

InvivoGen Insight

As the leading supplier of TLR products, InvivoGen is constantly seeking to expand its product line by developing new tools to study the TLRs. This issue features three new synthetic molecules that act as TLR7 and/or TLR8 ligands, psiRNA-TLR a family of plasmids designed for the silencing of TLRs, and two new TLR-expressing cell lines. THP1-Blue™ and THP1-Blue™-CD14 are human monocytic cells stably transfected with an NF-κB-inducible reporter plasmid. These cells represent a useful model for TLR studies and can be utilized for the detection of endotoxins and pyrogens.

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Review

TLR7 and TLR8: Key players in the antiviral response

InvivoGen™

TLR7 and TLR8: Key players in the antiviral response

TLR7 and TLR8 are phylogenetically and structurally related. TLR7 is predominantly expressed in lung, placenta, and spleen, while TLR8 is predominantly expressed in lung and peripheral blood leukocytes, in particular monocytes.

Using TLR7-deficient mice and HEK293 cells transfected with human TLR7, Hemmi *et al.* have demonstrated that TLR7 is involved in the recognition of the imidazoquinoline compounds imiquimod (R837) and resiquimod (R848)¹. Human TLR8 also mediates the recognition of R848 but surprisingly not R837. Mouse TLR8 recognizes neither of these compounds suggesting that TLR8 is nonfunctional in mice². Furthermore, Lee *et al.* reported that guanosine analogs, such as loxoribine, activate immune cells via TLR7 but not TLR8³.

Recently, the natural ligands of TLR7 and TLR8 were identified as single-stranded RNA (ssRNA)^{4,6}. Mouse TLR7, human TLR8, and to a lesser extent human TLR7, recognize ssRNA viruses such as the influenza^{5,6}, Sendai⁷ and Coxsackie B⁸ viruses. This recognition requires the internalization of the virus and its replication to release the viral RNA into endosomes, where TLR7 and TLR8 reside. The interaction between the ssRNA and TLR7/8 triggers the recruitment of the adapter molecule MyD88 leading to the activation of NF-κB and other transcription factors and the production of proinflammatory cytokines and chemokines.

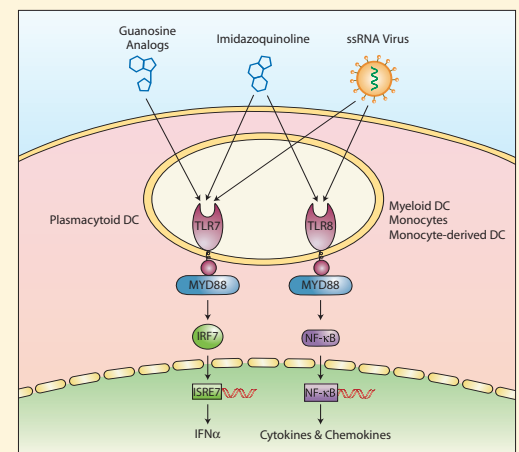
Purified viral ssRNAs or synthetic ssRNAs, complexed with cationic lipids to protect them from degradation and facilitate their internalization, can substitute for viruses. Although sequence specificity remains poorly defined, ssRNA containing poly(U)- or GU-rich sequences can stimulate TLR7 and TLR8^{4,5}. Synthetic siRNA duplexes containing a 5'-UGUGU-3' internal motif were also found to stimulate TLR7/8⁹. According to Hornung *et al.*, some siRNA motifs are stimulatory independently of their GU content suggesting the existence of specific sequences that are recognized by TLR7/8¹⁰. Since poly(U)- or GU-rich sequences are likely to be found in non-viral RNAs, it seems that TLR7 and TLR8 are able to discriminate between self and viral RNAs by recognizing the endocytic location of viral RNAs which serves as a molecular signature. Another factor that distinguishes mammalian RNA from viral and also bacterial RNA is the level of nucleoside modification. Microbial RNA which is rarely modified is a potent activator of dendritic cells (DCs) through TLR7, TLR8, and TLR3 in contrast to mammalian RNA which is abundant in modified nucleosides¹¹.

