

InvivoGen Insight

WINTER
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Inside this issue:

REVIEWS

Deciphering the STING Paradox

PRODUCTS

STING Product Line

- ❖ STING Ligands
- ❖ STING Reporter Cells
- ❖ STING Variants

Luciferase Assays

- ❖ Streptavidin-Luciferase
- ❖ QUANTI-Luc™

TLR9 Ligands

- ❖ Stimulatory CpG ODNs
- ❖ Inhibitory ODNs
- ❖ TLR9 Agonist Discovery Kits

Endotoxin Detection

- ❖ HEK-Blue™ LPS Detection Kit 2

Deciphering the STING Paradox

STING (stimulator of interferon genes), alternatively known as MPYS, TMEM173, MITA and ERIS, is a key sensor of cytosolic nucleic acids. In the past year, an incredible amount has been revealed on the biology of STING. As the studies were published, the complexity of STING became apparent. STING, initially thought to serve solely as an adaptor protein for mediating signaling by cytosolic DNA sensors (CDS), was recently found to be a direct sensor of cyclic dinucleotides (CDNs)¹.

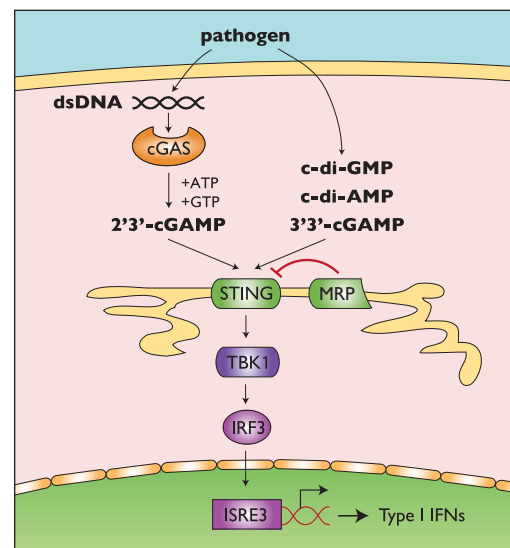
CDNs are ubiquitous second messenger molecules used in bacterial signal transduction and are defense triggers in mammalian cells. Upon bacterial pathogen attack, CDNs released into cells bind directly to STING leading to TBK1-mediated IRF3 activation and type I IFN production. Cyclic diguanylic acid (c-di-GMP) is the most prevalent intracellular signaling intermediate in bacteria. Other functionally important CDNs include cyclic diadenylic acid (c-di-AMP)² and the recently identified cyclic adenylyguanylic acid (cGAMP)³. A highlight of the year was the back-to-back discovery of metazoan cGAMP and the enzyme cyclic cGAMP synthase (cGAS)^{3,4}. Mammalian cells synthesize cGAMP in response to cytosolic DNA interaction with cGAS. Increasing evidence now places cGAS as the critical cytosolic DNA sensor.

Surprisingly, certain cells are able to respond to cytosolic DNA and the cGAS product but are poorly responsive to direct administration of cGAMP. With several close structural examinations, another key discovery was made. Metazoan cGAMP is structurally distinct to the bacterial cGAMP^{5,6}. Metazoan cGAMP contains [G(2',5')-pA(3',5')p] phosphodiester linkages, whereas bacterial cGAMP contains [G(3',5')pA(3',5')p] linkages. Thus arose the nomenclature 3'3'-cGAMP for the bacterial "canonical" cyclic dinucleotide, and 2'3'-cGAMP relating to the mammalian "noncanonical" cGAS-produced cGAMP. The latter cGAS-produced cGAMP was found to bind STING with stronger affinity than bacterial cGAMP, inducing a robust IFN response^{7,8}.

Interestingly, a variety of natural variants of human STING (hSTING) have been identified⁹. The presence of non-synonymous variants of hSTING, some in high frequencies is indicative of its implicit role in disease. It is important to be aware that variant hSTING alleles differentially respond to cGAMPs. Several of the aforementioned studies were performed on the hSTING allele containing the H232 allele and not R232 that is now known as the wild type hSTING prevalent in ~60% of the population. Furthermore, the variant hSTING haplotype (HAQ) found

in the THP1 monocytic cell line has low intrinsic activity but responds to bacterial and metazoan CDNs. Very recently, a splice variant of hSTING, named MRP, has been identified in HeLa and 293T cells that acts as a dominant negative regulator of STING-mediated IFN production¹⁰.

The STING variants (alleles and mutants) and different forms of CDNs are powerful tools to further understand STING biology. Important lessons have been learned the hard way. A small molecule DMXAA (Vadimezan or ASA404) that failed in phase III clinical trials in combination with chemotherapy, was found to stimulate STING signaling in mice but not in humans¹¹. Together, these studies underscore the careful attention required when it comes to conducting experiments and interpreting the role of STING.



1. Burdette DL, et al., 2011. STING is a direct innate immune sensor of cyclic di-GMP. *Nature* 478(7370):515-8. 2. Woodward JJ, et al., 2010. c-di-AMP secreted by intracellular *Listeria monocytogenes* activates a host type I interferon response. *Science* 328(5986):1703-5. 3. Wu J, et al., 2013. Cyclic GMP-AMP is an endogenous second messenger in innate immune signaling by cytosolic DNA. *Science* 339(6121):826-30. 4. Sun L, et al., 2013. Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type I interferon pathway. *Science* 339(6121):786-91. 5. Ablasser A, et al., 2013. cGAS produces a 2'-5'-linked cyclic dinucleotide second messenger that activates STING. *Nature* 498(7454):380-4. 6. Civril F, et al., 2013. Structural mechanism of cytosolic DNA sensing by cGAS. *Nature* 498(7454):332-7. 7. Gao P, et al., 2013. Cyclic [G(2',5')pA(3',5')p] is the metazoan second messenger produced by DNA-activated cyclic GMP-AMP synthase. *Cell* 153(5):1094-107. 8. Zhang X, et al., 2013. Cyclic GMP-AMP Containing Mixed Phosphodiester Linkages Is An Endogenous High-Affinity Ligand for STING. *Mol Cell* 52(2):226-35. 9. Yi G, et al., 2013. Single Nucleotide Polymorphisms of Human STING Can Affect Innate Immune Response to Cyclic Dinucleotides. *PLoS One* 8(10):e77846. 10. Chen H, et al., 2014. An Alternative Splicing Isoform of MITA Antagonizes MITA-Mediated Induction of Type I IFNs. *J Immunol* doi: 10.1094. 11. Conlon J, et al., 2013. Mouse, but not human STING, binds and signals in response to the vascular disrupting agent 5,6-dimethylxanthenone-4-acetic acid. *J Immunol* 190(10):5216-25.



