InvivoGen Insight

As summer approaches, mycoplasma contamination of cell cultures is becoming an alarming concern. For the past five years, InvivoGen has provided Plasmocin™ that is now recognized as the most efficient mycoplasma removal agent allowing you to save your valuable cell lines. InvivoGen continues to expand its collection of TLR ligands. In this issue you will find new ligands for TLR2 and TLR7/8, labeled ODNs for TLR9 and a kit that contains a ligand for each TLR. In addition, we provide a set of RT-PCR primers to detect the expression of human TLRs. The featured product in this newsletter is pCpG, a CpG-free plasmid designed for sustained expression *in vivo*. Lastly, we provide pFUSE-Fc the first plasmid commercially available to facilitate the construction of Fc-fusions. More information regarding these products is available on our redesigned website launched a few weeks ago. **Check our new website and get 20% off your first online order.**

Inside this issue:

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Mycoplasma Eradication

Mycoplasma contamination of cultured cells is a major problem in both basic research and industrial production. Up to 87% of cell lines may be contaminated by mycoplasma. Mycoplasma infection can affect virtually any function and activity of eukaryotic cells leading to experimental artifacts and unreliable results.

It is usually recommended that an infected cell culture be immediately autoclaved to prevent the infection from spreading and to use only mycoplasma-free cultures. However, some cell lines are irreplaceable and require an effective eradication treatment. The use of specific antibiotics can efficiently eliminate mycoplasma contaminations. Antibiotics commonly used in cell culture are inactive on mycoplasma (e.g. penicillins and streptomycin). Three classes of antibiotics have been shown to kill mycoplasma at relatively low concentrations: tetracyclines, macrolides and quinolones. Tetracyclines and macrolides block the protein synthesis by interfering with ribosome translation, while quinolones inhibit the replication of bacterial DNA.

Several antibiotics are commercially available for the removal of mycoplasma: BM-Cyclin (Roche) contains a macrolide and a tetracycline, Ciprobay (Bayer, available only with a prescription) and MRA (ICN) are both quinolones. Plasmocin™ (InvivoGen) is the only antimycoplasma reagent that combines a macrolide and a quinolone. Unlike BM-Cyclin that requires the sequential and cyclic use of two antibiotics, Plasmocin™ is ready-to-use and can be added to the culture medium directly. Furthermore, both components in Plasmocin™ act on separate targets blocking protein synthesis and DNA replication, whereas the two antibiotics in BM-Cyclin are both inhibitors of protein synthesis. Therefore, Plasmocin™ is more effective in removing mycoplasma and prevents the appearance of resistant strains. In contrast to other anti-mycoplasma compounds, Plasmocin™ is active on both free mycoplasma as well as intracellular forms. This advantage is conferred by one component of Plasmocin™ which is actively transported into mammalian cells. It ensures that following treatment with Plasmocin™ a cell culture is not reinfected by mycoplasma released from intracellular compartments of infected cells. To date, no consistent and permanent alterations that affect the eukaryotic cells during and after the treatment have been detected[1].

Plasmocin™ Kills Mycoplasma

1. Uphoff CC, Drexler HG., 2005. Eradication of mycoplasma contaminations. Methods Mol Biol. 290:25-34.
2. Somasundaram C. *et al.*, 1992. Use of ciprofloxacin and BM-Cyclin in mycoplasma decontamination. In vitro Cell Dev Biol. 28A(11-12):708-10.
3. Drexler HG. *et al.*, 1994. Treatment of mycoplasma contamination in a large panel of cell cultures. In vitro Cell Dev Biol Anim. 30A(5):344-7

Comparison of the most common anti-mycoplasma agents

Product	Supplier	Treatment	Ease of use	Efficacy	Cytotoxicity	Resistance
BM-Cyclin	Roche	3 weeks	-	+++	+	+/-
Ciprobay	Bayer	12 to 20 days	+	++	+/-	+
MRA	ICN	1 to 2 weeks	+	++	+/-	+
Plasmocin	InvivoGen	2 weeks	+	++++	+/-	-

Plasmocin for treatment: Catalog code: ant-mpt - Quantity: 50 mg (treats 2 liters) - Price: $125

TLR Ligands

• TLR2 Ligand - Purified LTA-SA

Lipoteichoic acid (LTA) is a major immunostimulatory component of Gram-positive bacteria. This new preparation of LTA from *S. aureus* (LTA-SA) is purified following the method described by Morath *et al*[1]. It contains 10 times less endotoxin according to the gel clot LAL Assay than the standard preparation. At concentrations ranging from 10 ng to 10 µg/ml, it highly activates TLR2 and no other TLRs including TLR4.

Working concentration: 10 ng - 10 µg/ml

• TLR2/6 Ligand - Pam2CSK4

Pam2CSK4 is a synthetic diacylated lipopeptide (LP). Bacterial lipoproteins are strong immune modulators that activate early innate host responses after infection. LP analogues of these lipoproteins signal either through TLR2/1 or TLR2/6 heterodimers. According to the current model, triacylated LP like Pam3CSK4, are recognized by TLR2/1, whereas diacylated LP, such as FSL1, induce signaling through TLR2/6. However, it was recently reported that diacylated LP, such as Pam2CSK4, induce signaling in a TLR6-independent manner[2]. This finding suggests that both the lipid and peptide part of lipoproteins take part in the specificity of recognition by TLR2 heterodimers.