TLR7 and TLR8 agonists differ in their target cell

selectivity and cytokine induction profile. TLR7-specific agonists activate plasmacytoid DCs (pDCs) and B cells and induce mainly IFN-α and IFN-regulated cytokines. TLR8-specific agonists activate myeloid DCs, monocytes and monocyte-derived DC leading primarily to the production of proinflammatory cytokines and chemokines, such as TNF-α, IL-12 and MIP-1α¹².

The ability of TLR7-8 agonists to activate DCs and thus elicit Th1 and CD8+ T cells responses can be exploited to enhance the efficacy of vaccination^{13, 14}. They may represent better adjuvants than CpG ODN as TLR7 and TLR8 are broadly expressed on DCs and other antigen presenting cells in contrast to TLR9 which is only expressed on pDCs and B cells.

Activation of TLR7 and/or TLR8 represents a powerful and novel therapeutic strategy for the treatment of various viral infections. IFN-α is the mainstay treatment for chronic hepatitis C virus (HCV) infection but with partial success. TLR7 agonists were shown to impede anti-HCV infection by inducing IFN-α production but remain too toxic for clinical use. Thus development of new agonists is a major focus of many pharmaceutical companies.



TLR7 and TLR8 selectivity in human PBMC

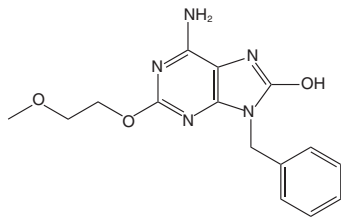
1. Hemmi H., Kaisho T. *et al.*, 2002. *Nat Immunol.* 3(2):196-200.
2. Jurk M., Heil F. *et al.*, 2002. *Nat Immunol.* 3(6):499.
3. Lee J., Chuang TH. *et al.*, 2003. *PNAS.* 100(11):6646-51.
4. Heil F, Hemmi H. *et al.*, 2004. *Science.* 303(5663):1526-9.
5. Diebold SS., Kaisho T. *et al.*, 2004. *Science.* 303(5663):1529-31.
6. Lund JM., Alexopoulou L. *et al.*, 2004. *PNAS.*101(15):5598-603.
7. Melchjorsen J., Jensen SB. *et al.*, 2005. *J Virol.* 79(20):12944-51.
8. Triantafyllou K., Orthopoulos G. *et al.*, 2005. *Cell Microbiol.* 7(8):1117-26.
9. Judge AD., Sood V. *et al.*, 2005. *Nat Biotechnol.* 23(4):457-62.
10. Hornung V., Guenther-Biller M. *et al.*, 2005. *Nat Med.* 11(3):263-70.
11. Kariko K, Buckstein M. *et al.*, 2005. *Immunity.*23(2):165-75.
12. Gorden KB., Gorski KS. *et al.*, 2005. *J Immunol.* 174(3):1259-68.
13. Craft N, Bruhn KW. *et al.*, 2005. *J Immunol.*175(3):1983-90.14.
14. Wille-Reece U, Flynn BJ. *et al.*, 2005. *PNAS* 102(42):15190-4.

New TLR7 and TLR8 Ligands

❖ CL087 - TLR7 ligand

CL087, an adenine derivative, induces the activation of NF-κB and the secretion of IFN-α in TLR7-expressing cells¹. CL087 is a TLR7-specific ligand, it does not stimulate TLR8 even at high concentrations (> 10 μg/ml). In TLR7-transfected HEK293 cells, CL087 induces the activation of NF-κB and other transcription factors at 0.3 μM (0.1 μg/ml).

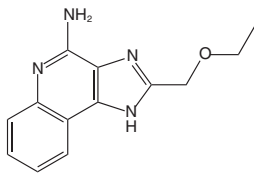
1. Lee J. *et al.*, 2006. Activation of anti-hepatitis C virus responses via Toll-like receptor 7. *Proc Natl Acad Sci U S A.* 103(6):1828-33.



