

# InvivoGen Insight

An Insightful Look At InvivoGen's Innovative Products

The main focus of this new issue of InvivoGen Insight is the Toll-like receptors (TLRs), in particular the various signaling pathways that they induce. Understanding these pathways at the molecular level is key to develop novel therapies for septic shock and immune diseases. To assist you in your research, InvivoGen provides a comprehensive collection of genes encoding the TLRs and related proteins, such as the interferon regulatory factors (IRFs), and the IKK-related kinases, IKK $\epsilon$  and TBK1. TLR4 signal transduction activated by lipopolysaccharide (LPS) is

the best understood. This knowledge has led InvivoGen to develop an innovative system to detect the presence of LPS in any sample. Another type of ligands that is being extensively studied is the CpG ODNs. Depending on their sequence and structure, these TLR9 ligands can be stimulatory or inhibitory. Interestingly, their effect seems to extend to the signaling of TLR7. InvivoGen provides a large choice of CpG ODNs. Lastly, as a leader in antibiotic production, InvivoGen offers two new promising analogues of geldanamycin, a potent antitumor agent.

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## Toll-Like Receptors and Viral Infection

Toll-like receptors (TLRs) are essential for the recognition of microbial pathogens. A subset of TLRs, TLR3, TLR7/8, and TLR9, is involved in antiviral responses by triggering the production of antiviral cytokines such as type I interferons (IFNs). TLR3 responds to double stranded RNA, a replication intermediary for many viruses. TLR7/8 recognize viral single-stranded RNAs, whereas TLR9 recognizes unmethylated CpG motifs within viral DNA<sup>1</sup>.

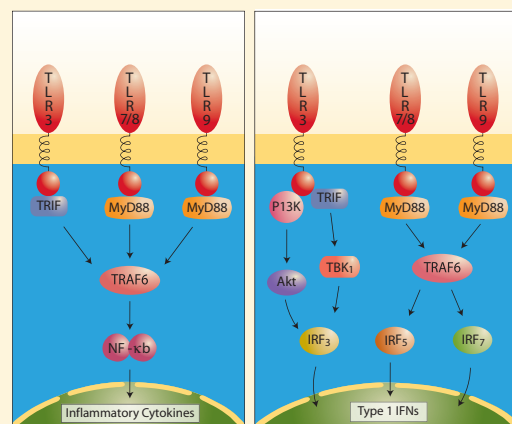
Expression of type I IFNs (IFN $\alpha/\beta$ ) is regulated by at least three families of transcription factors: c-jun/ATF-2, NF- $\kappa$ B and the IFN regulatory factors (IRFs). IFN $\alpha$  promoter contains IRF3, IRF5 and IRF7 binding sites while IFN $\beta$  promoter has mainly IRF3 and IRF7 binding sites. In unstimulated cells, IRFs reside in the cytoplasm at low levels in an inactive form. Upon viral infection their production is enhanced, followed by their activation by phosphorylation allowing them to translocate to the nucleus. Activated IRFs form homo- or heterodimers: IRF3/IRF5 and IRF3/IRF7 heterodimers are active while the IRF5/IRF7 heterodimer is inactive and acts as a repressor of IFN $\alpha/\beta$  expression<sup>2</sup>.

TLRs involved in virus recognition are expressed on endosomal membranes and can be separated according to their requirement for the adaptor protein MyD88: TLR3 activity is MyD88-independent while TLR7/8/9 depend on MyD88. These two families mobilize a different set of adaptor molecules to activate different IRFs. Upon stimulation, TLR3 recruits TRIF which in turn activates TBK1 and/or IKK $\epsilon$ , two IKK-related kinases responsible for IRF3 activation by phosphorylation. The full activation of IRF3 requires the recruitment by TLR3 of an additional kinase, PI3 kinase, which interacts with a second kinase, Akt<sup>3</sup>. TLR7/8/9 use MyD88 to mobilize the signaling proteins TRAF6 and IRAK4 leading to the activation of IRF7<sup>4</sup>. Recent data suggest that IRF3 can be activated independently of TLR3 through two cytoplasmic RNA helicases: RIG-I and mda5<sup>5</sup>. These helicases might be connected through their CARD domain to the death

domain containing proteins FADD and RIP1 resulting in TBK1 induced activation of IRF3<sup>6</sup>.