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STING Ligands

Cyclic dinucleotides (CDNs) and xanthenone derivatives, such as DMXAA, were recently found to bind to and activate STING leading to a potent type I IFN response. CDNs are important messengers in bacteria, affecting numerous responses of the prokaryotic cell, but also in mammalian cells, acting as agonists of the innate immune response. CDNs represent a promising new class of vaccine adjuvants. To assist your research needs, InvivoGen provides a large collection of CDNs available in two grades:

- **InvivoGen Standard Grade:** purity \geq 95 %, validated using cell-based assays
- **VacciGrade™:** sterility guaranteed and endotoxin level $<$ 1 EU/mg, in addition to the above-mentioned

➤ Cyclic Dinucleotides

2'3'-cGAMP - Mammalian cGAMP **NEW**

2'3'-cGAMP (cyclic [G(2',5')pA(3',5')p]) is the only isomer of cGAMP produced by the mammalian DNA sensor cGAMP synthase (cGAS) in response to cytosolic DNA¹. This isomer contains two distinct phosphodiester linkages, a non-canonical (2',5') linkage at the GpA step and a canonical (3',5') linkage at the ApG step¹⁻³. Mammalian 2'3'-cGAMP binds to STING with high affinity and is a potent inducer of IFN- β ³.

3'3'-cGAMP - Bacterial cGAMP

3'3'-cGAMP (cyclic [G(3',5')pA(3',5')p]) is the initially proposed isomer of cGAMP produced by cGAS⁴. This cyclic dinucleotide is not produced in mammals but only in bacteria, thus is a pathogen associated molecular pattern. Bacterial 3'3'-cGAMP contains two conventional (3',5') phosphodiester linkages. It is differentially recognized by STING variants and induces mainly the type I IFN pathway.

2'2'-cGAMP - Unnatural cGAMP **NEW**

2'2'-cGAMP (cyclic [G(2',5')pA(2',5')p]) is a synthetic cyclic dinucleotide not found in nature. It contains two non-canonical (2',5') phosphodiester linkages. Compared to 2'3'-cGAMP, it binds with lower affinity to STING³ but induces similar IFN- β response in cell-based assays^{3,5}.

➤ Xanthenone Analog

DMXAA - Mouse-specific **NEW**

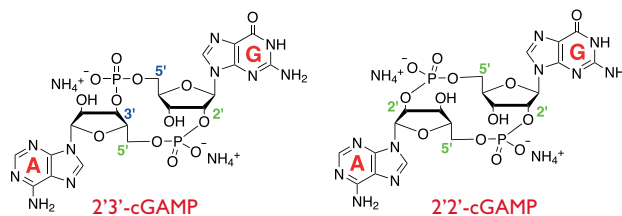
DMXAA (5,6-dimethylxanthenone-4-acetic acid, also known as Vadimezan or ASA404) was initially identified as a potent tumor vascular disrupting agent in mice through the induction of cytokines, notably IFN- β . Recent studies have demonstrated that DMXAA targets the STING pathway⁶, and this in a mouse-specific manner; DMXAA has no effect on human STING^{7,8}.

1. **Gao P. et al., 2013.** Cyclic [G(2',5')pA(3',5')p] is the metazoan second messenger produced by DNA-activated cyclic GMP-AMP synthase. *Cell*. 153(5):1094-107. 2. **Ablasser A. et al., 2013.** cGAS produces a 2'-5'-linked cyclic dinucleotide second messenger that activates STING. *Nature*. 498(7454):380-4. 3. **Zhang X. et al., 2013.** Cyclic GMP-AMP containing mixed phosphodiester linkages is an endogenous high-affinity ligand for STING. *Mol Cell*. 2013 Jul 25;51(2):226-35. 4. **Wu J. et al., 2012.** Cyclic GMP-AMP Is an Endogenous Second Messenger in Innate Immune Signaling by Cytosolic DNA. *Science*. 339(6121):826-30. 5. **Gao P. et al., 2013.** Structure-function analysis of STING activation by c[G(2',5')pA(3',5')p] and targeting by antiviral DMXAA. *Cell*. 154(4):748-62. 6. **Prantner D. et al., 2012.** 5,6-Dimethylxanthenone-4-acetic acid (DMXAA) activates stimulator of interferon gene (STING)-dependent innate immune pathways and is regulated by mitochondrial membrane potential. *J Biol Chem*. 287(47):39776-88. 7. **Conlon J. et al., 2013.** Mouse, but not human STING, binds and signals in response to the vascular disrupting agent 5,6-dimethylxanthenone-4-acetic acid. *J Immunol*. 190(10):5216-25. 8. **Kim S. et al., 2013.** Anticancer Flavonoids Are Mouse-Selective STING Agonists. *ACS Chem Biol*. 8(7): 1396-1401.

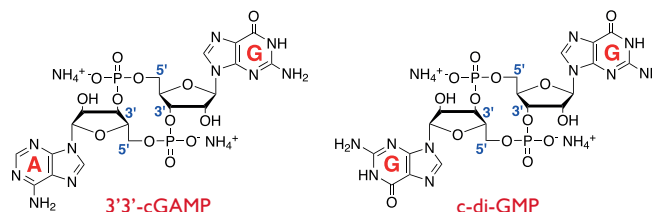
Contents

STING ligands are provided lyophilized. Cyclic dinucleotides are ammonium salts. Each product is supplied with endotoxin-free water.

Non-canonical cGAMP isomers



Canonical cyclic dinucleotides



Xanthenone analog

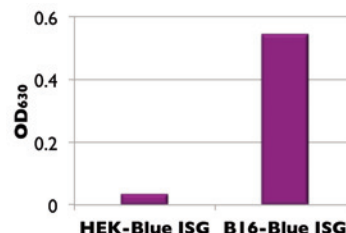
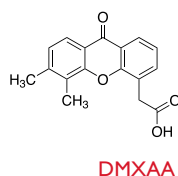


Figure 1 - Response of HEK-Blue™ ISG and B16-Blue™ ISG cells to 30 µg/ml DMXAA.