$C_{15}H_{17}N_5O_3$
MW: 315.33

❖ CL097 - TLR7/8 ligand

CL097 is a highly water-soluble derivative of the imidazoquinoline compound R848. Similarly to R848, CL097 is a TLR7 and TLR8 ligand. It induces the activation of NF-κB at 0.4 μM (0.1 μg/ml) in TLR7-transfected HEK293 cells and at 4 μM (1 μg/ml) in TLR8-transfected HEK293 cells.

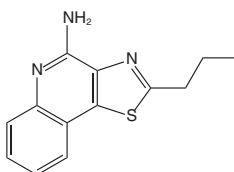


$C_{13}H_{14}N_4O$
MW: 242.28

❖ CL075 - TLR8/7 ligand

CL075 (3M002) is a thiazoloquinolone derivative that stimulates TLR8 in human PBMC. It activates NF-κB and triggers preferentially the production of TNF-α and IL-12². CL075 seems also to induce the secretion of IFN-α through TLR7 but to a lesser extent. It induces the activation of NF-κB at 0.4 μM (0.1 μg/ml) in TLR8-transfected HEK293 cells, and ~ 10 times more CL075 is required to activate NF-κB in TLR7-transfected HEK293 cells.

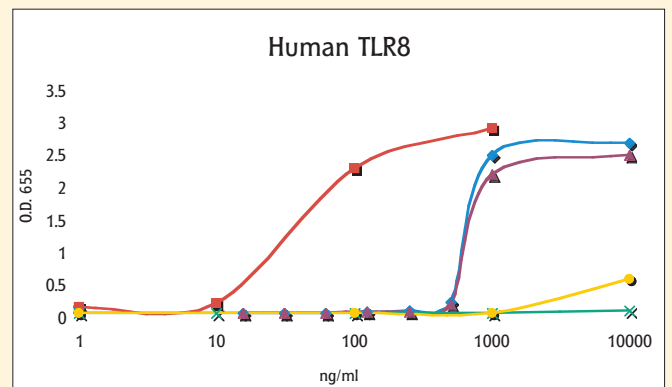
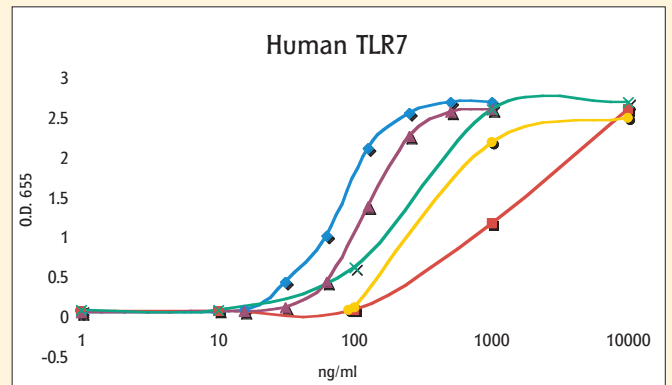
2. Gorden KB. *et al.*, 2005. Synthetic TLR agonists reveal functional differences between human TLR7 and TLR8. *J Immunol.* 174(3):1259-68.



$C_{13}H_{13}N_3S$
MW: 243.33

CL075 (3M002) references:

- Gorski KS. *et al.*, 2006. Distinct indirect pathways govern human NK-cell activation by TLR-7 and TLR-8 agonists. *Int Immunol.* 18(7):1115-26.
- Levy O. *et al.*, 2006. Unique efficacy of Toll-like receptor 8 agonists in activating human neonatal antigen-presenting cells. *Blood.* 108(4):1284-90.
- Qin J. *et al.*, 2006. TLR8-mediated NF-κB and JNK activation are TAK1-independent and MEK3-dependent. *J Biol Chem.* 281(30):21013-21.
- Wille-Reece U. *et al.*, 2006. Toll-like receptor agonists influence the magnitude and quality of memory T cell responses after prime-boost immunization in nonhuman primates. *J Exp Med.* 203(5):1249-58.



