PlasmoTest™

Colorimetric HEK-Blue™ *Mycoplasma* contamination test

Catalog code: rep-pt1

https://www.invivogen.com/plasmotest-mycoplasma-detection

A cell-based assay for the detection of *mycoplasma* in cell cultures

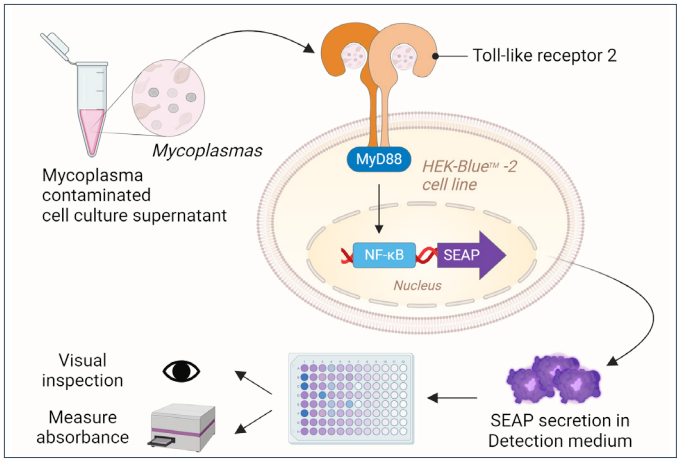
For research use only

Version 24J31-AK

INTRODUCTION

InvivoGen introduces **PlasmoTest™**, a simple, rapid, and reliable assay to recognize *mycoplasmas* and other cell-walled bacteria in cell cultures. *Mycoplasma* contamination of cultured cells is a major problem in scientific research. It cannot be detected by visual inspection and may remain totally undetected for long periods, thus affecting virtually any function of eukaryotic cells and leading to unreliable experimental results and potentially unsafe biological products.

PlasmoTest™ is the first cellular assay, designed as a routine method for the visual, colorimetric detection of *mycoplasma* contamination in cell cultures. It is based on the activation of the Toll-like receptor 2 (TLR2), the preferential pattern recognition receptor for *mycoplasma* lipoproteins. Our proprietary HEK-Blue™-2 cells stably express human TLR2 and an NF-κB-inducible SEAP (secreted embryonic alkaline phosphatase) reporter gene. They are cultured in HEK-Blue™ Detection medium, making them ideal for routine tests. The simple addition of test samples to these cells provides colorimetric results with sensitivity similar to luminescence-based biochemical assays. SEAP activity can also be measured using a spectrophotometer for a more precise and semi-quantitative result.



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

FOR RESEARCH USE ONLY

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Diagrams in this protocol are created with BioRender.com.

WHAT'S IN THE BOX

ITEMS	QUANTITY	CONCENTRATION	STORAGE UPON ARRIVAL
HEK-Blue™-2 cells	1 ml	3 - 7 x 10^6 cells/ml	Start cell propagation direct *(see page 5)*
HEK-Blue™ Selection	2 x 1 ml	250X	- 20°C up to 12 months
Normocin™	1 ml	50 mg/ml (500X)	- 20°C up to 12 months
HEK-Blue™ Detection	1 pouch	-	4°C up to 6 months
Positive and negative control	lyophilized	100X	4°C up to 12 months
HEK-Blue™ water (endotoxin-free)	2 x 50 ml	-	RT up to 6 months

HEK-Blue™-2 Cells: Engineered HEK293-derived *mycoplasma* sensor cells. These cells stably express TLR2 and multiple genes from the TLR2 pathway. Additionally, they coexpress an NF-κB-inducible secreted embryonic alkaline phosphatase (SEAP) reporter gene.

HEK-Blue™ Selection: a solution containing the required selection antibiotics. These antibiotics guarantee the persistent expression of the various transgenes introduced in HEK-Blue™-2 cells.

Normocin™: an anti-microbial reagent to protect HEK-Blue™-2 cells from any potential contamination, whether caused by *mycoplasma*, bacteria or fungi.

HEK-Blue™ Detection: a cell culture medium specifically designed for the detection of SEAP. In the presence of *mycoplasma*-contaminated samples, HEK-Blue™-2 cells secrete SEAP 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).

Positive and negative controls: a 100X lyophilized preparation of positive and negative controls. When used as 1X solution, it allows to perform 200 tests. Please note, that the positive control is not a living *mycoplasma*.

