cells in growth medium supplemented with 10% sterile DMSO at a concentration of 0.5-1 $x10^7$ cells/ml.

4. Dispense 1 ml of cell suspension per cryotube.
5. Freeze the cells using a freezing container or by placing them successively at -20 °C for 3 hours and at -70 °C overnight.
6. Store the vials in a liquid nitrogen tank.

Note: To ensure a maximal efficiency of the HEK-Blue™-2 cell line, thaw a new tube when the cultured cell line has reached 30 passages.

MYCOPLASMA DETECTION PROCEDURE

The following protocol refers to the use of 96-well plates. Vary your procedure accordingly depending on volumes of reagents needed based on the size of your wells.

Reagents required

- HEK-Blue™ Detection (see preparation page 9)
- 10X Positive and Negative controls (see preparation page 9)
- HEK-Blue™ water (sterile endotoxin free water)
- Phosphate buffered saline (PBS)

Prewarm the reagents at 37 °C before use.

All the steps must be realized under sterile conditions according to good cell culture practices (see "troubleshooting").

Sample preparation

1. Collect 500 µl of supernatant of cell culture to be tested and transfer into a 1.5 ml microtube.
    - **For adherent cell cultures,** scrape the bottom of the petri dish/flask to collect some cells.
    - **For suspension cell cultures**, homogenize the cells before collecting the supernatant.
2. Close tightly the top of the sample-containing microtubes to prevent opening during the subsequent heating step.
3. Boil or heat all the samples in a water bath or in a heating block 15 min at 100 °C.
4. Let the samples cool down at room temperature for a few minutes.

Notes:

- For optimal sensitivity and specificity we strongly recommend to test your cell cultures at 100% confluence grown without antibiotics at least 48 hours before the test.

- Samples can be tested immediately or stored at 4 °C for several weeks before being tested.

- Do not forget to heat each sample 15 min at 100 °C before test. Heating allows the elimination of alkaline phosphatase eventually present in the sample.

PlasmoTest assay

• Deposit of samples and controls in the 96-well plate

5. Mix vigorously each sample by vortexing.
6. Add 50 µl of each sample per well of a flat-bottom 96-well plate.
7. Add 50 µl of 1X Negative control in one well.
8. Add 50 µl of 1X Positive control in one well.

Note: Use new sterile filter tips for each well to avoid cross-contamination.

• Cell suspension preparation

9. Carefully rinse the cell monolayer with PBS (5 ml PBS per 25 cm^2 flask). Remove PBS.
10. Detach cells in presence of PBS (1.5 ml PBS per 25 cm^2 flask) by tapping the flask.
11. Carefully homogenize the cell suspension by gentle pipetting. Avoid the formation of air bubbles.
12. Estimate the cell concentration by using a counting cell.
13. Dilute the cells with pre-warmed HEK-Blue™ detection medium at a concentration 1-3.5 x 10^5 cells/ml (ideally 2.5 x10^5 cells/ml).
14. Mix the cell suspension by gentle pipetting.
15. Transfer the cell suspension into a sterile reagent reservoir if using a multichannel pipette.
16. Add 200 µl (~50,000 cells) of cell suspension to each well containing the samples or controls and in one well without any sample.
17. Incubate the plate at 37 °C in a CO_2 incubator for 16-24 hours.

Notes:

- *We strongly recommend to use a pipette with filter tips to avoid any contamination of the cell suspension.*
- *Before detaching cells, do not forget to rinse cell monolayer with PBS to eliminate all alkaline phosphatases eventually present in the FBS of the growth medium.*

To ensure the best results of the test:

- *Do not use a cell suspension containing more than 3.5 x 10^5 cells/ml as it may result in a loss of sensitivity of the kit.*
- *Use HEK-Blue™ -2 cells that have been passaged less than 30 times.*
- *Use a culture showing 50-80% confluency and that has been passaged at least 48 hours before the test.*
- *All cell cultures showing signs of suffering, characterized by the presence of adherent or floating round cells should not be used for the test. The cells should be flat, adherent and healthy.*
- *Preparation of the cells should be as short as possible to prevent any damage resulting from the prolonged stay at room temperature without 5% CO_2.*

Reading and Interpretation

After 16-24 hours incubation read the plate with the naked eye:

- the positive control should appear purple or blue
- the negative control should be pink or light purple
- the well containing only cell suspension should appear pink or light purple

All samples resulting in a violet or blue color must be considered as positive. The blue color of HEK-Blue™-2 cells is the evidence of microbial contamination of the cell lines tested (Figure 1).