TLR7 and TLR8 stimulation: HEK293 cells transfected with human TLR7 or TLR8 and cotransfected with an IRF-NF-κB-inducible SEAP plasmid were incubated with CL075, CL087, CL097, gardiquimod and the control ligand R848 at the indicated concentrations. After overnight incubation, TLR7 and TLR8 stimulation was assessed by measuring the levels of SEAP secreted in the supernatant by using HEK-Blue™ Detection, a SEAP detection cell culture medium. Legend: ◆ R848 ■ CL075 ▲ CL097 × CL087 ● Gardiquimod

Check out invivogen.com
for more comparative data on
TLR7 and TLR8 ligands.

Product	Quantity	Catalog Code
CL075 NEW	1 mg	tlrl-c75
CL087 NEW	1 mg	tlrl-c87
CL097 NEW	1 mg	tlrl-c97
Gardiquimod	1 mg	tlrl-gdq
HEK-Blue Detection	2 x 50 ml	hb-det1

Monocyte Reporter Cells

THP1-Blue™ Clones

NEW

THP-1 are human peripheral blood monocytic cells. Monocytes play a key role in innate immunity and express most TLRs at various levels¹⁻³ (figure 1). As for the primary cells, THP-1 cells activate NF-κB and other transcription factors in response to TLR ligands and a variety of stimuli including phorbol esters (PMA) and cytokines. Unlike HEK293 cells that were engineered to respond to TLR agonists, THP-1 cells naturally express the TLR genes and all the genes involved in the signaling cascade. To facilitate analysis of TLR response in monocytes, InvivoGen provides two THP-1 clones stably transfected with an NF-κB-inducible reporter system, called THP1-Blue™. One of these clones, THP1-Blue™-CD14, overexpresses the cell surface protein CD14 for enhanced sensitivity. THP1-Blue™ and THP1-Blue™-CD14 clones are a useful and convenient model system for TLR studies. Furthermore they can be used for the detection of endotoxins and pyrogens in biological samples.

Description

Stably Transfected with an Inducible Reporter System

THP-1 cells were stably transfected with a reporter plasmid expressing a secreted embryonic alkaline phosphatase (SEAP) gene under the control of a promoter inducible by several transcription factors such as NF-κB and AP-1. The resulting THP1-Blue™ cells are resistant to the selectable marker Zeocin™. Upon TLR stimulation, THP1-Blue™ cells activate transcription factors and subsequently the secretion of SEAP which is easily detectable when using QUANTI-Blue™ a medium that turns purple/blue in the presence of SEAP.

Overexpression of CD14

CD14, a macrophage-specific differentiation antigen, interacts with several TLRs in the induction of the signaling cascade upon TLR stimulation. THP1-Blue™ cells were cotransfected with a CD14-expression plasmid selectable with blasticidin. THP1-Blue™-CD14 are 2 to 4 times more sensitive to TLR2, TLR4 and TLR5 ligands than THP1-Blue™ cells which express CD14 at a lower level.

Preactivation with PMA

Treatment with phorbol myristate acetate (PMA) induces differentiation of THP-1 cells into adherent macrophage-like cells. PMA-activated THP-1/CD14-SEAP cells become significantly more sensitive to TLR agonists than undifferentiated cells (figure 2).

Quality Control

TLR expression (TLR1 to TLR10), and CD14 expression were determined by RT-PCR in THP1-Blue™ and THP1-Blue™-CD14 cells (Figure 1). All TLRs were detected. Although TLR3, TLR7 and TLR9 seem to be expressed their cognate ligands did not activate NF-κB (Figure 2) at the concentrations used.

Contents and Storage

THP1-Blue™ and THP1-Blue™-CD14 clones are grown in RPMI medium with 10% FBS and 3 mM L-glutamine supplemented with 100 µg/ml Zeocin™ or 100 µg/ml Zeocin™ and 10 µg/ml Blasticidin respectively. Each vial contains 3-5 x 10⁶ cells and is supplied with 10 mg Zeocin™ (and 1 mg blasticidin). Cells are shipped on dry ice.