Also Available

- c-di-GMP, 1 mg (tlrl-cdg)
- c-di-GMP VacciGrade™, 1 mg (vac-cdg)
- c-di-AMP VacciGrade™, 1 mg (vac-cda)
- c-di-IMP, 1 mg (tlrl-cdi)
- c-di-AMP, 1 mg (tlrl-cda)
- c-di-UMP, 1 mg (tlrl-cdu)

PRODUCT	QUANTITY	CAT. CODE
2'3'-cGAMP	1x 500 µg	tlrl-cga23-s
2'3'-cGAMP VacciGrade™	2x 500 µg	vac-cga23
2'2'-cGAMP	2x 500 µg	tlrl-cga22
2'2'-cGAMP VacciGrade™	2x 500 µg	vac-cga22
3'3'-cGAMP	2x 500 µg	tlrl-cga
3'3'-cGAMP VacciGrade™	2x 500 µg	vac-cga
DMXAA	5 mg	tlrl-dmx

STING Reporter Cells

STING ligands trigger the production of type I IFNs and the induction of interferon stimulated genes (ISG) through interferon regulatory factors (IRFs). In order to facilitate their study, InvivoGen has developed stable reporter cells in which STING has been knocked out. These cells are either of human or mouse origin and feature IRF-inducible secreted reporter proteins SEAP (secreted embryonic alkaline phosphatase) or Lucia luciferase as convenient read-outs.

All InvivoGen's KO-STING cell lines were generated through knock out, close to the START codon, of the *sting* gene expressed endogenously by their parental cell line. These cell lines express a secreted reporter gene, either SEAP or Lucia luciferase, under the control of an IRF-inducible promoter. This composite promoter (I-ISG54) is comprised of five IFN-stimulated response elements (ISRE) fused to an ISG54 minimal promoter. IRF induction can be monitored by measuring the levels of SEAP or Lucia luciferase present in the supernatant using QUANTI-Blue™ or QUANTI-Luc™ (see p. 5), respectively.

► Human Reporter Cells

HEK-Blue™ ISG-KO-STING NEW

HEK-Blue™ ISG-KO-STING cells and their parental cell line HEK-Blue™ ISG were derived from the PEAKrapid cell line (similar to ATCC® CRL-2828™) which itself was derived from the HEK293 cell line.

HEK-Blue™ ISG cells respond poorly to cytosolic DNA, DMXAA (Fig. 1) and canonical cyclic dinucleotides (CDNs) but strongly to non-canonical CDNs (Fig. 2A). HEK-Blue™ ISG-KO-STING cells fail to respond to non-canonical CDNs but respond to type I IFNs comparable to their parental cell line.

HEK-Blue™ ISG-KO-STING and HEK-Blue™ ISG cells are resistant to Zeocin™.

► Mouse Reporter Cells

BI6-Blue™ ISG-KO-STING NEW

BI6-Blue™ ISG-KO-STING cells and their parental cell line BI6-Blue™ ISG were derived from the B16 F1 murine melanoma cell line.

BI6-Blue™ ISG cells respond to cytosolic DNA, DMXAA (Fig. 1), canonical and non-canonical CDNs (Fig. 2B). BI6-Blue™ ISG-KO-STING cells have lost the ability to respond to these molecules while retaining the ability to respond to type I IFNs.

BI6-Blue™ ISG-KO-STING and BI6-Blue™ ISG cells are resistant to Zeocin™.

RAW-Lucia™ ISG-KO-STING NEW

RAW-Lucia™ ISG-KO-STING cells were generated from the RAW-Lucia™ ISG cell line which is derived from the RAW 264.7 murine macrophage cell line. This cell line is a well established immune murine cell model.

RAW-Blue™ ISG cells respond to cytosolic DNA, DMXAA, canonical and non-canonical CDNs (Fig. 2B), in contrast to RAW-Blue™ ISG-KO-STING cells. Both cell lines exhibit comparable IFN responses.

RAW-Blue™ ISG-KO-STING and RAW-Blue™ ISG cells are resistant to Zeocin™.

Contents

HEK-Blue ISG-KO-STING, HEK-Blue™ ISG, RAW-Lucia ISG-KO-STING and RAW-Lucia ISG cells are grown in DMEM medium. BI6-Blue™ ISG-KO-STING and BI6-Blue™ ISG cells are grown in RPMI medium. DMEM and RPMI media contain 2mM L-glutamine, 10% FBS and are supplemented with 100 µg/ml Normocin™ and 100 µg/ml Zeocin™. Cells are provided frozen in a cryotube containing 3-7 × 10⁶ cells and supplied with 50 mg of Normocin™, 10 mg Zeocin™ and 1 pouch of QUANTI-Blue™. Cells are guaranteed mycoplasma-free.

Also Available

- THPI-Blue ISG, 3-7 × 10⁶ cells (thp-isg)
- THPI-Blue ISG-KD-STING, 3-7 × 10⁶ cells (thp-kdstg)
- THPI-Dual (NF-κB-SEAP IRF-Luc), 3-7 × 10⁶ cells (thp-nfis)