To differentiate easily the mycoplasmal contamination from other microbial contaminations we strongly recommend to test your cell culture grown at 100% confluence without antibiotics for several days (at least 48 hours) before the test:

- If the cell line (grown several days without antibiotics) does not present any visible signs of contamination and if PlasmoTest™ gives a positive result, the cell line tested is contaminated by *Mycoplasma*.
- If the cell line (grown several days without antibiotics) presents visible signs of contamination and if PlasmoTest™ gives a positive result, the test confirms a contamination by cell walled bacteria.

Test results can be validated only if the controls give the expected results. The negative control might appear as a light purple color without altering the interpretation of the test. However, if the negative control results in a deep purple color, the test cannot be validated and should be repeated. For a more precise and semi-quantitative result, reading of the test can be performed with a spectrophotometer set on 620-655 nm.

PlasmoTest™ is designed as a routine method for screening cell cultures for the presence of mycoplasmas and other cell walled bacteria. When the procedure is accurately followed, PlasmoTest™ is highly sensitive for the detection of mycoplasma infection in cell cultures. As it is intended as a presumptive screening tool, any positive results should be confirmed by repeating the test or by using another detection method.

Column 1: Positive and negative controls
Column 2: Uninfected cell line supernatants and *M. hyorhinis* infected 293PR
Column 3: Uninfected cell line supernatants
Column 4: Dilutions of 293PR supernatant infected with *M. hyorhinis*

Figure 1: Vizualisation of Mycoplasma contamination using PlasmoTest™.

SENSITIVITY AND SPECIFICITY

Sensitivity

To determine the sensitivity of PlasmoTest™, the five *Mycoplasma* species most frequently isolated were grown in pure culture, serially diluted in cell culture medium, numbered and tested after heating for 15 min at 100 °C with HEK-Blue™-2 cells and HEK-Blue™ detection medium.
PlasmoTest™ is highly sensitive and the detection limit depends on the species of mycoplasmas: 5.10^2-5.10^5 CFU/ml as shown below. (CFU= Colony Forming Units)

Mycoplasma species	Sensitivity (CFU/ml)
Acholeplasma laidlawii	2.5 x 10^3
Mycoplasma arginini	2 x 10^3
Mycoplasma fermentans	5 x 10^2
Mycoplasma hyorhinis	7 x 10^2
Mycoplasma orale	5 x 10^5

Specificity

PlasmoTest™ detects all *Mycoplasma* and *Acholeplasma* species known to infect cell cultures.
PlasmoTest™ cannot be used for mycoplasma species identification.
PlasmoTest™ can detect other cell culture contaminants such as bacteria (*E. coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus)*.

To differentiate easily the mycoplasmal contamination from other microbial contaminations we strongly recommend to test your cell culture grown at 100% confluence without antibiotics for several days (at least 48 hours) before the test.
If the cell culture (grown several days without antibiotics) does not present perceptible changes (e.g turbidity, pH), and if PlasmoTest™ gives a positive result, the cell line tested is contaminated by *Mycoplasma*.

TECHNICAL HINTS

Maintain optimal functions of HEK-Blue™-2 cells

- Do not forget to supplement growth medium with HEK-Blue™- Selection, a solution of selective antibiotics that guarantee the persistent expression of the transgenes introduced in HEK-Blue™-2 cells.
- Do not use trypsin to detach HEK-Blue™-2 cells: trypsin may alter HEK-Blue™-2 cells. Detach HEK-Blue™-2 cells in presence of PBS by tapping the flask.
- Do not use HEK-Blue™-2 cells that have been passaged more than 30 times to remain fully efficient. Thaw a new tube of your frozen stock.