Note: This kit allows to test up to 250 samples. Components can be purchased separately (see "Related Products" page 9).

ATTENTION!!

HANDLING FROZEN CELLS UPON ARRIVAL

Cells are shipped in dry ice, and upon receipt should immediately be thawed for culture or stored below -130°C, preferably in liquid nitrogen vapor, for long-term storage.
IMPORTANT: Do not store cell vials at -80°C as this will decrease cell viability and performance. Contact technical support if the cells are not frozen or in dry ice upon arrival. To insure the highest level of viability and best assay performance, we strongly recommend that you thaw the cells and initiate the culture as soon as possible upon receipt.

Additional materials required:

REAGENTS
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) **without microbial debris**
Trypsin-EDTA (0.05% Trypsin, EDTA.4Na)
Phosphate buffered saline (PBS) without Mg^{2+} or Ca^{2+}
Dimethylsulfoxide (DMSO)

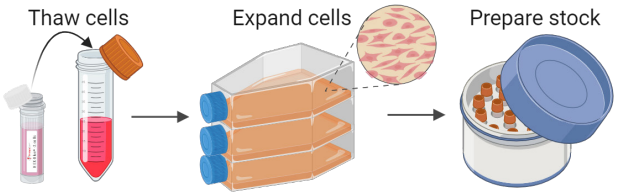
SUPPLIES
Laminar flow hood, CO_2 incubator
Boiling water bath or heating block (100°C)
Water bath (37°C), centrifuge
Inverted microscope
Sterile cell culture plasticware (e.g. 96-well plates, tips, etc.)
Counting cell (e.g. Malassez)
Multichannel pipettes (200/300 µl) and reagent reservoirs

ASSAY OUTLINES

Cell Handling Procedures *(p.5)*

Cell handling upon arrival

1. Thaw and expand HEK-Blue™-2 cells.
2. Make your frozen stock of HEK-Blue™-2 cells.

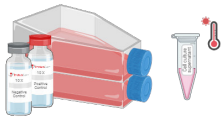


Assay Workflow - *Mycoplasma* Detection *(p.6 - 7)*

Step 1 - Preparation *(day 1)*

1. Prepare all needed reagents, controls, and cell suspension.
2. Collect and heat cell culture supernatant(s) that needs to be checked.

Step 1 - Preparation

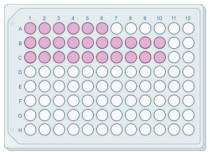


Prepare
- Positive and Negative control
- Heated sample(s)
- HEK-Blue™-2 cell suspension

Step 2- Procedure *(day 1)*

3. Add HEK-Blue™-2 cells to the heated test sample.
4. Culture overnight in HEK-Blue™ Detection medium.

Step 2 - Test procedure

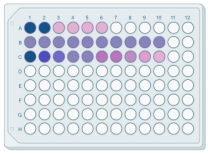


Start test
- Combine samples with HEK-Blue™-2 cells
- Incubate overnight

Step 3 - Interpretation & reading *(day 2)*

5. Inspect with the naked eye:
   → purple/blue = positive
   → pink = negative
6. *Optional*: Read the absorbance detection plate at 620-655 nm.

Step 3 - Detection



Naked eye Blue, violet = positive
Pink = negative

Read absorbance

SAFETY CONSIDERATION

HEK-Blue™-2 cells were derived from HEK293 cells (transformed with adenovirus 5 DNA) that require Biosafety level 2 according to the American Centers 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/safelabs/resources-tools/biosafety-resources-and-tools.html.

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

CELL LINE WARRANTIES

- InvivoGen's cells are provided 'AS IS' and their viability is guaranteed upon shipment from our facilities for a period of 30 days, provided that the customer has properly stored and handled the product.
- Our cell lines are guaranteed free of *mycoplasma* contamination.
- The stability of our cell lines is guaranteed for 20 passages.

PREPARATION OF REAGENTS

All reagents should be prepared under sterile conditions according to good laboratory practices. All materials coming in contact with the samples or test reagents must be free of microbial contamination. These materials should be tested before use. Bacteria are lurking everywhere. Special caution should be applied to the following potential sources:

WATER
Use high-purity water to prepare media and solutions, and clean glassware.