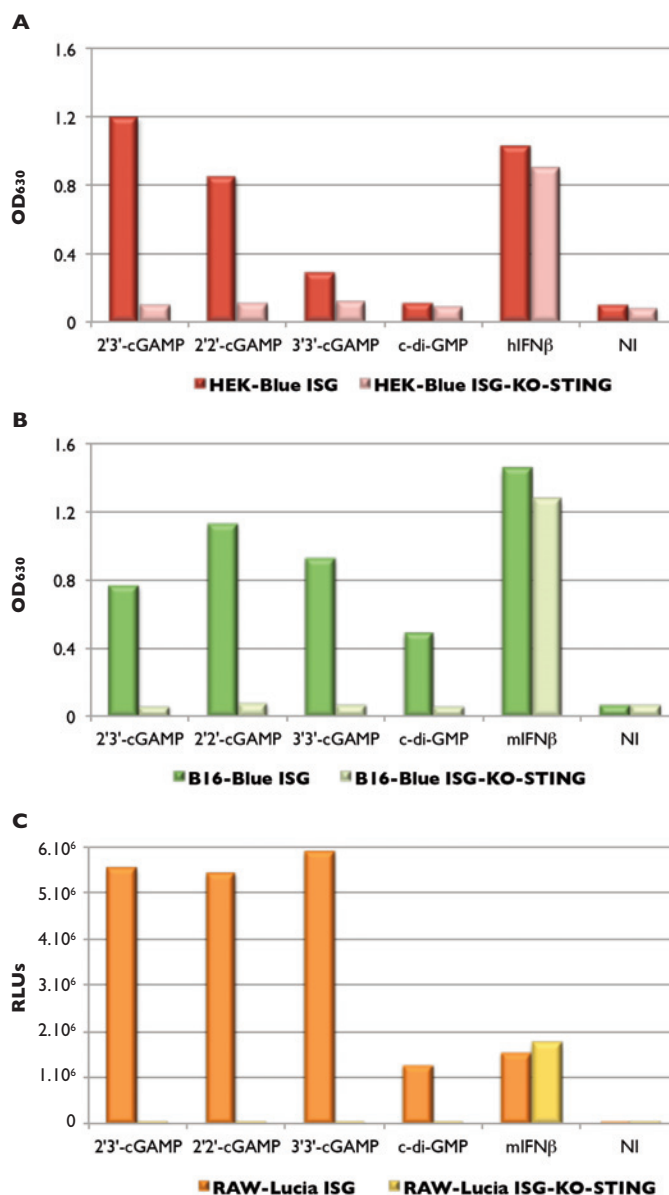


Figure 2 - Response of STING Reporter Cells to CDNs and IFN-β: Cells were stimulated with 30 µg/ml of the cyclic dinucleotides, 10⁴ U/ml of hIFN-β or 10³ U/ml of mIFN-β. Cells were not permeabilized. After 24h incubation, the levels of IRF-induced SEAP or Lucia luciferase were determined using QUANTI-Blue™ or QUANTI-Luc™, respectively.

PRODUCT	QUANTITY	CAT. CODE
BI6-Blue™ ISG	3-7 × 10 ⁶ cells	bb-ifnabg
BI6-Blue™ ISG-KO-STING	3-7 × 10 ⁶ cells	bb-kostg
HEK-Blue™ ISG	3-7 × 10 ⁶ cells	hkb-isg
HEK-Blue™ ISG-KO-STING	3-7 × 10 ⁶ cells	hkb-kostg
RAW-Lucia™ ISG	3-7 × 10 ⁶ cells	rawl-isg
RAW-Lucia™ ISG-KO-STING	3-7 × 10 ⁶ cells	rawl-kostg

STING Variants

Several non-synonymous variants of STING have been described in the human population, as well as various induced mutants of the human and mouse STING genes. Studies have revealed that STING variation can affect cyclic dinucleotide (CDN) recognition and signal transduction. InvivoGen provides most of the STING variants described, cloned and fully-sequenced into the expression plasmid pUNO1.

➤ Human STING Isoforms

hSTING-WT - The prevalent human STING isoform (~60% of the human population) contains an arginine at position 232 (R232) and is thus considered as wild-type^{1,2}. The hSTING-WT isoform is preferentially activated by 2',5'-linkage-containing cGAMP isomers³.

hSTING-H232 (R232H) - The H232 isoform of STING occurs in ~14% of the human population². It appears to respond similarly to the WT allele to metazoan CDNs but weakly to bacterial CDNs^{2,4}. Most of the hSTING proteins used for structural studies contained the R232H allele.

hSTING-A230 (G230A) - G230 is located in the flexible loop that forms a lid above the c-di-GMP binding pocket. The G230A variant is able to respond to lower concentrations of CDNs due to a different binding to the ligand².

hSTING-HAQ (R71H-G230A-R293Q) - STING-HAQ is a common haplotype (~20% of the human population and found in THP1 cells) that contains three non-synonymous single nucleotide polymorphisms. It expresses a STING protein that displays reduced intrinsic IFN- β stimulating activity^{1,2} but retains the ability to respond to metazoan and bacterial CDNs².

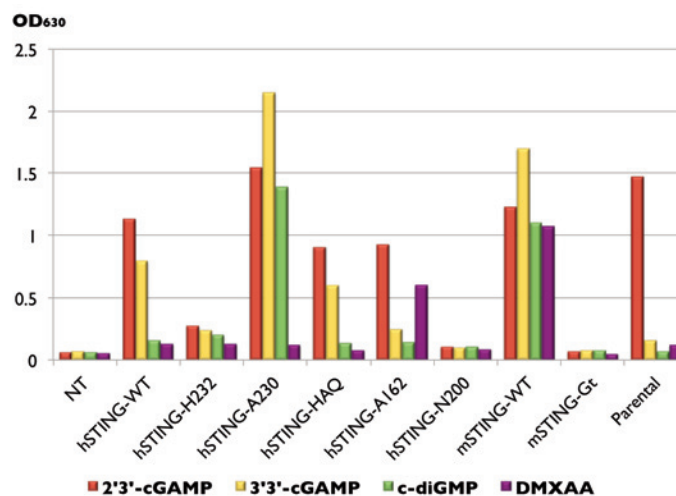
hSTING-A162 (S162A) - Human STING fails to bind DMXAA, a potent tumor vascular disrupting agent in mice⁵. A unique point mutation (S162A) placed at the cyclic-dinucleotide-binding site was found to confer DMXAA sensitivity to hSTING³.

hSTING-N200 (I200N) - The hSTING-N200 isoform harbors a missense mutation (I200N) equivalent to I199N mutation of the *Goldenticket* (*Gt*) mouse strain^{6,7}. The I200N mutation results in a null-phenotype with no detectable STING activity⁶.

➤ Mouse STING Isoforms

mSTING-WT - Wild-type mouse STING (mSTING-WT) contains an arginine at position 231, similarly to hSTING-WT. Unlike hSTING-WT, mSTING-WT appears to have no preference for the cGAMP isomers³ and efficiently binds DMXAA to produce type I IFNs⁵.

mSTING-Gt (I199N) - The *Goldenticket* (*Gt*) mutant mice carries a I199N missense mutation in exon 6 of the mSting gene and fails to display detectable activity⁷.