Interestingly, the contact between a virus and a TLR-expressing cell is often sufficient to induce type I IFN production without need for infection, allowing uninfected cells to participate in the antiviral responses<sup>2</sup>. This feature can be exploited to study virus-recognizing TLRs by mimicking virus-TLR interactions using synthetic ligands such as poly(I:C) (TLR3 ligand), ssRNA40 (TLR7/8 ligand) or CpG ODNs (TLR9 ligands). To facilitate your discoveries in this area, InvivoGen offers a large selection of such ligands as well as an expanding collection of genes involved in these signaling pathways.

1. Boehme KW. & Compton T., 2004. Innate sensing of viruses by toll-like receptors. *J Virol* 78, 7867-7873
2. Malmgaard L., 2004. Induction and regulation of IFNs during viral infections. *J Interferon Cytokine Res* 24, 439-454
3. Sen GC. & Sarkar SN., 2005. Transcriptional signaling by double-stranded RNA: role of TLR3. *Cytokine Growth Factor Rev* 16, 1-14
4. Kawai T., Sato S., Ishii KJ. *et al.*, 2004. Interferon-alpha induction through Toll-like receptors involves a direct interaction of IRF7 with MyD88 and TRAF6. *Nat Immunol* 5, 1061-1068
5. Li K. *et al.*, 2005. Distinct poly-I: C and virus-activated signaling pathways leading to interferon-beta production in hepatocytes. *J Biol Chem* [Epub ahead of print]
6. Balachandran S. *et al.*, 2004. A FADD-dependent innate immune mechanism in mammalian cells. *Nature* 432, 401-405



# HEK-Blue™ LPS Detection Kit

## Detection based on Signaling through Toll-Like Receptor 4

The HEK-Blue™ LPS Detection Kit is a simple, rapid and reliable system to detect the presence of lipopolysaccharide (LPS) in a sample. This system is based on the ability of TLR4 and its accessory co-receptors to bind LPS and to trigger intracellular signaling through MyD88 and TRIF leading to the activation of a set of transcription factors. Presence of LPS is evidenced by the secretion of alkaline phosphatase induced by these transcription factors.

### • Easy-to-use

- **Only basic cell culture knowledge required** - Can be easily established as a routine procedure in the lab.
- No need for specific lab equipment - Follow the reaction over time with the naked eye or a spectrophotometer.

### • Rapid

- **Hand-on time less than 1 hour**
- Selection and detection media included in the kit allow rapid expansion and use of HEK-Blue™-4 cells

### • Cost-effective

- **Up to 1000 tests with one kit.** Additional tests can be performed by purchasing only the needed components of the kit.
- HEK-Blue™-4 cells can be expanded and frozen for further use.

### Key Features

**HEK-Blue™-4 cells** are engineered HEK293 cells stably transfected with multiple genes involved in LPS recognition that include TLR4 and the co-receptors MD2 and CD14. In addition, HEK-Blue™-4 cells stably express an optimized secreted alkaline phosphatase gene (sAP), placed under the control of a modified human IL12p40 promoter inducible by several transcription factors such as NF-κB, AP-1 and IRFs. TLR4-induced production of sAP reflects the presence of LPS in the sample tested.

**HEK-Blue™-4 Selection** combines several selective antibiotics to guarantee the persistent expression of the various transgenes introduced in HEK-Blue™-4 cells. Furthermore, Normocin™ is included in the kit to protect HEK-Blue™-4 cells from any potential microbial contamination.

