HEK-Blue™ LPS Detection Kit 2

Colorimetric cell-based assay

For the detection of biologically active endotoxin

Catalog code: rep-lps2
https://www.invivogen.com/hek-blue-lps-detection-kit

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

FOR RESEARCH USE ONLY
Version 23K16-MM

Product covered by a Limited Use License

InvivoGen
www.invivogen.com

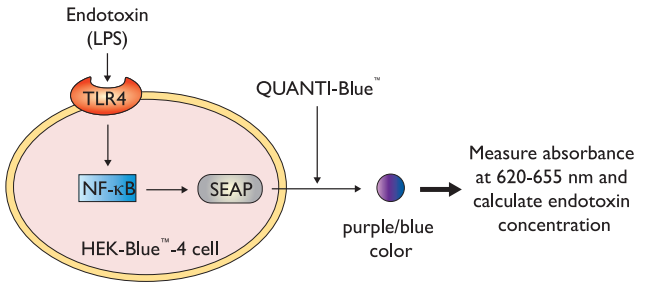
TABLE OF CONTENTS

INTRODUCTION

Lipopolysaccharide (LPS), also known as endotoxin, is the major cell wall component of Gram-negative bacteria. LPS is a potent stimulator of the vertebrate innate immune system and can cause fever, septic shock and eventually death. *In vitro*, it can introduce a bias in experiments involving cells sensitive to LPS. Thus, monitoring the presence of LPS in biological reagents is crucial. Current methods for the detection of endotoxins rely on the Limulus Amebocyte Lysate (LAL), an extract of blood cells from an horseshoe crab, that reacts with endotoxin. A major drawback of the LAL test is overcoming assay inhibition. InvivoGen introduces the HEK-Blue™ LPS Detection Kit 2, a simple, rapid and reliable assay to detect the presence of endotoxin in virtually all biological samples, including particulate compounds, such as vaccine adjuvants, and inhibitors of the LAL test. The HEK-Blue™ LPS Detection Kit 2 is a cell-based colorimetric assay for the detection of biologically active endotoxin that offers a sustainable alternative to the LAL test.

KIT DESCRIPTION

The HEK-Blue™ LPS Detection Kit 2 is a new assay intended for the detection and quantification of biologically active LPS for research purposes. It is based on the activation of Toll-like receptor 4 (TLR4), the mammalian endotoxin sensor (Beutler B., 2002). TLR4 recognizes structurally different LPS from gram-negative bacteria and in particular lipid A, their toxic moiety. Proprietary cells engineered to become extremely sensitive to LPS, called HEK-Blue™-4 cells, are the main feature of this endotoxin detection kit. The presence of minute quantities of LPS, starting as low as 0.01 EU/ml, are detected by the HEK-Blue™-4 cells leading to the activation of NF-κB. Using QUANTI-Blue™, a specific detection medium, NF-κB activation can be observed with the naked eye or measured at 620-655 nm. Since the absorbance is in direct proportion to the amount of endotoxin present, the concentration of endotoxin can be calculated from a standard curve (see page 8).



Beutler B. 2002. TLR4 as the mammalian endotoxin sensor. Curr Top Microbiol Immunol. 270:109-20.

HEK-Blue™-4 Cells

HEK-Blue™-4 cells, the endotoxin sensor cells, are engineered HEK293 cells. These cells stably express TLR4 and multiple genes from the TLR4 pathway. Additionally, they coexpress an NF-κB-inducible secreted embryonic alkaline phosphatase (SEAP) reporter gene.

HEK-Blue™ Endotoxin Standard

HEK-Blue™ Endotoxin Standard is a preparation of *E. coli* 055:B5 LPS standardized against FDA approved control standard endotoxin (CSE).

QUANTI-Blue™

QUANTI-Blue™ Solution is a reagent specifically designed for the detection of SEAP. It contains a color substrate that produces a purple/blue color following its hydrolysis by SEAP.
QUANTI-Blue™ is provided as 1 ml of QB reagent and 1 ml of QB buffer which is sufficient to prepare 100 ml of QUANTI-Blue™ Solution, a SEAP detection reagent.