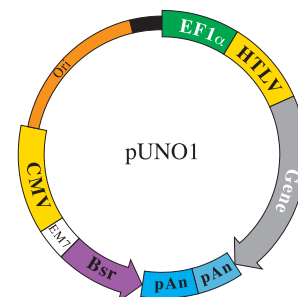


Response to CDNs and DMXAA of STING variants: Pools of HEK-Blue™ ISG KO-STING cells (see p. 3) transfected with WT or mutant STING and HEK-Blue™ ISG cells (parental) were stimulated with 10 μ g/ml of 2'3'-cGAMP, 3'3'-cGAMP, c-di-GMP or DMXAA. After 24h incubation, the levels of IRF-induced SEAP were determined using QUANTI-Blue™.

hSTING-MRP is an alternatively spliced isoform of hSTING lacking exon 7 that acts as a dominant negative mutant of STING. It was recently reported to block STING-mediated IFN response while retaining the ability to activate NF- κ B⁸.

1. Jin L. et al., 2011. Identification and characterization of a loss-of-function human MPYS variant. *Genes Immun.* 12(4):263-9. 2. Yi G. et al., 2013. Single Nucleotide Polymorphisms of Human STING Can Affect Innate Immune Response to Cyclic Dinucleotides. *PLoS One.* 8(10):e77846. 3. Gao P. et al., 2013. Structure-function analysis of STING activation by c[G(2',5')pA(3',5')p] and targeting by antiviral DMXAA. *Cell.* 154(4):748-62. 4. Diner E.J. et al., 2013. The innate immune DNA sensor cGAS produces a noncanonical cyclic dinucleotide that activates human STING. *Cell Rep.* 3(5):1355-61. 5. Conlon J. et al., 2013. Mouse, but not human STING, binds and signals in response to the vascular disrupting agent 5,6-dimethylxanthenone-4-acetic acid. *J Immunol.* 190(10):5216-25. 6. Yin Q. et al., 2012. Cyclic di-GMP sensing via the innate immune signaling protein STING. *Mol Cell.* 46(6):735-45. 7. Sauer J.D. et al., 2011. The N-ethyl-N-nitrosourea-induced Goldenticket mouse mutant reveals an essential function of Sting in the in vivo interferon response to *Listeria monocytogenes* and cyclic dinucleotides. *Infect Immun.* 79(2):688-94. 8. Chen H. et al., 2014. An Alternative Splicing Isoform of MITA Antagonizes MITA-Mediated Induction of Type I IFNs. *J Immunol.* [Ahead of print]

ISOFORM	RESIDUE POSITION	QTY	CAT. CODE
	h 71 162 200 230 232 293 m 70 161 199 229 231 292		
hSTING-WT	R S I G R R	20 μ g	puno1-hstingwt
hSTING-H232	R S I G H R	20 μ g	puno1-hsting-h232
hSTING-A230	R S I A R R	20 μ g	puno1-hsting-a230
hSTING-HAQ	H S I A R Q	20 μ g	puno1-hsting-haq
hSTING-A162	R A I G R R	20 μ g	puno1-hsting-a162
hSTING-MRP	R S I G R -	20 μ g	puno1-hsting-mrp
hSTING-N200	R S N G R R	20 μ g	puno1-hsting-n200
mSTING-WT	C S I I R R	20 μ g	puno1-mstingwt
mSTING-Gt	C S N I R R	20 μ g	puno1-msting-gt



Contents

STING variants are provided in the pUNO1 plasmid as 20 μ g of lyophilized DNA. Each plasmid is supplied with 4 pouches of Fast-media® Blas and 1 ml blasticidin at 10 mg/ml.

Streptavidin-Lucia - A new bioluminescent conjugate of Streptavidin

The common techniques of ELISA and Western Blot analysis widely use the Streptavidin-Biotin system for signal amplification. InvivoGen introduces Streptavidin-Lucia, a new bioluminescent streptavidin-conjugate featuring the Lucia luciferase. Streptavidin-Lucia has been optimized for ELISA and can be adapted for use in a variety of assays requiring the detection of biotin and biotinylated proteins.

- **Highly sensitive** - Detect low levels of target with low background
- **Accurate** - Tight linear correlation over several logs
- **Convenient** - Reveal using one-step QUANTI-Luc™ detection reagent
- **Rapid** - Measure 96 samples in a microplate in less than a minute

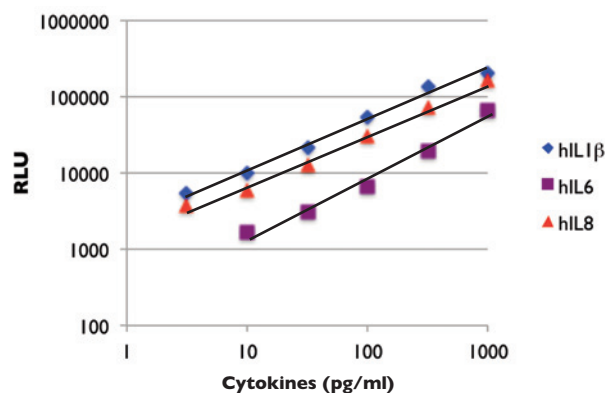
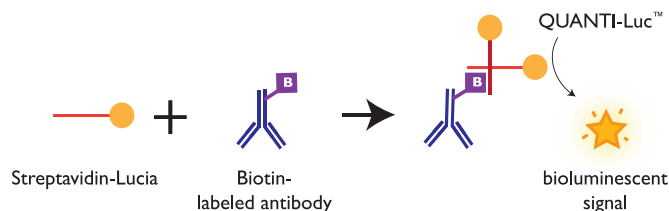
Streptavidin-Lucia consists of a streptavidin peptide fused to the Lucia luciferase, a small secreted coelenterazine-utilizing luciferase with strong and stable bioluminescence properties. Streptavidin-Lucia uses the advantages of two systems, streptavidin for strength of binding to biotin labeled proteins, together with an optimized luciferase with a strong and stable bioluminescence for high sensitive detection with low background. Thus, this product is used for the detection of streptavidin-biotin interactions with high sensitivity and accuracy.

The unique long-lasting bioluminescence property of Lucia luciferase allows for ease in reading measurements. After addition of the QUANTI-Luc™ assay reagent to each well of a 96-well plate, results can be obtained in less than a minute using a luminometer without injectors.

Contents

Streptavidin-Lucia is provided lyophilized in 2 vials, allowing to prepare 100x 96-well plates. Once reconstituted, each vial contains 500 µl each.