Product	Quantity	Catalog Code
THP1-Blue™	3-5 x 10 ⁶ cells	thp-sp
THP1-Blue™-CD14	3-5 x 10 ⁶ cells	thp-cd14sp
TLR RT-Primer Set	20 x 2.5 nmol	rts-htlrs
Blasticidin	100 mg	ant-bl-1
Zeocin™	1 g	ant-zn-1
QUANTI-Blue™	5 x 100 ml	rep-qb1

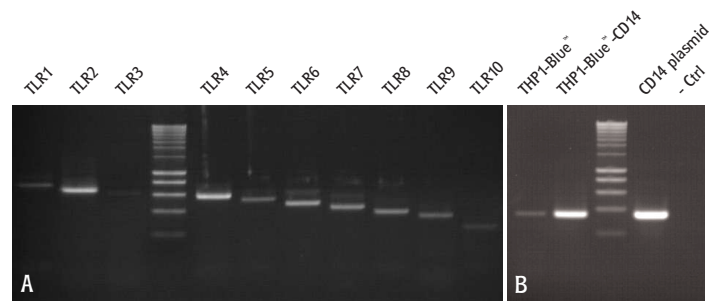


Figure 1: Expression of TLR1-10 mRNAs in THP1-Blue™ cells (A), and expression of CD14 mRNA in THP1-Blue™ and THP1-Blue™-CD14 cells (B). RT-PCR was performed using the InvivoGen's TLR RT-PCR Primer Set.

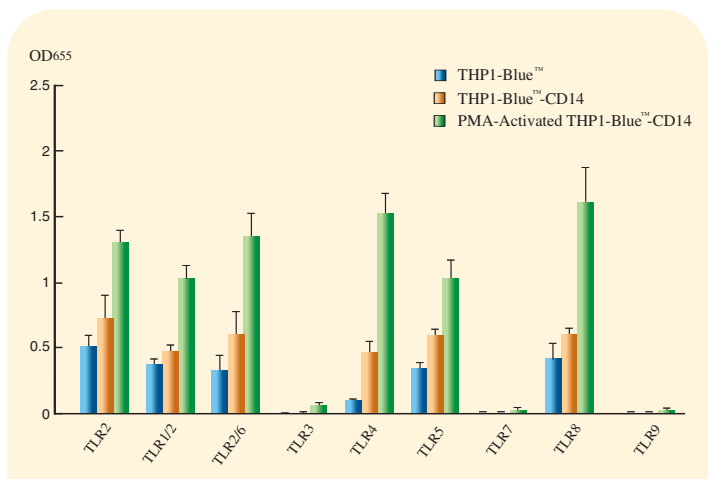
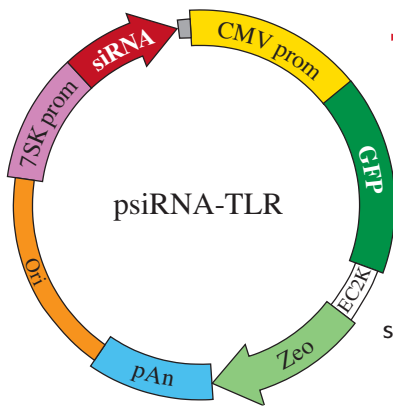


Figure 2: TLR stimulation profile in THP-1/SEAP, THP-1/CD14-SEAP and PMA-activated THP-1/CD14-SEAP cells. THP-1/SEAP, THP-1/CD14-SEAP and PMA-activated THP-1/CD14-SEAP cells were incubated with TLR agonists: TLR2 (HKLM, 5.10⁷ cells/ml), TLR1/2 (Pam3CSK4, 0.5 ng/ml), TLR2/6 (FSL-1, 5 ng/ml), TLR3 (poly(I:C), 100 µg/ml), TLR4 (LPS-EK, 10 ng/ml), TLR5 (ST-FLA, 2 ng/ml), TLR7 (CL087, 1 µg/ml), TLR8 (CL075, 0.5 µg/ml), TLR9 (ODN2006, 10 µg/ml). After 24h incubation, TLR stimulation was assessed by measuring the levels of SEAP secreted in the supernatant by using QUANTI-Blue™, a SEAP detection medium.