HEK-Blue™ Selection & Normocin™

HEK-Blue™ Selection is a solution containing the required selection antibiotics. These antibiotics guarantee the persistent expression of the various transgenes introduced in HEK-Blue™-4 cells. Further, Normocin™ is included in the kit to protect HEK-Blue™-4 cells from any potential microbial contamination, whether caused by mycoplasma, bacteria or fungi.

KIT INFORMATION

Contents

The HEK-Blue™ LPS Detection Kit2 contains the following:

- 1 vial of HEK-Blue™-4 cells (3-7x 10^6 cells)
- 8 tubes of 250X HEK-Blue™ Selection (1 ml each)
- 4 tubes of 500X Normocin™ (1 ml each)
- 1 ml of QB reagent and 1 ml of QB buffer
- 2 tubes of HEK-Blue™ Endotoxin Standard (50 EU each)
- 2 bottles of endotoxin-free water (50 ml)

Note: Components of the HEK-Blue™ LPS Detection kit can be purchased separately (see "Related Products" page 11).

Storage and stability

- The HEK-Blue™ LPS Detection Kit 2 is shipped on dry ice.
- Upon receipt HEK-Blue™-4 cells must be thawed **immediately** and grown according to handling procedures described on page 5.
- Store unopened HEK-Blue™ Selection, Normocin™, and HEK-Blue™ Endotoxin Standard at -20°C for up to 12 months.
- Store QB reagent and QB buffer at -20 °C for up to 12 months.
- Resuspended QUANTI-Blue™ is stable for 2 weeks at 2-8°C and for 2 months at -20°C when properly stored. Avoid repeated freeze-thaw cycles.

ADDITIONAL MATERIALS REQUIRED

Reagents required

- 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 endotoxin**
- Trypsin-EDTA (0.05% Trypsin, EDTA.4Na)
- Endotoxin-free water
- Phosphate buffered saline (PBS)
- Dimethylsulfoxide (DMSO)

Supplies required

- Laminar flow hood, CO_2 incubator
- Water bath (37°C), centrifuge
- Inverted microscope
- Microplate reader with 620-655 nm filter
- Sterile cell culture plasticware: cell culture flasks, 96-well round and flat bottom plates, tubes, pipettes, tips.
- Freezing container, 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

SAFETY CONSIDERATION

The HEK-Blue™ LPS Detection Kit 2 contains antibiotics and products of biological and bacterial origins that must be handled observing the usual safety precautions (wear appropriate protective equipment, do not ingest, do not inhale).
HEK-Blue™-4 cells were derived from HEK293 cells (transformed with adenovirus 5 DNA) that require **Biosafety Level 2** according to CDC guidelines. The biosafety level may vary depending on the country.
HEK-Blue™-4 cells are sent with the condition that you are responsible for its safe storage, handling and use. InvivoGen is not liable for damages or injuries resulting from receipt and/or use of an InvivoGen culture. Detailed discussions of laboratory safety procedures are provided in 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.html.
Note: InvivoGen recommends that protective gloves and clothing always be used and a full mask always be worn when handling frozen vials.

PROCEDURES SUMMARY

Handling procedures of HEK-Blue™-4 cells

1. Thaw HEK-Blue™-4 cells.
2. Expand HEK-Blue™-4 cells in the presence of HEK-Blue™ Selection.
3. Make your frozen stock of HEK-Blue™-4 cells.

Endotoxin detection procedure

1. Prepare serial dilutions of the sample and the HEK-Blue™ Endotoxin Standard.
2. Prepare cell suspension of HEK-Blue™-4 cells in growth medium.
3. Add sample or HEK-Blue™ Endotoxin Standard dilutions to cell suspension into wells of a 96-well microplate.
4. Incubate 16-24h at 37°C in 5% CO_2.
5. Add HEK-Blue™-4 cell supernatants to QUANTI-Blue into wells of a 96-well microplate.
6. Incubate 1-3h at 37°C.
7. Read the absorbance of each microplate well at 620-655 nm.
8. Calculate the endotoxin concentration of the sample.

PREPARATION OF REAGENTS

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

Cell culture medium for HEK-Blue™ -4 Cells

• **Growth Medium** (for thawing and recovery of the frozen cell line)**:**

DMEM high glucose supplemented with 10% FBS, Penicillin-Streptomycin and 1X Normocin™ (growth medium). Warm at 37°C before use and store at 2-8°C.