PRODUCT	QUANTITY	CAT. CODE
Streptavidin-Lucia	2 x 500 µl	rep-strlc



Typical data obtained using Streptavidin-Lucia for the detection of cytokines with commercially available ELISA. Graph shows detection of hIL1β, hIL8 and hIL6. Streptavidin-Lucia was detected using QUANTI-Luc™. Linear regression indicates detection range over at least 3 logs.

QUANTI-Luc™ - Coelenterazine-utilizing luciferase assay reagent

- **Ready to use** - Just add water
- **Cost effective** - One pouch prepares 5 x 96 well plates
- **Practical** - Working reagent stable at least one month

QUANTI-Luc™ is an assay reagent containing all the components required to quantitatively measure the activity of Lucia luciferase and other coelenterazine-utilizing luciferases. QUANTI-Luc™ is optimized for use with Lucia luciferase reporter cell lines for fast and accurate real-time measurements, directly from the cell culture media. QUANTI-Luc™ is also suited for any applications using Streptavidin-Lucia. QUANTI-Luc™ contains the coelenterazine substrate for the luciferase reaction, which produces a light signal that is quantified using a luminometer and expressed as relative light units (RLU). The signal produced correlates to the amount of luciferase protein expressed, indicating promoter activity in the reporter assay.

Contents

QUANTI-Luc™ is provided in a 2- or 5-pouch unit. Each pouch makes 25 ml of reagent allowing the preparation of 500 wells of a 96-well plate.

PRODUCT	QUANTITY	CAT. CODE
QUANTI-Luc™	2 pouches (2 x 25 ml)	rep-qlc1
	5 pouches (5 x 25 ml)	rep-qlc2

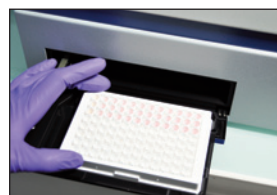
QUANTI-Luc™ Procedure



1. Prepare QUANTI-Luc by adding 25 ml water to the contents of one pouch.



2. Transfer aliquots of cell culture medium to opaque 96-microwell plate.



3. Set up the luminometer prior to addition of 50 µl QUANTI-Luc™ reagent to each well either manually or by automated injection. Measure luminosity in endpoint mode when using Lucia™ or in kinetic mode depending on the coelenterazine-luciferase used.

TLR9 Ligands

Synthetic oligodeoxynucleotides containing CpG motifs (CpG ODNs) are widely used to induce TLR9-dependent immune responses that vary according to their class (see below). Prototype CpG ODNs exist for human and mouse. InvivoGen introduces new CpG ODNs that are active in many species and also a new inhibitory ODN very efficient in blocking TLR9 activation. To compare the activities of stimulatory or inhibitory ODNs, InvivoGen offers a choice of TLR9 Ligand Discovery Kits. Each kit contains six ODNs that are either stimulatory, control or inhibitory of the TLR9 response. InvivoGen's TLR9 ligands are high quality products.

- **Functionally tested**, the activity of each lot of ODN is validated using cell-based assays
- **Guaranteed contamination-free** to avoid TLR9-independent immune responses

➤ Stimulatory CpG ODNs

ODN BW006

NEW

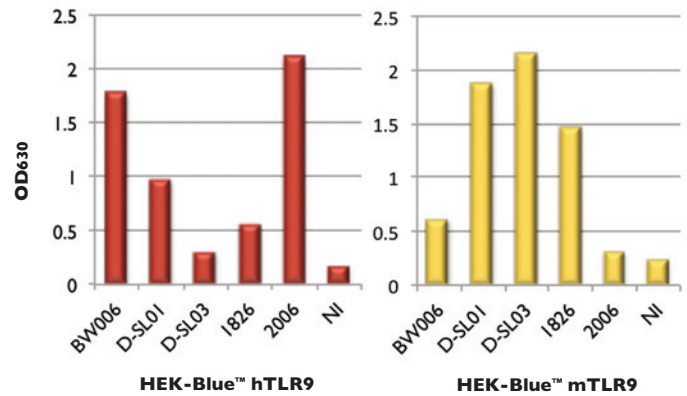
ODN BW006 (also known as ODN 684) is a class B ODN containing twice the optimal motif in human, GTCGTT¹. ODN BW006 is capable of inducing the proliferation of human PBMC and mouse splenocytes as vigorously as ODN 2006, a class B prototype ODN. It was found to improve the rabies vaccine by inducing an earlier and more vigorous protective response. ODN BW006 promotes strong Th1 responses^{2,3}.

ODN BW007 is a control ODN that contains GpC dinucleotides instead of CpGs and can be used as a negative control together with ODN BW006.

ODN D-SL01 & ODN D-SL03

NEW

ODN D-SL01 and ODN D-SL03 are double stem loop ODNs belonging to the B class and C class CpG ODNs, respectively⁴. Both of them have been shown to potently activate human B cells, NK cells and mononuclear cells as well as PBMC/splenocytes obtained from diverse vertebrate species (mouse, rat, rabbit, guinea pig, swine and dog).



Response of human and mouse TLR9 reporter cells to stimulatory CpG ODNs: HEK-Blue™ hTLR9 and HEK-Blue™ mTLR9 were stimulated with 1 µg/ml or 0.01 µg/ml CpG ODNs, respectively. After 24h incubation, the levels of NF-κB-induced SEAP were determined using QUANTI-Blue™.

➤ Inhibitory ODNs

ODN INH-18

NEW

ODN INH-18 is a linear and class R ('restricted') inhibitory ODN. It contains an inhibitory DNA motif consisting of two nucleotide triplets, a proximal CCT and a more distal GGG, spaced from each other by four nucleotides. ODN INH-18 is a potent inhibitor of TLR9-induced B cells and macrophages⁵. ODN INH-18 strongly blocks TLR9 activation in both human and mouse TLR9-expressing cells (see figure p. 7).