**HEK-Blue™ Detection** is a medium specifically designed for the detection of sAP produced by activated HEK-Blue™ cells. This medium turns into a purple/blue color in the presence of sAP allowing TLR4 signaling to be observed with the naked eye or quantified spectrophotometrically. sAP being a secreted protein, kinetic studies can be performed.

Presence of concentrations of LPS as low as 0.3 ng/ml can be detected using the HEK-Blue™ LPS Detection Kit.

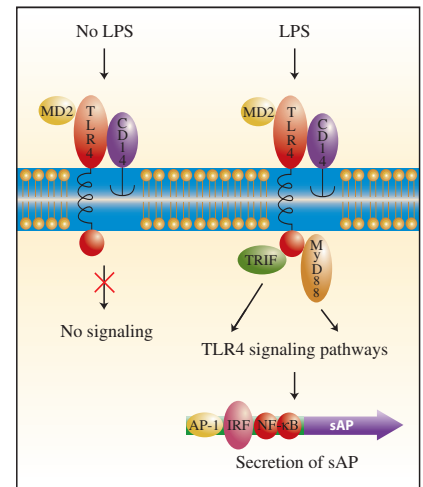
This system is highly specific to LPS or any TLR4 ligand. Ligands to other TLRs will not induce the signaling pathways leading to sAP secretion.

### Kit Contents

- HEK-Blue™-4 cells (3-5 x 10<sup>6</sup> cells)
- HEK-Blue™-4 Selection (4 x 2 ml)
- Normocin™ (4 x 1 ml)
- HEK-Blue™ Detection (2x 100 ml)
- *E. coli* K12 LPS (100 µg), positive control
- Endotoxin-free water (1.5 ml), negative control

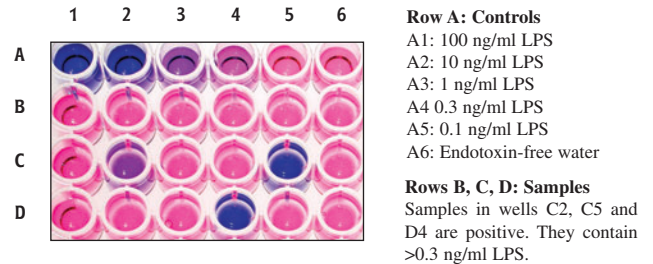
Most components of the HEK-Blue™ LPS Detection Kit can be purchased separately.

Product	Quantity	Code	Price
HEK-Blue™ LPS Detection Kit	1 kit	rep-lps	\$1,300



### Procedure

- 1- Grow HEK-Blue™-4 cells in DMEM medium supplemented with FBS, Pen/Strep, Normocin™ and HEK-Blue™-4 Selection solution.
- 2- Add your sample in a well of a multi-well plate.
- 3- Trypsinize HEK-Blue™-4 cells and resuspend them in HEK-Blue™ Detection medium.
- 4- Add HEK-Blue™-4 cells to the wells containing the samples.
- 5- Incubate 18-24h at 37°C in 5% CO<sub>2</sub> before assessing the blue colour by the naked eye or read the OD at 620-655 nm with a microplate reader.



[www.invivogen.com/TLR/LPS\\_detection.htm](http://www.invivogen.com/TLR/LPS_detection.htm)

## IRFs, TBK1 & IKKε Intermediaries of the TLR Signaling Pathways

Interferon Regulatory Factors (IRFs) and IKK-related kinases play an important role in the intracellular signaling pathways of TLRs involved in antiviral responses. Upon virus stimulation, TLRs trigger the activation of IRF3, 5, 7 through phosphorylation leading to the production of type I IFNs. IRF phosphorylation was shown recently to be mediated by two IKK-related kinases, IKKε and TANK-1-binding kinase 1 (TBK1).