Note: The use of some FBS might affect the functionality of HEK-Blue™-4 Cells as they may contain endotoxins. Make sure the FBS used is endotoxin-free.

• **Selection Medium** (for cell culture maintenance)**:**

Growth medium supplemented with 1X HEK-Blue™ Selection. Warm at 37°C before use and store at 2-8°C.

• **Freezing Medium:**

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

• **Test Medium :**

Growth medium containing 10% heat-inactivated FBS (heated 30 min at 56°C).

QUANTI-Blue™

- Prepare 100 ml of QUANTI-Blue™ Solution by adding 1 ml of QB reagent and 1 ml of QB buffer to 98 ml of sterile water in a sterile glass bottle or flask.
- Mix well by vortexing and incubate at room temperature for 10 min before use.
- Use QUANTI-Blue™ Solution immediately or store at 2-8 °C or -20 °C.

HEK-Blue™ Endotoxin Standard

- Reconstitute by adding 1 ml of endotoxin-free water to the content of the tube to obtain an initial stock solution at X EU/ml, where X is the amount of EU per vial.

Note: Each vial contains approximatively 40-100 EU lyophilized endotoxin. The actual value of X is stated on the Certificate of Analysis. For example, if the X value of the vial is 55 EU, when reconstituted with 1 ml endotoxin-free water it will yield a concentration of 55 EU/ml.

- Mix vigorously by vortexing as endotoxin tends to attach to glass.
- Prepare a 1.0 EU/ml HEK-Blue™ Endotoxin Standard stock solution by diluting 1/X. For example, if the initial stock solution is at 55 EU/ml, the dilution is 1/55 or 0.1 ml of initial stock solution into 5.4 ml of endotoxin-free water.
- Store at 2-8°C. Initial and 1.0 EU/ml HEK-Blue™ Endotoxin Standard stock solutions are stable for 1 week.

Preparation of diluted Trypsin-EDTA solution

- Mix 10 ml of Trypsin-EDTA (0.05% Trypsin, EDTA.4Na) with 20 ml of PBS.
- Warm at 37°C before use and store at 2-8°C.

The solution is stable for 3 days at 2-8°C and 6 months at -20°C.

Note: HEK-Blue™-4 cells functions are altered by the action of trypsin unless the solution is diluted. We strongly recommend the use of diluted trypsin to pass HEK-Blue™-4 cells. Before the test, use PBS to detach the cells.

HANDLING PROCEDURES OF HEK-Blue™-4 CELLS

HEK-Blue™-4 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™-4 cells

- Thaw the HEK-Blue™ -4 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 (approximately 2 minutes).
- 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.

- Gently transfer the contents of the vial in a sterile tube containing 20 ml of pre-warmed growth medium and spin at 300 x g (RCF) for 5 minutes.
- Remove the supernatant and resuspend the cells with 5 ml of growth medium.
- Transfer the contents of the vial to a 25 cm^2 tissue culture flask or a 75 cm^2 tissue culture flask containing 15 ml of pre-warmed 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.

- Place the flask at 37°C in a CO_2 incubator overnight.
- Follow the growth of the cells by daily observation of the culture with an inverted microscope. When 50-80% confluency is reached, trypsinize the cells using diluted trypsin (as described above) and grow them in selection medium (growth medium supplemented with 1X HEK-Blue™ Selection).

Cell maintenance

- Maintain and subculture the cells in selection medium.
- Renew growth medium 2 to 3 times a week.
- Cells should be passaged when a 60-80% confluency is reached. Do not let the cell grow to 100% confluency.

Note: The HEK-Blue™-4 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™ -4 cells in 175 cm² tissue culture flasks containing growth medium supplemented with 1X HEK-Blue™ Selection. Those cells can be frozen according to the following procedure to make your own frozen stock.