PRODUCT	QUANTITY	CAT. CODE
ODN D-SL01	200 µg	tlr-ds101
ODN D-SL03	200 µg	tlr-ds103
ODN BW006	200 µg	tlr-bw006
ODN BW007	200 µg	tlr-bw007
ODN INH-18	200 µg	tlr-inh18

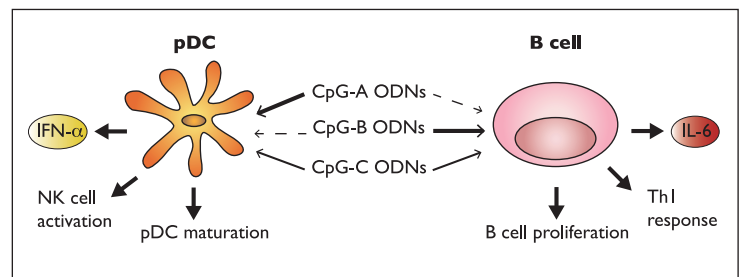
CpG ODN Classes

Bacterial DNA contains unmethylated "CpG motifs" that are recognized by the pattern recognition receptor Toll-like receptor (TLR) 9 and induce strong immunostimulatory effects in mammals. Synthetic oligodeoxynucleotides containing such CpG motifs (CpG ODNs) stimulate B cells, natural killer (NK) cells and professional antigen-presenting cells to proliferate and/or secrete a variety of cytokines, chemokines and immunoglobulins. Three major classes of stimulatory CpG ODNs have been identified based on structural characteristics and activity on human peripheral blood mononuclear cells (PBMCs), in particular B cells and plasmacytoid dendritic cells (pDCs). These three classes are Class A (Type D), Class B (Type K) and Class C.

- **Class A** CpG ODNs are characterized by a PO central CpG-containing palindromic motif and a PS-modified 3' poly-G string. They induce high IFN-α production from pDCs but are weak stimulators of TLR9-dependent NF-κB signaling and pro-inflammatory cytokine production.

- **Class B** CpG ODNs contain a full PS backbone with one or more CpG dinucleotides. They strongly activate B cells and TLR9-dependent NF-κB signaling but weakly stimulate IFN-α secretion.

- **Class C** CpG ODNs combine features of both classes A and B. They contain a complete PS backbone and a CpG-containing palindromic motif. C-Class CpG ODNs induce strong IFN-α production from pDC as well as B cell stimulation.



➤ TLR9 Ligand Discovery Kits **NEW**

Human TLR9 Agonist Kit

This kit features prototype CpG ODNs of the A, B or C class that function best in human. These CpG ODNs are often cited in the literature. This kit will help you choose the most appropriate class of CpG ODNs for studies in human cells.

- ODN 2006 and ODN 2006 control - B-class
- ODN 2216 and ODN 2216 control - A class
- ODN 2395 and ODN 2395 control - C-class

Mouse TLR9 Agonist Kit

This kit contains prototype CpG ODNs of the A-, B- or C-class described to work well in mice studies. This kit allows to compare their effectiveness and select the most suitable one for a given application in murine cells or mice.

- ODN 1585 and ODN 1585 control - A-class
- ODN 1826 and ODN 1826 control - B class
- ODN 2395 and ODN 2395 control - C-class

B-Class TLR9 Agonist Kit - Multispecies

CpG ODNs of this kit belong exclusively to the B class and are active in human and/or mouse and other species. The kit contains prototype CpG ODNs as well as less popular but worth testing CpG ODNs.

- ODN 1668 and ODN 1826 - B-class, mouse preferred
- ODN 2006 - B class, human preferred
- ODN BW006 - B class, human/mouse
- ODN 2007 - B-class, bovine/porcine
- ODN D-SL01 - B-class, multispecies

A&C-Classes TLR9 Agonist Kit - Multispecies

This kit contains CpG ODNs that belong to the A or C class. They are active in several species.

- ODN 1585 - A-class, mouse preferred
- ODN 2216 and ODN 2336 - A class, human preferred
- ODN 2395 and ODN M362 - C-class, human/mouse
- ODN D-SL03 - C-class multispecies

TLR9 Antagonist Kit - Multispecies

This kit contains inhibitory ODNs, that are active in human and/or mouse, and a control ODN, that can be used with each inhibitory ODN.

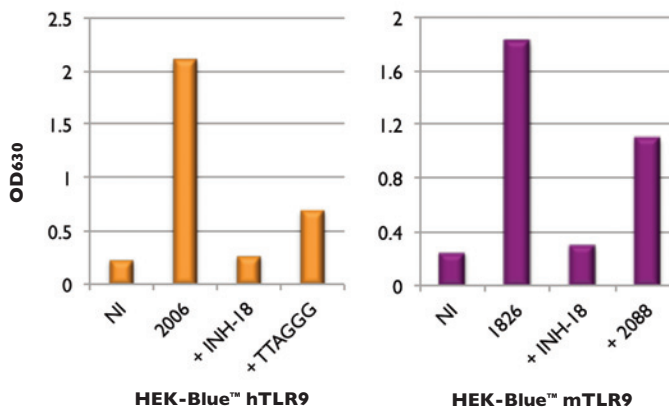
- ODN 2088 - Mouse preferred
- ODN 4084-F - Human/mouse
- ODN INH-I - Human/mouse
- ODN INH-18 - Human/mouse
- ODN TTAGGG - Human preferred
- Neutral ODN - Control ODN

1. Wang X. et al., 2008. A CpG oligodeoxynucleotide acts as a potent adjuvant for inactivated rabies virus vaccine. *Vaccine*. 26(15):1893-901. 2. Yan Y. et al., 2012. A CpG oligodeoxynucleotide potentiates the anti-tumor effect of HSP65-Her2 fusion protein against Her2 positive B16 melanoma in mice. *Int Immunopharmacol*. 12(2):402-7. 3. Zhang X. et al., 2011. Enhanced specific immune responses by CpG DNA in mice immunized with recombinant hepatitis B surface antigen and HB vaccine. *Virology*. 418:78-84. 4. Yang L. et al., 2013. CpG oligodeoxynucleotides with double stem-loops show strong immunostimulatory activity. *Int Immunopharmacol*. 15(1):89-96. 5. Lenert P. et al., 2009. DNA-like class R inhibitory oligonucleotides (INH-ODNs) preferentially block autoantigen-induced B-cell and dendritic cell activation in vitro and autoantibody production in lupus-prone MRL-Fas(lpr/lpr) mice in vivo. *Arthritis Res Ther*. 11(3):R79.

Contents

Stimulatory and inhibitory ODNs are provided lyophilized. Larger quantities are available: 1 mg, 5 mg and bulk. Each ODN is supplied with sterile endotoxin-free water. Sequences are available online.