Avoid false positive results

PlasmoTest™ is based on the detection of alkaline phosphatase secreted by HEK-Blue™-2 cells after recognition of various microbial molecules by TLR2 expressed on their surface.
Therefore false positive results can be due to:

1. Microbial contamination during the assay
2. Phosphatases initially present in samples or growth medium of HEK-Blue™-2 cells
3. Incorrect manipulation of the HEK-Blue™-2 cells

1. Avoid microbial contamination during reagent and sample preparation and while running the assay:

Non-sterile conditions will give you false positives as HEK-Blue™-2 cells are very sensitive.
- The use of gloves and a labcoat is obligatory.
- Do not forget to add Normocin™ to the growth medium to prevent HEK-Blue™-2 cells from mycoplasmal, bacterial and fungal contaminations.
- Use only sterile reagents (such as PBS) that have been cell culture tested.
- We recommend to use ultrapure water (commercialized as sterile endotoxin free water) to prepare HEK-Blue™ Detection medium. Nevertheless if you want to use your own lab water to prepare HEK-Blue™ detection medium, verify first that your water does not activate HEK-Blue™-2 cells: you can test your water as sample without heating it 15 min at 100 °C.
- Do not use trypsin to detach HEK-Blue™-2 cells as trypsin may be a potential source of mycoplasmal contamination.

- If using a water bath to heat samples, wipe the vials and decontaminate by spraying with 70% ethanol the outer wall of the vials before opening them.
- Use only sterile materials such as sterile filter tips for each addition of samples, controls or HEK-Blue™-2 cells suspension to avoid cross-contamination.
- Use only a sterile reagent reservoir if you use a multichannel pipette (with sterile filter tips) to add HEK-Blue™-2 cells suspension.

2. Eliminate all external sources of phosphatases (no secreted by HEK-Blue™-2 cells)

- Do not forget to heat each sample 15 min at 100 °C before test. Heating avoids false positive by eliminating phosphatases eventually present in the sample.
- Rinse HEK-Blue™-2 cells with PBS very well to eliminate all the phosphatases potentially present in the growth medium of these detection cells.

3. Avoid false manipulation of HEK-Blue™-2 cells

- Do not use for the test HEK-Blue™-2 cell cultures showing signs of suffering, characterized by the presence of adherent or floating round cells. The cells should be flat, adherent and healthy.
- Use HEK-Blue™-2 cell culture showing 50-80% confluency and that has been passaged at least 48 hours before the test.
- Preparation of the HEK-Blue™-2 cell suspension should be as short as possible to prevent any damage resulting from the prolonged stay at room temperature without 5% CO_2.
- Do not use a HEK-Blue™-2 cell suspension containing more than 3.5 x 10^5 cells/ml.

Optimize the sensitivity or specificity of PlasmoTest™

- Before testing any cell cultures for mycoplasma contamination, let them grow at 100% confluence without antibiotics several days (at least 48-72 hours). The contamination will be then higher and easier to detect. Moreover it may help you to distinct mycoplasmal contamination from other bacterial contaminations as these other bacterial contaminations induce visible change in the cell culture without antibiotics contrary to mycoplasmal contamination.
- When you collect your samples, do not forget to scrape the bottom of the petri dish/flask to collect some cells.

Mycoplasmas stick on cell surface and some species can penetrate in the cell.

- More than 50 µl of sample can be tested if 48 well-plates, 24 well-plates or 12 well-plates are used. Vary your procedure accordingly depending on volumes of reagents needed based on the size of your wells.

TROUBLESHOOTING GUIDE

Problem	Reason	Solution
All the wells (including the negative controls) become blue in HEK-Blue™ Detection medium	• HEK-Blue™ -2 cells used for the test have been under stress before or during the test.	• Repeat the test following strictly the instructions. • Start a new culture from the frozen stock if cells have been subcultured more than 30 times. • Use healthy cells that have been passaged at least 48 hours before the test. • Use cells at 60-80% confluence. • Prepare the cell suspension as fast as possible to limit the period at 20-25 °C without 5% of CO_2 and avoid excessive pipetting. • Do not use an excessive number of cells per well: no more than 70,000 cells per well of a 96-well plate. If the problem persists, explore the other reasons.
	• HEK-Blue™-2 cells have been insufficiently rinsed with PBS and phosphatases present in the growth medium have been detected.	• Test the presence of a phosphatase activity in your growth medium by adding 50 µl of your medium to 200 µl of HEK-Blue™ Detection medium. If a blue color appears after 18-24 hours at 37°C your growth medium contains a phosphatase activity. Then repeat the test after rinsing very well the cells with PBS to eliminate all the phosphatases present in the growth medium. If the problem persists, explore the other reasons.