- When the culture has reached 80% confluency, harvest the cells by trypsinization using diluted trypsin (see page 5).
- Resuspend the cells in growth medium and estimate the cell concentration by using a counting cell.
- Centrifuge the cells at 300 x g (RCF) for 5 minutes.
- Resuspend the cells in freezing medium (growth medium supplemented with 10% sterile DMSO) at a concentration of 0.5-0.8 x10^7 cells/ml.
- Dispense 1 ml of cell suspension per cryotube.
- Freeze the cells using a freezing container.
- Store the vials in a liquid nitrogen tank.

Note: To ensure a maximal efficiency of the HEK-Blue™-4 cell line, thaw a new tube when the cultured cell line has reached 30 passages.

ENDOTOXIN DETECTION PROCEDURE

Reagents and materials required

- 1.0 EU/ml HEK-Blue™ Endotoxin Standard stock solution (see preparation page 5)
- Endotoxin-free water
- PBS
- Microplates: 96-well round and flat bottom plates
- Cell culture plasticware

Warm the samples and all the test reagents at 37°C before use.

Sample preparation

Careful technique must be used to avoid microbiological or endotoxin contamination. All materials coming in contact with the samples or test reagents must be endotoxin-free. These materials should be tested before use.

All powdered samples should be resuspended in endotoxin-free water.

Samples to be tested should be stored at 2-8°C or frozen to stop all bacteriological activity otherwise the endotoxin level may increase with time.

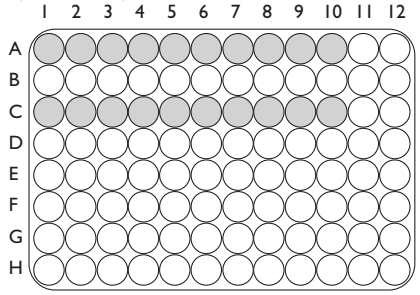
Note: Samples containing a phosphatase activity cannot be tested as they can result in false positive results.

We recommend to prepare a three-fold serial dilution (other dilution factors may be chosen) in a round bottom 96 well-plate (Dilution plate) using endotoxin-free water.

A three-fold serial dilution of the test sample may be prepared as indicated below:

Well	Sample	Endotoxin-free water	Sample dilution
A1	210 µl of sample	-	Direct
A2	70 µl from well A1	140 µl	1/3
A3	70 µl from well A2	140 µl	1/9
A4	70 µl from well A3	140 µl	1/27
A5	70 µl from well A4	140 µl	1/81
A6	70 µl from well A5	140 µl	1/243
A7	70 µl from well A6	140 µl	1/729
A8	70 µl from well A7	140 µl	1/2187
A9	70 µl from well A8	140 µl	1/6561
A10	-	210 µl	-

Cytotoxicity



Dilution plate

A1-A10: sample dilutions
C1-C10: endotoxin standard dilutions

We recommend to test the absence of toxicity of the test sample on HEK-Blue™-4 cells before running the endotoxin detection test. If a cytotoxic effect is observed, the sample may require further dilution until the cytotoxicity is overcome.

Note: Avoid testing of pure samples soluble only in ethanol or DMSO. These solutions are toxic to the cell line and can result in false negative results.

Inhibition/enhancement

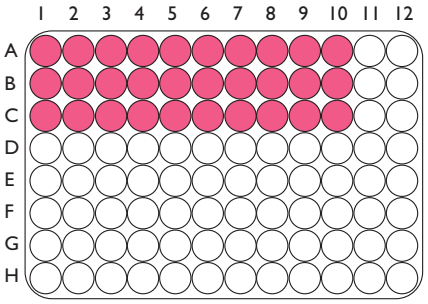
Sample inhibition or enhancement occurs when substances in the test sample interfere with the activation of the HEK-Blue™-4 cells. This interference affects the final Δ absorbance, suggesting lower or higher levels of endotoxin than those actually present in the test sample. The lack of sample interference should be determined for each specific sample. To verify the lack of interference, a serial dilution of the test sample is spiked with a known amount of endotoxin (e.g. 0.1 EU/ml). The spiked sample dilutions are assayed along the unspiked sample dilutions. The absorbances of the spiked sample dilutions should equal to 50-200% of the absorbance of the spike (endotoxin diluted in endotoxin-free water).