GLASSWARE
To destroy microbes, heat glassware to 250°C for≥30 min, or 180°C for 3 hrs.

PLASTICWARE
Ask for manufacturer certification of microbial levels, or test before use.

MEDIA, SERA & ADDITIVES
Ask for manufacturer certification of microbial levels, or test before use.

Cell culture medium for HEK-Blue™-2 Cells

• **Growth Medium** (for thawing and recovery of the frozen cell line)**:**
DMEM, 4.5 g/l glucose, 2 mM L-glutamine, 10% (v/v) heat-inactivated fetal bovine serum (FBS; 30 min at 56 °C), Pen-Strep (100 U/ml-100 µg/ml), 100 µg/ml Normocin™. Do not add selection antibiotics. Warm at 37°C before use and store at 2-8°C.
• **Required Selective Antibiotic(s):** HEK-Blue™ Selection
• **Freezing Medium:** Growth medium supplemented with 10% (v/v) DMSO. Prepare extemporaneously, no storage. You may also use commercial available serum-free freezing media.

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 HEK-Blue™ Water.
3. Vortex vigorously until powder is completely dissolved.
4. Warm reconstituted HEK-Blue™ Detection to 37 °C for at least 3 hours.
5. Filter the medium through a 0.2 µm bottle-top vacuum filter into a sterile vial/bottle.
6. Keep the HEK-Blue™ Detection medium at 37 °C before use. Store at 2-8°C for up to 2 weeks or 2 months at -20 °C. Protect from light.

Preparation of positive and negative controls (lyophilized powder)

1. Solubilize the positive and negative controls by adding 1 ml of HEK-Blue™ water (sterile endotoxin-free water) in each tube.
2. A 10X solutions of the controls are obtained.
3. Mix vigorously by vortexing as the 10X positive and negative controls may stick to the tube wall.
4. Prepare a 1/10 dilution of 10X positive and negative controls with HEK-Blue™ water.
   Solutions of the positive control are stable for 1 month at 4 °C and for 6 months at -20 °C.
   Solutions of the negative control are stable for 6 months at 4 °C when stored properly.

HANDLING PROCEDURES OF HEK-BLUE™-2 CELLS

The first propagation of cells should be for generating stocks for future use. This ensures the stability and performance of the cells for subsequent experiments. During the handling process, please note the following points:

CELL RECOVERY
Do not add selective antibiotics until the cells have been passaged twice.

CELL PASSAGE
The cells should be passaged when 60 - 80 % confluency is reached.

CELL PERFORMANCE
Thaw a new vial when cultured cells have reached 20 passages.

CELL STORAGE
Do not store cells at -80°C. Transfer cells in liquid nitrogen for long term storage.

Thawing

1. Thaw the vial in a 37°C water bath. Keep the O-ring and cap out of the water. Thawing should be rapid (~ 2 min).
2. Remove the vial from the water bath as soon as the tube content is thawed, and decontaminate by dipping in or spraying with 70% (v/v) ethanol.
3. Transfer the vial content in a sterile tube containing 20 ml of pre-warmed growth medium and spin at 300 x g (RCF) for 5 min.
4. Remove the supernatant and resuspend the cells with 5 ml of growth medium.
5. Transfer to a 25 cm^2 tissue culture flask or a 75 cm^2 tissue culture flask containing 15 ml of pre-warmed growth medium.
   Do not add selective antibiotics until the cells have been passaged twice.
6. Place the flask at 37°C in a CO_2 incubator overnight.
7. Observe the culture daily with an inverted microscope. When 60 - 80% confluency is reached, continue with cell maintenance.

Cell maintenance

HEK-Blue™-2 cells grow as adherent cells in a monolayer.

1. Detach the cells in the presence of pre-warmed 1X PBS at 37°C or room temperature (RT) by tapping the flask.
   Note: The response of HEK-Blue™-2 cells can be altered by the prolonged action of trypsin. Do not use trypsin to detach these cells.
2. Maintain and subculture the cells in growth medium supplemented with 1X HEK-Blue™ Selection.
3. Renew selection medium 2 to 3 times a week.
4. **Cells should be passaged when a 60-80% confluency is reached.** Do not let them grow to 100% confluency as it might affect cell performance.

Frozen stock preparation

Use cells at 80% confluency to prepare frozen stocks.