# TLR Ligands

## CpG ODNs: Inducers of TLR9 signaling but inhibitors of TLR7 signaling?

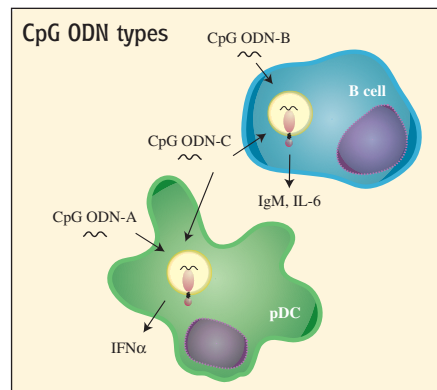
Bacterial and viral DNA induce strong immunostimulatory effects through the activation of TLR9 due to the presence of unmethylated CpG dinucleotides in particular sequence contexts. TLR9 activation can be mimicked by synthetic CpG oligodeoxynucleotides (ODNs).

Two types of immunostimulatory CpG-ODNs have been described. Type A (or D) ODNs preferentially activate plasmacytoid dendritic cells (pDC) to produce IFN $\alpha$ , whereas type B (or K) ODNs induce the proliferation of B cells and the secretion of IgM and IL-6. Another type has been generated that combines features of both types A and B termed type C<sup>1</sup>.

Type A ODNs are characterized by a central CpG-containing palindromic motif and a 3' poly-G string that further increases their activity. They are chimeric ODNs with a central phosphodiester (PD) backbone and flanking phosphorothioate (PTO) modifications. Type B ODNs contain multiple CpGs, preferentially located at the 5' end, and a PTO backbone.

Cellular uptake is a prerequisite for CpG-induced signal transduction as TLR9 is expressed in the endosome. Uptake of CpG-ODNs in mice is independent of CpG motifs while signaling is strictly dependent on such structures. Indeed, PTO ODNs are taken up much more efficiently than their PD counterparts. However, PTO ODNs are associated with CpG-independent immunostimulatory effects and seem to induce a slightly different profile than PD ODNs. Type B prototype ODN 2006 in its PD form is poorly internalized. Addition of a 3' poly-G string (ODN 2006-G5) was reported to improve its internalization which was correlated with increased IL-6 secretion and PBMC proliferation<sup>2</sup>. Thus, internalization of PD ODNs can be improved by adding a 3' poly-G string with no need for PTO modifications.

Changing as few as two bases in an



immunostimulatory (IS) ODN can convert it into an inhibitory (IN) ODN. The optimal structure for IN-ODNs (ODN2088) is a single-stranded DNA containing a pyrimidine-rich triplet, preferably CCT, positioned in 5' position to the GGG sequence<sup>3</sup>. This structure is similar for inhibition of both IS-ODN types. An additional IN-ODN, derived from a motif found in mammalian telomeres (TTAGGG), has been described. It prevents the colocalization of IS-ODNs with TLR9 within endosomal vesicles. Potentially, IN-ODNs could be a way to control overactivation of the immune system observed in autoimmune diseases and thereby are of great interest for pharmaceutical companies.

Recently, Barrat FJ *et al.* suggested that some ODNs that inhibit TLR9 could also block TLR7

ODN 2216 (A)	<u>ggGGG</u> <u>CGA</u> : <u>TCGT</u> Cgggggg
ODN 2006 (B)	<u>tcg</u> tctgttttgcgttttgcgtt
ODN 2006-G5 (B)	<u>TCGTCG</u> TTTTGT <u>CG</u> TTTTGT <u>CG</u> TTGGGGG
ODN M362 (C)	<u>tcg</u> tctgttcggtcgaacgacggtgat
ODN 2088 (IN)	tctggcggggaagt

Bases in capital letters are phosphodiester, and those in lower case are phosphorothioate. Palindromes are underlined.

and TLR8 signaling. Furthermore, they found ODN inhibitors of TLR7 and 8 but not of TLR9 (communication, Keystone symposia). In-house experiments using HEK293 cells expressing TLR7, 8 or 9 have confirmed these data. We found that all our ODNs, whether stimulatory or inhibitory, containing or not CpGs, were inhibitory on TLR7 but interestingly not on TLR8. Our results suggest that the ODNs could exert their TLR7-specific inhibitory effect by affecting the binding of the ligand to TLR7, directly or through a still unknown accessory molecule. Further experiments are needed to understand the mechanism underlying this inhibitory effect.