HEK-Blue™ Endotoxin Standard preparation

- Mix vigorously by vortexing the 1.0 EU/ml HEK-Blue™ Endotoxin Standard stock solution.
- Prepare a two-fold serial dilution of the 1.0 EU/ml HEK-Blue™ Endotoxin Standard stock solution in the dilution microplate (96-well round bottom plate used for the preparation of sample dilutions) using endotoxin-free water as indicated below:

Well	Sample	Endotoxin-free water	Final concentration
C1	200 µl of 1.0 EU/ml endotoxin standard	-	1 EU/ml
C2	100 µl from well C1	100 µl	0.5 EU/ml
C3	100 µl from well C2	100 µl	0.25 EU/ml
C4	100 µl from well C3	100 µl	0.125 EU/ml
C5	100 µl from well C4	100 µl	0.062 EU/ml
C6	100 µl from well C5	100 µl	0.031 EU/ml
C7	100 µl from well C6	100 µl	0.016 EU/ml
C8	100 µl from well C7	100 µl	0.008 EU/ml
C9	100 µl from well C8	100 µl	0.004 EU/ml
C10	-	200 µl	-

- To spike the test sample, prepare a 0.1 EU/ml HEK-Blue™ Endotoxin Standard spike solution (50 µl of 1.0 EU/ml HEK-Blue™ Endotoxin Standard stock solution in 450 µl of endotoxin-free water).



Test plate

A1-A10: unspiked sample dilutions with cells
B1-B10: spiked sample dilutions with cells
C1-C10: endotoxin standard dilutions with cells

HEK-Blue™-4 cell suspension preparation

To ensure the best results of the test:
- Use HEK-Blue™ -4 cells that have been passaged less than 30 times.
- Use a culture showing 50-80% confluency and that has been passaged at least 48 h before the test.

Notes:

• *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.*

- Remove the medium by aspiration.
- Carefully rinse the cell monolayer with PBS pre-warmed at 37°C. This step is intended to remove round cells and all trace of culture medium. Use 10 ml of PBS for a 75 cm^2 tissue culture flask.
- Remove PBS by aspiration.
- Detach the cells from the flask in the presence of PBS. Use 7 ml of PBS for a 75 cm^2 tissue culture flask. If necessary incubate the cells 2-5 minutes at 37°C in a CO_2 incubator.
- Carefully homogenize the cell suspension by gentle pipetting using a 1 ml pipet tip. Avoid the formation of air bubbles.
- Estimate the cell concentration by using a counting cell.
- Dilute the cells with pre-warmed test medium at a concentration of 1-5 x10^5 cells/ml.
- Homogenize the cell suspension by gentle pipetting.
- Transfer the cell suspension into a sterile reagent reservoir if using a multichannel pipette.

Test procedure (for a 96-well flat bottom plate)

Day 1:
- Prepare sample dilutions and HEK-Blue™ Endotoxin Standard dilutions (see page 5) in a round bottom 96 well-plate (dilution plate).
- In the first two rows of a 96-well flat bottom plate (test plate), dispense 20 µl of the sample serial dilution (20 µl from well A1 of dilution plate into wells A1 and B1 of test plate, 20 µl from well A2 of dilution microplate into wells A2 and B2 of test plate, and until wells A10 and B10).
- In the third row of the test plate, dispense 20 µl of the HEK-Blue™ Endotoxin Standard serial dilution (20 µl from well C1 of dilution plate into well C1 of test plate, 20 µl from well C2 of dilution plate into well C2 of test plate, and until well C10).
- Add 20 µl of endotoxin-free water into wells A1 to A10 of test plate. This row corresponds to the unspiked sample dilutions.
- Add 20 µl of 0.1 EU/ml HEK-Blue™ Endotoxin Standard spike solution into wells B1 to B10 of test plate. This row corresponds to the spiked sample dilutions.
- Add 20 µl of endotoxin-free water into wells C1 to C10 of test plate. This row corresponds to the HEK-Blue™ Endotoxin Standard dilutions.

Note: *We recommend to run the sample and HEK-Blue™ Endotoxin Standard dilutions in duplicate.*

- *Prepare the* HEK-Blue™ -4 cell suspension as described in the previous section
- Add 160 µl (50,000 cells max) of the HEK-Blue™-4 cell suspension to the test plate wells. Use new tips for each well to avoid cross-contamination.
- Incubate the test plate at 37°C in a CO_2 incubator for 18-24 h.