1. Detach the cells in the presence of pre-warmed 1X PBS at 37°C or RT.
2. Wash cells in growth medium by centrifuging at 300 x g (RCF) for 5 min.
3. Resuspend cells at 5 - 8 x 10^6 cells/ml in freezing medium.
   Note: A T-75 culture flask typically yields for 3-4 frozen vials.
4. Dispense 1 ml of cell suspension per cryotube.
5. Place vials in a freezing container and store at -80 °C overnight.
6. **Transfer vials to liquid nitrogen for long term storage.**
   *Note: If properly stored, cells should remain stable for years. To ensure a maximal efficiency of the HEK-Blue™-2 cell line, thaw a new tube when the cultured cell line has reached **20 passages.***

ASSAY WORKFLOW - MYCOPLASMA DETECTION PROCEDURE

This protocol details the procedure to be performed in 96-well plates. Vary your procedure accordingly depending on volumes of reagents needed based on the size of your wells. Prepare all reagents as described on page 4: HEK-Blue™-2 cell culture medium, 1X HEK-Blue™Detection medium, and 1X positive and negative controls. Warm the samples and all reagents at 37°C before use.

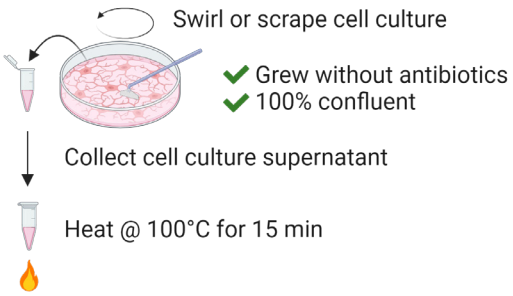
Step 1: Preparation of samples - Day 1

REAGENTS	SUPPLIES
HEK-Blue™-2 cells (60 - 80% confluent; passage < 20)	Pre-warmed PBS, 1X HEK-Blue™ Detection medium
Sample(s) of interest (~500 µl)	96-well flat bottom plate
10X positive and negative controls *(preparation see page 4)*	Cell culture plasticware (pipette tips, 1.5 ml tubes, etc.)
HEK-Blue™ water (endotoxin-free)	Lab equipment (vortex machine, heater, etc.)

Preparation of sample(s)

1. Collect 500 µl of supernatant of the cell culture to be tested and transfer into a 1.5 ml microtube.
   - **Adherent cells:** scrape the bottom of the petri dish/flask to collect some cells.
   - **Suspension cells:** homogenize cells before collecting the supernatant.

   Note: 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.
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 for 15 min at 100 °C.



Do not forget this heating step as it allows the elimination of alkaline phosphatase potentially present in the sample.

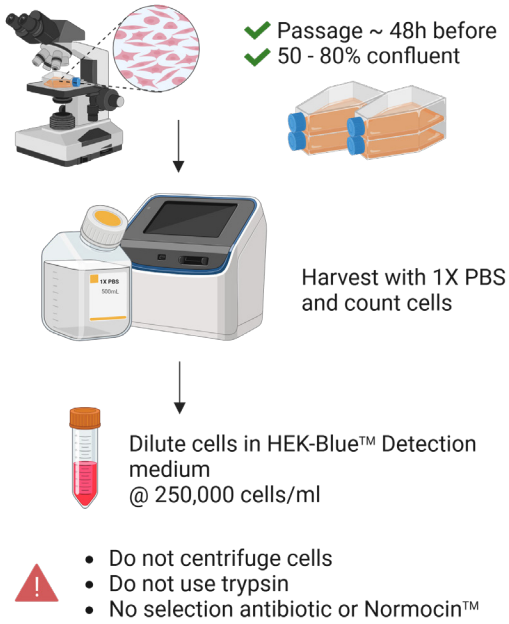
Let the samples cool down at room temperature for a few minutes. Samples can be tested immediately or stored at 4 °C for several weeks before being tested.

HEK-Blue™-2 cell suspension preparation

To ensure the best results of the test:

- Preparation of the cells should be as short as possible to prevent any damage resulting from the prolonged stay at RT without 5% CO_2
- Prepare all reagents, plate setup and samples as described in Step 2 before preparing cell suspension.
- Use HEK-Blue™ -2 cells that have been **passaged less than 20 times.**
- 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 assay.
- Use a culture showing 50-80% confluency and that has been passaged at least 48 h before the test.