References:

- Hornung V, Rothenfusser S, *et al.*, 2002. J Immunol. 168(9):4531-7.
- Bekeredjian-Ding I, Roth SI, *et al.*, 2006. J Immunol. 2006 Jun 15;176(12):7438-46.
- Remer KA, Brcic M, *et al.*, 2006. J Immunol Methods. 313(1-2):1-10



Toll-Like Receptor Silencing

psiRNA-TLR - shRNAs Targeting the TLR Genes

psiRNA-TLR is a family of plasmids expressing short hairpin RNAs (shRNAs) that target the Toll-like receptor (TLR) genes and induce their successful silencing through RNAi. psiRNA-TLR plasmids can be used to generate cell lines that are stably silenced for a given TLR and thus represent powerful tools to study TLR responses.

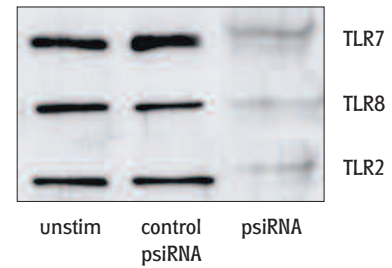
Description

psiRNA-TLR plasmids express shRNAs from the human RNA Pol III promoter 7SK. The efficacy of the shRNAs to induce silencing of the TLR genes was evaluated using the psiTEST system (catalog code #ksitest) and for some by Western blot analysis (see Figure)^{1,2}.

psiRNA-TLR features a GFP::Zeo fusion gene that allows to monitor transfection efficiency and standardize gene silencing efficiency. It also enables the selection of stable cell lines which are silenced for a given TLR that can be useful to study TLR responses.

psiRNA-TLR express shRNAs *in situ* and thus do not activate an interferon response unlike some siRNAs that have been shown to be immunostimulatory in a sequence-specific manner³.

psiRNA plasmids are also available for many genes that are involved in the TLR signaling cascade. Check our website for more information.



Silencing of human TLR2, TLR7 and TLR8 genes using psiRNA-TLR plasmids. Human primary cardiac cells were transfected with 0.5 µg of psiRNA using a lipid-based transfection reagent. After 48h, the level of silencing was determined by Western Blotting. (Data from reference 1)

References:

1. Triantafilou K., Orthopoulos G. *et al.*, 2005. *Cell Microbiol.* 7(8):1117-26.
2. Triantafilou K., Vakakis E. *et al.*, 2005. *Eur J Immunol.* 35(8):2416-23.
3. Hornung V., Guenther-Biller M. *et al.*, 2005. *Nat Med.* 11(3):263-70.

Contents and Storage

psiRNA-TLR are provided in a kit that contains the following components:

- 20 µg of a psiRNA-TLR plasmid
- 20 µg of a control psiRNA plasmid targeting Luciferase GL3
- 1 vial of LyoComp GT116, an *E. coli* strain engineered to be more compatible with hairpin structures.
- 4 pouches of Fast-Media® Zeo, a microwaveable *E. coli* selection medium.

Products are shipped at room temperature. Store at -20°C.

psiRNA-TLR are also provided alone as 20 µg of lyophilized DNA.

Product	Code (human)	Code (mouse)
psiRNA-TLR1	ksirna42-htr1	ksirna42-mtr1
psiRNA-TLR2	ksirna42-htr2	ksirna42-mtr2
psiRNA-TLR3	ksirna42-htr3	ksirna42-mtr3
psiRNA-TLR4	ksirna42-htr4	ksirna42-mtr4
psiRNA-TLR5	ksirna42-htr5	ksirna42-mtr5
psiRNA-TLR6	ksirna42-htr6	ksirna42-mtr6
psiRNA-TLR7	ksirna42-htr7	ksirna42-mtr7
psiRNA-TLR8	ksirna42-htr8	ksirna42-mtr8
psiRNA-TLR9	ksirna42-htr9	ksirna42-mtr9
psiRNA-TLR10	ksirna42-htr10	