Day 2:
- Prepare QUANTI-Blue™ (see page 8) and warm at 37°C for 30 min before use.
- Dispense 20 µl of supernatant from each well of the test plate into the corresponding well of a new plate (detection plate).
- Add 180 µl of QUANTI-Blue™ and incubate 1 to 6 hours at 37°C.
- Read the absorbance of the plate at 620-655 nm.

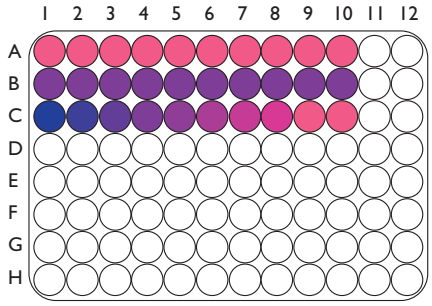
Reading and Interpretation

Presence of endotoxin in a sample can be observed with the naked eye and the concentration calculated by measuring the absorbance.

The test is validated if after 1-6 hours incubation:

- the HEK-Blue™ Endotoxin Standard (row C1-C10) displays a blue/purple/pink gradient
- the blank (wells A10 and C10) 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 purple or blue color should be considered as positive and containing ≥ 0.01 EU/ml endotoxin.



Detection plate

A1-A10: unspiked sample dilutions in QUANTI-Blue™
B1-B10: spiked sample dilutions in QUANTI-Blue™
C1-C10: endotoxin standard dilutions in QUANTI-Blue™

Calculation of Endotoxin Concentration

Under the standard conditions, the absorbance at 620-655 nm is linear in the concentration range of 0.01 to 0.1 EU/ml endotoxin. Substract the mean absorbance of the blank from the mean absorbance value of the HEK-Blue™ Endotoxin Standard dilutions and sample dilutions to calculate mean Δ absorbance.

- Plot the mean Δ absorbance for the HEK-Blue™ Endotoxin Standard dilutions on the y-axis versus the corresponding endotoxin concentration in EU/ml on the x-axis.
- Fit a linear trendline to these points and display the equation and the coefficient of correlation (R-squared value) on chart.

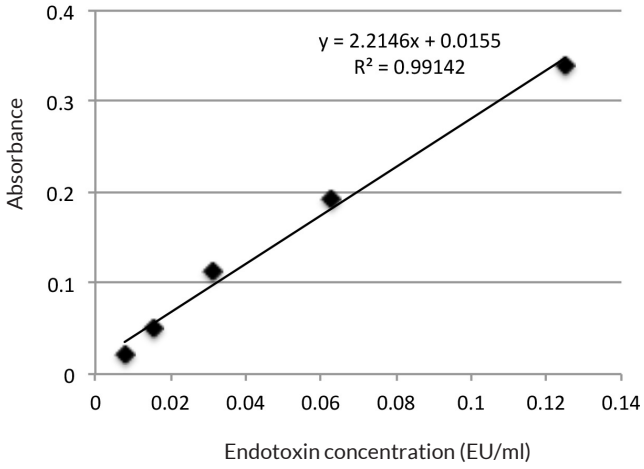
Note: The linearity of the HEK-Blue™ Endotoxin Standard curve, within the concentration range used to predict endotoxin values, is reliable when the coefficient of correlation (R2 value) is ≥ 0.98. No less than 4 standard concentrations spanning the desired concentration range should be assayed.

- Choose one or several y values of the sample dilutions that are in the linear range of the HEK-Blue™ Endotoxin Standard curve and calculate the corresponding x value(s) using the equation (see example data).
- To determine the endotoxin concentration of the sample, multiply the x value(s) obtained by their respective dilution factor(s) (see example data).