Invivogen commercializes a large panel of CpG ODNs continuously updated according to the literature.

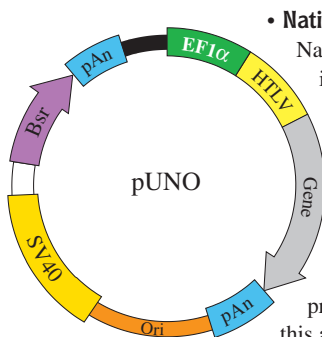
CpG-ODNs are provided as 200  $\mu$ g units.

**Price \$150**

1. Marshall JD. *et al.*, 2005. Superior activity of the type C class of ISS in vitro and in vivo across multiple species. *DNA Cell Biol.* 24(2):63-72.
2. Bartz H. *et al.*, 2004. Poly-guanosine strings improve cellular uptake and stimulatory activity of phosphodiester CpG oligonucleotides in human leukocytes. *Vaccine.* 23(2):148-55.
3. Ashman RF. *et al.*, 2005. Sequence requirements for oligodeoxyribonucleotide inhibitory activity. *Int Immunol.* 2005 Mar 3 [ahead of print]

Product	Code
ODN 2006 (type B)	tlrl-hodnb
ODN 2006-G5 (type B)	tlrl-hodnbg
ODN 2216 (type A)	tlrl-hodna
ODN M362 (type C)	tlrl-hodnc
ODN 2088	tlrl-minhodn
ODN TTAGGG	tlrl-hinhodn

InvivoGen provides native or engineered versions of these genes in pUNO, an expression plasmid selectable with blasticidin in *E. coli* and mammalian cells. The expression of the gene of interest is strong, constitutive and ubiquitous.



### • Native Genes

Native genes containing the wild-type, full-length sequence are intronless genes cloned from the ATG to the Stop codon.

### • Super-Activated Genes

- Super activated IRF3 (saIRF3) was generated by creating a single point mutation of Ser<sup>396</sup> to Asp and presents a >10-fold enhanced transactivating potential over the wild-type IRF-3 for the IFN- $\alpha$  and IFN- $\beta$  promoters<sup>1</sup>.
- Super activated IRF7 (saIRF7 $\Delta$ ) was constructed by deleting the auto-inhibitory domain ( $\Delta$ <sup>238-410</sup>) producing a protein extremely more active than full-length IRF7, even in this absence of viral infection<sup>2</sup>.

1. Servant MJ. *et al.*, 2003. Identification of the minimal phosphoacceptor site required for in vivo activation of interferon regulatory factor 3 in response to virus and double-stranded RNA. *J Biol Chem.* 278(11):9441-7.
2. Marie I. *et al.*, 2000. Phosphorylation-induced dimerization of interferon regulatory factor 7 unmasks DNA binding and a bipartite transactivation domain. *Mol Cell Biol.* 20(23):8803-14.

Product	Species	Code	Price
<b>Native Genes</b>			
pUNO-hIKK $\epsilon$	human	puno-hikke	\$435
pUNO-hIRF3	human	puno-hirf3	\$435
pUNO-mIRF3	mouse	puno-mirf3	\$435
pUNO-hIRF5b	human	puno-hirf5	\$435
pUNO-mIRF5	mouse	puno-mirf5	\$435
pUNO-hIRF7	human	puno-hirf7	\$435
pUNO-mIRF7	mouse	puno-mirf7	\$435
pUNO-hTBK1	human	puno-htbk1	\$435
<b>Super-Activated Genes</b>			
pUNO-sahIRF3	human	puno-sahirf3	\$455
pUNO-sahIRF7 $\Delta$	human	puno-sahirf7d	\$455

**More dominant-positive and negative genes coming soon.**

[www.invivogen.com/TLR/TLR\\_genelist.htm#TLR-asso](http://www.invivogen.com/TLR/TLR_genelist.htm#TLR-asso)

# Geldanamycin Analogues

## A Family of Potent Antitumoral Agents

- **Largest Collection Available**

InvivoGen provides Geldanamycin and five of its more promising analogs including two new ones.