4. Prepare a suspension of HEK-Blue™-2 cells by gently rinsing the cells once with pre-warmed 1X PBS. Detach the cells from the flask in the presence of 1X PBS. *Note: Do not use trypsin to detach the cells. Do not centrifuge cells.*
5. Estimate the cell concentration using a counting cell. Avoid air bubbles.
6. Dilute the cells with pre-warmed HEK-Blue™ Detection medium at 250,000 cells/ml.
7. Transfer cell suspension into a sterile reagent reservoir if using a multichannel pipette and continue with Step 2.



Step 2: Test procedure - Day 1

PlasmoTest™ assay

1. Mix vigorously each test samples by vortexing.
2. Add 50 µl of each sample per well of a **flat-bottom 96-well plate**.
3. Add 50 µl of 1X negative control in one well.
4. Add 50 µl of 1X positive control in one well.
   Note: Use new sterile filter tips for each well to avoid cross-contamination.

5. Prepare the HEK-Blue™ -2 cell suspension as described above.
6. Add 200 µl/well (max. 50,000 cells/well) of the HEK-Blue™-2 cell suspension to each well containing the samples or controls and in two wells without any sample (=blank).
7. Incubate the plate at 37°C in a CO_2 incubator for 18-24 hours.

Step 3: Interpretation and reading - Day 2

Presence of *mycoplasma* in a sample can be observed with the naked eye. For a more precise and semi-quantitative result, the concentration can be calculated by measuring the absorbance using a spectrophotometer @ 620 - 655 nm.

Naked eye interpretation

The test was successful if after 16 - 24 hours incubation:

- the positive control displays a blue/purple/pink color.
- the negative control should be pink or light purple.
- the blank well containing only HEK-Blue™ -2 cell remains pink or light purple. The blank might appear as a light purple color without altering the interpretation of the test. However, if the blank results in a deep purple color, the test cannot be validated and should be repeated.

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, as shown below:

Row 1: Dilutions of 293PR supernatant infected with *M. hyorhinis*

Row 2: Uninfected cell line supernatants

Row 3: Uninfected cell line supernatants and *M. hyorhinis* infected 293PR

Row 4: Positive, negative controls, and «cells only» blank sample

Vizualisation of a typical PlasmoTest™ plate.

To easily differentiate the *mycoplasma* 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 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 presents visible signs of contamination and if PlasmoTest™ gives a positive result, the test confirms a contamination by cell walled bacteria.

WHAT IF MY TEST IS POSITIVE ?

Your culture is easily treatable with InvivoGen's anti-*mycoplasma* reagents.

Treat your culture and eradicate the contamination using Plasmocin™ or Plasmocure™. Upon completion of the treatment (~2 weeks), re-test using PlasmoTest™ comparing your newly treated culture with your previous sample .

TECHNICAL HINTS

Maintain optimal functions of HEK-Blue™-2 cells

- Supplement growth medium with HEK-Blue™- Selection to guarantee the persistent expression of the transgenes in HEK-Blue™-2 cells.
- Do not use trypsin to detach 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 20 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. 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. How to avoid microbial contamination during 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.
- Add Normocin™ to the growth medium to prevent HEK-Blue™-2 cells from *mycoplasma*, 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 *mycoplasma* 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.
- 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. How to eliminate all external sources of phosphatases

- Heat each sample 15 min at 100 °C before test. Heating avoids false positive by eliminating phosphatases present in the sample.
- Rinse HEK-Blue™-2 cells with PBS very well to eliminate all the phosphatases potentially present in the growth medium.

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 distinguish *mycoplasma* contamination from other bacterial contaminations as these other bacterial contaminations induce visible changes in the cell culture without antibiotics, in contrary to *mycoplasma* 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.

Sensitivity

To determine the sensitivity of PlasmoTest™, the *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 *mycoplasma* species: 5 x 10^2 - 5 x 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	2.0 x 10^3
Mycoplasma fermentans	5.0 x 10^2
Mycoplasma hominis	6.0 x 10^3
Mycoplasma hyorhinis	7.0 x 10^2
Mycoplasma orale	5.0 x 10^5
Mycoplasma salivarium	1.0 x 10^4

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 (*Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*).