Example Data

EU/ml	Absorbance	Mean Absorbance	Mean Δ Absorbance
1	1.217 / 1.305	1.261	1.093
0.5	0.914 / 1.001	0.958	0.790
0.25	0.676 / 0.730	0.703	0.535
0.125	0.483 / 0.533	0.508	0.340
0.062	0.352 / 0.369	0.361	0.193
0.031	0.254 / 0.307	0.281	0.113
0.016	0.207 / 0.231	0.219	0.051
0.008	0.181 / 0.192	0.191	0.023
0.004	0.180 / 0.192	0.186	0.018
0 (Blank)	0.165 / 0.170	0.168	-

HEK-Blue™ Endotoxin Standard curve



Equation

$y = 2.2146x + 0.0155$ or $x = (y - 0.0155)/2.2146$

→ X = x × dilution factor

y : Mean Δ Absorbance value
x : Endotoxin concentration of dilution
X : Endotoxin concentration of sample in EU/ml

Endotoxin concentration of sample 1

Calculation using example data:

Dilution Factor	Absorbance	Mean Absorbance	Mean Δ Absorbance
1	2.584 / 2.508	2.546	2.378
1/3	2.520 / 2.461	2.491	2.323
1/9	2.411 / 2.329	2.370	2.202
1/27	2.088 / 2.056	2.072	1.904
1/81	1.516 / 1.462	1.489	1.321
1/243	1.104 / 1.036	1.070	0.902
1/729	0.613 / 0.577	0.595	0.427
1/2187	0.485 / 0.441	0.463	0.295
1/6561	0.302 / 0.276	0.289	0.121
Blank	0.171 / 0.165	0.168	-

• 1/2187 sample dilution

$x = (0.295 - 0.0155)/2.2146 = 0.126 \rightarrow X = 0.126 \times 2187 = 276$

• 1/6561 sample dilution

$x = (0.121 - 0.0155)/2.2146 = 0.048 \rightarrow X = 0.048 \times 6561 = 315$

Sample 1 contains an average of 276 + 315 = 295 EU/ml.

Endotoxin concentration of sample 2

Dilution Factor	Absorbance	Mean Absorbance	Mean Δ Absorbance
1	0.646 / 0.570	0.608	0.440
1/2	0.458/ 0.404	0.431	0.263
1/4	0.317 / 0.251	0.284	0.116
1/8	0.258 / 0.214	0.236	0.068
1/16	0.201 / 0.173	0.187	0.019
1/32	0.180 / 0.162	0.171	0.003
1/64	0.174 / 0.156	0.165	-
1/128	0.177 / 0.151	0.164	-
1/256	0.172 / 0.158	0.165	-
Blank	0.175 / 0.161	0.168	-

Calculation using example data

• 1/2 sample dilution

$x = (0.263 - 0.0155)/2.2146 = 0.112 \rightarrow X = 0.112 \times 2 = 0.224$

• 1/4 sample dilution

$x = (0.116 - 0.0155)/2.2146 = 0.045 \rightarrow X = 0.045 \times 4 = 0.18$

• 1/8 sample dilution

$x = (0.068 - 0.0155)/2.2146 = 0.024 \rightarrow X = 0.024 \times 8 = 0.192$

Sample 2 contains an average of 0.224 + 0.18 + 0.192 = 0.2 EU/ml.

Inhibition/enhancement assay

To determine if a sample interferes with the performance of HEK-Blue™ LPS Detection assay, we recommend to test dilutions of your sample that have been spiked with endotoxin (e.g. 0.1 EU/ml). The spiked samples are assayed along with the unspiked samples. Spiked samples with absorbance values equal to 50-200% of the absorbance of the spike are considered non-interfering and can be used for the determination of the endotoxin concentration.

Sample Dilutions	Absorbance of unspiked dilutions	Absorbance of spiked dilutions	Difference	Pourcentage
1	0.18	0.24	0.06	15%
1/2	0.15	0.23	0.08	20%
1/4	0.14	0.35	0.21	52%
1/8	0.15	0.46	0.31	77%
1/16	0.13	0.49	0.36	90%
1/32	0.09	0.47	0.38	95%
Blank	0.10	0.50	0.40	100%

Example: Determination of non-inhibitory sample dilutions

Undiluted sample and first sample dilution are inhibitory. Dilutions 1/4, 1/8, 1/16 and 1/32 are non-inhibitory.

Limitations

• Toxic products

Toxic compounds may interfere with the HEK-Blue™-4 cells at high concentrations. The lack of product cytotoxicity should be determined for each specific sample, either undiluted or at different dilutions.