Problem	Reason	Solution
All the wells (**including the negative controls**) become blue in HEK-Blue™ Detection medium.	• HEK-Blue™-2 cells have secreted alkaline phosphatase consecutively to a stimulation due to a microbial contamination that occurred before or during the assay.	• Start a new culture from the frozen stock following strict aseptic conditions. Add Normocin™ to the growth medium to prevent microbial contamination. Do not use trypsin a potential source of mycoplasma. If the problem persists, contamination of HEK-Blue™-2 cells occurs during the assay. • Test for the presence of secreted phosphatase at the different steps of the assay to determine when the cells were contaminated and secrete alkaline phosphatase. Repeat the assay and test a sample of HEK-Blue™-2 cells suspended in sterile PBS by adding 50 µl of your sample to 200 µl of HEK-Blue™ detection medium (do not heat your sample). Then incubate for 18h-24 hours at 37 °C. Take 250 µl of HEK-Blue™-2 cells suspended in HEK-blue™ detection medium just before adding cells to the 96-well plate and incubate them 18-24 hours at 37°C. A sample in which a blue color appears after 18-24 hours at 37°C contains alkaline phosphatase. Verify that all the materials used during this step are sterile and that good cell culture practices are followed (see technical hints page 16-17).
	• HEK-Blue™ 2 cells have been activated with the growth medium (some FBS although sterile may contain microbial debris).	• Use another FBS.

Problem	Reason	Solution
The negative control gives a blue color.	• The negative control has been contaminated.	• Use a new negative control and use only sterile endotoxin-free water to prepare it.
The positive control does not give a blue color.	• The cell line is not HEK-Blue™-2 cells. • The detection medium is not HEK-Blue™ Detection. • Positive control adheres to the inner surface of the tube. • Component omitted or error in the preparation of the positive control.	• Start a new culture from the frozen stock. • Use a new pouch of HEK-Blue™ Detection medium. • Vortex extensively the positive control before use. • Read protocol thoroughly before repeating the assay.
False positives	• The sample has been contaminated during the assay. • Presence of a phosphatase activity in the sample. • The sample contains an NF-κB inducer.	• Avoid cross contamination by using pipette with sterile filter tips; change filter tips for each sample. • If using a water bath to heat samples, wipe the vials and decontaminate with spraying with 70% ethanol the outer wall of the vials before opening them. • Verify you do not forget to heat sample for 15 min at 100 °C. • Test for the presence of phosphatase activity in your sample by adding 50 µl of sample to 200 µl of HEK-Blue™ Detection medium. If a blue color appears after 18-24 hours at 37°C, your sample contains a phosphatase activity. Re-heat your sample to eliminate the phosphatase. • The sample cannot be tested with PlasmoTest™.

Problem	Reason	Solution
False negatives	• Cell cultures have been grown at low confluence in presence of antibiotics before the test.	• Let your cell cultures grow to 100% confluence without antibiotics several days (at least 48-72 hours) before testing them for mycoplasma contamination.
	• The level of cell contamination is lower than the response threshold.	• Repeat test after a further 48-72 hours culture without antibiotics.

InvivoGen guarantees 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. InvivoGen shall not be liable for any damage or injury to persons or property arising from the purchase or use of the product.

USE RESTRICTIONS

PlasmoTest™ is distributed for research purposes only.

Third party distribution of the HEK-Blue™-2 cell line is discouraged, since this practice has resulted in the unintentional spreading of cell lines contaminated with inappropriate animal cells or microorganisms.

Information for European customers: HEK-Blue™-2 cells are genetically modified cells. As a condition of sale, use of this product must be in accordance with all applicable local legislation and guidelines including EC directive 90/219/EEC on the contained use of genetically modified organisms.

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