PRODUCT	QUANTITY	CAT. CODE
Human TLR9 Agonist Kit		
- ODN 2006	100 µg	tlr1-kit9h
- ODN 2006 control (ODN 2137)	100 µg	
- ODN 2216	100 µg	
- ODN 2216 control (ODN 2243)	100 µg	
- ODN 2395	100 µg	
- ODN 2395 control	100 µg	
Mouse TLR9 Agonist Kit		
- ODN 1585	100 µg	tlr1-kit9m
- ODN 1585 control	100 µg	
- ODN 1826	100 µg	
- ODN 1826 control (ODN 2138)	100 µg	
- ODN 2395	100 µg	
- ODN 2395 control	100 µg	
B-Class TLR9 Agonist Kit		
- ODN 1668	100 µg	tlr1-kit9b
- ODN 1826	100 µg	
- ODN 2006	100 µg	
- ODN 2007	100 µg	
- ODN BW006	100 µg	
- ODN D-SL01	100 µg	
A&C-Class TLR9 Agonist Kit		
- ODN 1585	100 µg	tlr1-kit9ac
- ODN 2216	100 µg	
- ODN 2336	100 µg	
- ODN 2395	100 µg	
- ODN D-SL03	100 µg	
- ODN M362	100 µg	
TLR9 Antagonist Kit		
- ODN 2088	100 µg	tlr1-kit9i
- ODN 4084-F	100 µg	
- ODN INH-I	100 µg	
- ODN INH-18	100 µg	
- ODN TTAGGG (ODNA151)	100 µg	
- Neutral ODN	100 µg	



Inhibition of TLR9 signaling in human and mouse TLR9 reporter cells: HEK-Blue™ hTLR9 and HEK-Blue™ mTLR9 were stimulated with 1 µg/ml ODN 2006 or ODN 1826 respectively, alone or with 1 µg/ml inhibitory ODN. After 24h incubation, inhibition of TLR9 activation was determined by measuring the levels of NF-κB-induced SEAP using QUANTI-Blue™.

Related products

- HEK-Blue™ hTLR9, 3-7 × 10⁶ cells (hkb-htlr9)
- HEK-Blue™ mTLR9, 3-7 × 10⁶ cells (hkb-mtlr9)
- QUANTI-Blue™, 10 pouches (rep-qb2)

Endotoxin Detection

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.

HEK-Blue™ LPS Detection Kit 2 NEW

- **Simple** - Requires basic cell culture knowledge and no specific lab equipment
- **Versatile** - Measure endotoxin level in virtually all biological reagents
- **Highly sensitive** - Detect as little as 0.01 EU/ml
- **Economical** - Up to 500 samples can be tested with the kit

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 (TLR) 4, the mammalian endotoxin sensor (Beutler B., 2002). TLR4 recognizes structurally different LPS from gram-negative bacteria. Proprietary cells engineered to become extremely sensitive to LPS, called **HEK-Blue™-4 cells**, are the main feature of this endotoxin detection kit. These cells stably express human TLR4 and an NF-κB-inducible secreted embryonic alkaline phosphatase (SEAP) reporter gene. 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 SEAP detection medium that produces a purple/blue color; 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 obtained using serial dilutions of the **HEK-Blue™ Endotoxin Standard** (a preparation of *E. coli* 055:B5 LPS standardized against FDA approved control standard endotoxin (CSE)).

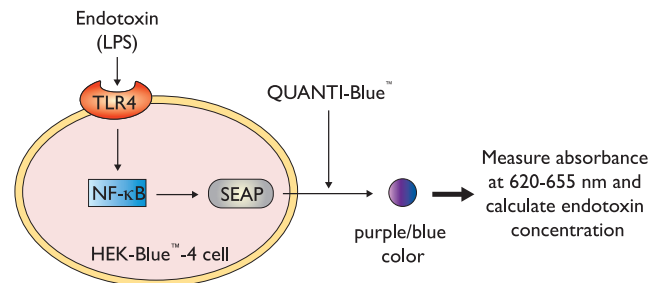
Contents

The HEK-Blue™ LPS Detection Kit is composed of the following components:

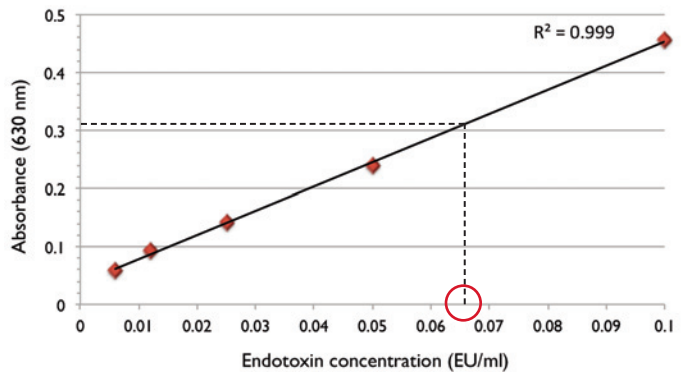
- 1 vial of HEK-Blue™-4 cells (3-7 × 10⁶ cells)
- 4 tubes of 250X HEK-Blue™ Selection (2 ml each)
- 4 tubes of 500X Normocin™ (1 ml each)
- 1 pouch of QUANTI-Blue™ (100 ml)
- 2 tubes of HEK-Blue™ Endotoxin Standard (50 EU each)
- 1 bottle of endotoxin-free water (50 ml)

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

Principle



Calculation of Endotoxin Concentration (Graphic Method)



Principle of the HEK-Blue™ LPS Detection Kit 2 - A small volume (20 μl) of the sample or a serial dilution of the HEK-Blue™ Endotoxin Standard is added to the HEK-Blue™-4 cells. Endotoxins present in the sample or standard are sensed by TLR4 leading to the activation of NF-κB and the production of SEAP in the supernatant. When a small volume (20 μl) of the supernatant is combined with QUANTI-Blue™, which contains a SEAP chromogenic substrate, a purple/blue color appears. SEAP is quantitated by measuring the absorbance at 620-655 nm and extrapolating against a standard curve.



PRODUCT	QTY	CAT. CODE
HEK-Blue™ LPS Detection Kit 2	1 kit	rep-lps2
HEK-Blue™ Selection	5 × 2 ml	hb-sel
Normocin™	10 × 1 ml	ant-nr-1
QUANTI-Blue™	5 pouches	rep-qb-1
HEK-Blue™ Endotoxin Standard	10 × 50 EU	rep-hbes-10

Buy the HEK-Blue™ LPS Detection Kit once then reorder only the reagents to perform further assays.