• Particulate samples

Particulate samples, such as vaccine adjuvants, should not affect the HEK-Blue™-4 endotoxin assay. Particulate samples are recognized by the HEK-Blue™-4 cells equally well as soluble samples.

• Colored samples

Samples which possess significant color may require special attention. A quick visual inspection of the Detection plate prior to incubation may be sufficient to determine if a product's intrinsic color is of concern. The undiluted sample should display a color similar to the blank. If the color of the undiluted sample is significantly greater than the color of the blank, the absorbance obtained after incubation of the Detection plate should not be considered for the final calculation of the endotoxin concentration of the sample. The endotoxin concentration of colored samples should be determined using the absorbance of the sample dilutions that have a color similar to the blank prior to the incubation of the Detection plate.

• NF-κB inducers

Compounds known to activate the NF-κB pathway may interfere with the HEK-Blue™-4 endotoxin assay by activating the HEK-Blue™-4 cells in a TLR4-independent manner. Such compounds include ionomycin, phorbol 12-myristate 13-acetate (PMA), cytokines, such as TNF-α and IL-1β, and ligands of TLR3, TLR5 or NOD1.

TROUBLESHOOTING GUIDE

Problem	Reason	Solution
All wells of the detection plate are blue	• 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 medium contains endotoxins (typically in the FBS)	• 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 • Use FBS certified endotoxin-free
Blank gives a blue color	• See above	• See above
HEK-Blue™ Endotoxin Standard dilutions stay pink	• Endotoxins adhere to the inner surface of the tube • QUANTI-Blue™ was stored for more than 2 weeks at 2-8°C • HEK-Blue™-4 cells are not in a healthy state • Cells are not HEK-Blue™-4 cells	• Warm and vortex extensively the endotoxin standards before use • Use a new pouch to prepare QUANTI-Blue™ • Start a new culture from the frozen stock • Start a new culture from the frozen stock
False negatives	• Samples are toxic to the HEK-Blue™-4 cells • Endotoxins in the samples adhere to the inner surface of the tube	• Dilute samples in endotoxin-free water and test their cytotoxicity on HEK-Blue™-4 cells before running the HEK-Blue™ LPS Detection kit 2 • Vortex extensively the sample before use
False positives	• Presence of a phosphatase activity in the sample • Sample contains an NF-κB inducer	• Test the presence of a phosphatase activity in your sample by adding 20 µl of your sample to 180 µl of QUANTI-Blue™. If a purple/blue color appears after 1-3h at 37°C your sample contains a phosphatase activity and cannot be tested using the HEK-Blue™ LPS Detection kit. • This sample cannot be tested with the HEK-Blue™ LPS Detection kit 2
Negative using the HEK-Blue™ endotoxin assay but positive using a LAL assay	• Sample contains a LPS that does not activate TLR4 (e.g. LPS from *Rhodobacter sphaeroides*)	• Such LPS may act as an endotoxin inhibitor

USE RESTRICTIONS

HEK-Blue™ -4 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.

<u>Limited Use License</u>

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 Pkwy, San Diego, CA 92121 USA.

Tel: 858-457-5873. Fax: 858-457-5843.

RELATED PRODUCTS

Product	Quantity	Cat. Code
HEK-Blue™ Selection	10 x 1 ml	hb-sel
QUANTI-Blue™ Solution	5 ml	rep-qbs
HEK-Blue™ Endotoxin Standard	10 x 50 EU	rep-hbes-10
LPS-RS Ultrapure (*R. sphaeroides*)	1 mg	tlrl-prslps

InvivoGen US
10515 Vista Sorrento Pkwy
San Diego, CA 92121, USA
Toll Free (US): 888.457.5873
Outside US: +1 858.457.5873
Email: info@invivogen.com

InvivoGen Europe
5, rue Jean Rodier
F-31400 Toulouse, France
Tel: +33 562.71.69.39
Fax: +33 562.71.69.30
Email: info@invivogen.fr

InvivoGen Asia
Unit 307; 3F, 8W Phase 2
Hong Kong Science Park, HK
Tel: +852 3622-3480
Fax: +852 3622-3483
Email: info.hk@invivogen.com