- **Highest Purity Achievable**

Purity >98% - Elimination of Nigericin a common contaminant of many commercially available Geldanamycin preparations.

- **Lowest Price Guaranteed**

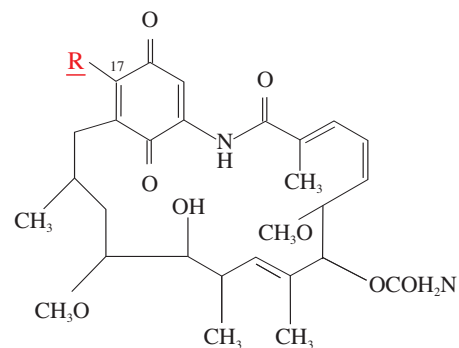
If you find a lower price for these compounds that is legitimate, InvivoGen will gladly match the price.

Geldanamycin (GA), a benzoquinone ansamycin antibiotic, interferes with the action of the heat shock protein 90 (Hsp90) leading to the degradation of Hsp90 client proteins. Since many of these clients proteins are involved in signal transduction and transcription, GA has a potential utility in cancer chemotherapy.

GA analogues with alkylamino groups in place of the methoxy moiety at C-17 are less cytotoxic and remain biologically active. Numerous 17-alkylamino-17-demethoxygeldanamycin analogues have been synthesized, including **17-AAG** which is currently in phase I clinical trial in several centers worldwide.

Recently, three potent GA analogues have been identified: **17-DMAG** (NSC707545), **17-AEP-GA** and **17-DMAP-GA**<sup>1,2</sup>. They showed significant growth inhibition of cancer cells (IC50 below 100 nM). Their binding affinity to Hsp90 was not significantly affected while their water solubility was greatly improved compared to 17-AAG.

GA cytotoxicity can be targeted to tumor cells by the conjugation of GA to a monoclonal antibody (mAb), such as Herceptin<sup>3</sup>. This conjugation is made possible by the addition of a maleimido group at C17 generating a new GA analogue termed **17-GMB-APA-GA**.



Compound	Radical (R)	Kd (μM)	Solubility (g/l)
Geldanamycin	MeO	-	-
17-AAG		1.3	0.1
17-DMAG		0.5	1.4
17-AEP-GA		0.4	3.7
17-DMAP-GA		0.6	4.6

### REFERENCE

1. Eiseman JL. *et al.*, 2005. Pharmacokinetics and pharmacodynamics of 17-demethoxy-17-[[2-(dimethylamino)ethyl]amino]geldanamycin (17DMAG, NSC 707545) in C.B-17 SCID mice bearing MDA-MB-231 human breast cancer xenografts. *Cancer Chemother Pharmacol.* 55(1):21-32.
2. Tian ZQ. *et al.*, 2004. Synthesis and biological activities of novel 17-aminogeldanamycin derivatives. *Bioorg Med Chem.* 12(20):5317-29.
3. Mandler R. *et al.*, 2004. Herceptin-geldanamycin immunoconjugates: pharmacokinetics, biodistribution, and enhanced antitumor activity. *Cancer Res.* 64(4):1460-7.

Product	Quantity*	Code	Price
Geldanamycin	1 mg	ant-gl-1	\$95
17-AAG	1 mg	ant-agl-1	\$135
17-GMB-APA-GA	1 mg	gmbapa-ga	\$175
17-DMAG	1mg	ant-dgl-1	\$165
17-AEP-GA	1mg	ant-egl-1	\$165
17-DMAP-GA	1mg	ant-mgl-1	\$165



\*Also provided in 5 mg - **Bulk quantities readily available**



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