RELATED PRODUCTS

Product	Description	Quantity	Cat. Code
HEK-Blue™ Selection	Selective antibiotic	10 x 1 ml	hb-sel
HEK-Blue™ Detection	SEAP detection medium	5 pouches	hb-det2
PlasmoTest™ Controls	Controls for the PlasmoTest™	200 tests	pt-ctr2
PlasmoTest™ Reagent Kit	Reagents for the PlasmoTest™	500 tests	rep-ptrk
Plasmocin™ treatment	*Mycoplasma* elimination reagent	25 mg (1 ml)	ant-mpt-1
Plasmocure™	*Mycoplasma* elimination reagent	100 mg (1 ml)	ant-pc

USE RESTRICTIONS

HEK-Blue™ -2 cells are distributed for research purposes only.

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

These products are covered by a Limited Use License. By use of these products you accept the terms and conditions of all applicable Limited Use Label Licenses.

Limited Use License

The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for-profit entity). The buyer cannot sell or otherwise transfer (a) this product (b) its components or (c) materials made using this product or its components to a third party or otherwise use this product or its components or materials made using this product or its components for Commercial Purposes.

The buyer may transfer information or materials made through the use of this product to a scientific collaborator, provided that such transfer is not for any Commercial Purpose, and that such collaborator agrees in writing (a) not to transfer such materials to any third party, and (b) to use such transferred materials and/or information solely for research and not for Commercial Purposes.

Commercial Purposes means any activity by a party for consideration and may include, but is not limited to: (1) use of the product or its components in manufacturing; (2) use of the product or its components to provide a service, information, or data; (3) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; or (4) resale of the product or its components, whether or not such product or its components are resold for use in research. If the purchaser is not willing to accept the limitations of this limited use statement, InvivoGen is willing to accept return of the product with a full refund. For information on purchasing a license to this product for purposes other than research, contact InvivoGen, 10515 Vista Sorrento Parkway, San Diego California 92121. Tel: 858-457-5873. Fax: 858-457-5843.

TROUBLESHOOTING GUIDE

PROBLEM	REASON	SOLUTION
All wells of the detection plate are blue (**including** the negative control)	• Cell culture was over confluent • Cells have been subcultured more than 30 times • Cells used for the test have been under stress before the test • Cells have been under stress during the test (e.g. long time at 20-25°C without 5% of CO_2, cold PBS, centrifugation) • Excessive number of cells per well in the test plate • Cell culture was contaminated before or during the assay • Cell culture medium contains *mycoplasma* (FBS or trypsin)	• Use cells at 60-80% confluence • Start a new culture from the frozen stock • Use healthy cells that have been passaged at least 48 h before the test • Prepare the cell suspension as fast as possible using warm reagents and avoid excessive pipetting and centrifugation • Do not use more than 50,000 cells per well of a 96-well plate • Start a new culture from the frozen stock and use Normocin™ • Use another FBS • Do not use trypsin to detach the cells
The negative control gives a blue color	• See above • The negative control has been contaminated	• See above • Start a new culture from the frozen stock
The positive control does not give a blue color (stays pink)	• Positive control adhere to the inner tube surface • HEK-Blue™ Detection was stored for more than 2 weeks at 2-8°C • HEK-Blue™-2 cells are not in a healthy state • Cells are not HEK-Blue™-2 cells	• Vortex extensively before use • Use a new pouch to prepare HEK-Blue™ Detection • Start a new culture from the frozen stock • Start a new culture from the frozen stock
False negatives	• Cell cultures have been grown at low confluence in presence of antibiotics before the test • The level of cell contamination is lower than the response threshold	• Let your cell cultures grow to 100% confluence without antibiotics several days (at least 48-72 hours) before testing them. • Repeat test after a further 48-72 hours culture without antibiotics
False positives	• Sample has been contaminated during the assay • Presence of a phosphatase activity in the sample • Sample contains an NF-κB inducer	• Avoid cross contamination by using pipette with sterile filter tips; change filter tips for each sample • Use 70% ethanol • Verify whether the sample was heated for 15 min at 100 °C • Test the presence of a phosphatase activity in your sample by adding 20 µl of your sample to 180 µl of HEK-Blue™ Detection. If a purple/blue color appears after 18 - 24h at 37°C your sample contains a phosphatase activity and cannot be tested using PlasmoTest™ • This sample cannot be tested with PlasmoTest™

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