Working concentration: 1-100 ng/ml

• TLR5 Ligand - Recombinant Flagellin from *S. typhimurium*

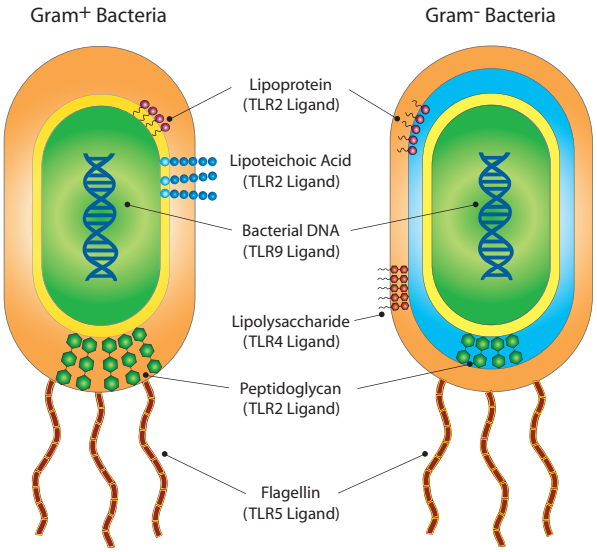
Flagellin is the major component of the bacterial flagellar filament and a potent stimulator of innate immune responses. FliC is one of the genes known to encode flagellin in *Salmonella*. The gene was expressed in HEK293 cells and the protein purified. The resulting recombinant flagellin is endotoxin-free according to the gel clot LAL Assay.

Working concentration: 10 ng - 1 µg/ml

• TLR7/8 Ligand - Single stranded RNA Double-Right (ssRNA-DR)

Long double stranded RNA (>30 bp) are known to induce type I interferons (IFNs) while siRNAs are thought to be short enough to bypass this immune response. However, Hornung *et al*. recently reported that certain siRNA sequences were potent stimuli of IFN-α[3]. This stimulation is sequence dependent and is triggered by the sense strand. The putative immunostimulatory motif was identified as a 9 mer sequence (5'-GUCCUUCAA-3') which when placed twice in a 19 mer RNA oligonucleotide (ssRNA-DR) was shown to be more immunostimulatory than when present as one copy. This sequence-specific recognition is mediated by TLR7/8, a sensor of ssRNA viruses. ssRNA-DR is provided pre-complexed with the cationic lipid LyoVec™ to facilitate its uptake.

Working concentration: 1-10 µg/ml



Labeled Ligands

• TLR9 Ligands - FITC CpG-ODNs

Unmethylated CpG-ODNs are recognized by TLR9 which is expressed in the endoplasmic reticulum. ODN 1826 (mouse) and ODN 2006 (human, type B) have been labeled with FITC at their 5' terminus. FITC-labeled CpG-ODNs are useful to study their cellular uptake and localization by confocal laser-scanning microscopy or flow cytometry.

Working concentration: 5 µM / 6 µg/ml

References:

1. Morath S. *et al*., 2001. Structure-function relationship of cytokine induction by lipoteichoic acid from Staphylococcus aureus. J.Exp.Med. 193:393-397.
2. Buwitt-Beckmann U. *et al*., 2005. Toll-like receptor 6-independent signaling by diacylated lipopeptides. Eur J Immunol. 35(1):282-9.
3. Hornung V. *et al*., 2005. Sequence-specific potent induction of IFN-alpha by short interfering RNA in plasmacytoid dendritic cells through TLR7.Nat Med. 11(3):263-70.

Ligand Kit

• TLR1-TLR9 Ligands - TLR Agonist Kit

This kit was developed to provide a known agonist for each TLR from TLR1 to TLR9. Each agonist allows to perform 100 tests (100 µl in a 96-well plate).

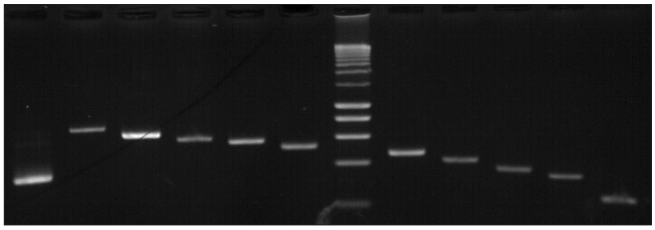
- TLR1/2: **Pam3CSK4** (10 µg)
- TLR2: **HKLM** (10^9 cells)
- TLR3: **Poly(I:C)** (250 µg)
- TLR4: **LPS *E. coli* K12** (100 µg)
- TLR5: **Flagellin *S. typhimurium*** (10 µg)
- TLR6/2: **FSL1** (10 µg)
- TLR7: **Imiquimod** (25 µg)
- TLR8: **ssRNA40** (25 µg)
- TLR9: **ODN2006** (100 µg)

Product	Quantity	Code	Price
TLR2 ligand - Purified LTA-SA	5 mg	tlrl-pslta	$140
TLR2/6 ligand - Pam2CSK4	100 µg	tlrl-pam2	$200
TLR5 ligand - Recombinant Flagellin	1 µg	tlrl-flic	$190
TLR7/8 ligand - ssRNA-DR	100 µg	tlrl-ssdr	$240
TLR1-9 ligands - TLR Agonist Kit	-	tlrl-kit1	$380
Labeled ligands - FITC-CpG ODNs			
- FITC ODN1826	50 µg	tlrl-fmodn	$110
- FITC ODN2006	50 µg	tlrl-fhodnb	$110

TLR Expression

TLR RT-Primers

Toll-like Receptors are predominantly expressed in tissues involved in immune function, such as spleen and peripheral blood leukocytes, as well as those exposed to the external environment such as lung and the gastrointestinal tract. Their expression profiles vary among tissues and cell types.
InvivoGen provides TLR RT-Primers, a collection of RT-PCR primers to determine the mRNA expression pattern of human TLRs. They can be used to analyze the expression of endogenous as well as transgenic human TLR genes. Each TLR RT-Primer pair is carefully designed and tested. TLR RT-Primers are provided as pairs for each individual TLR or as a set containing a primer pair for all ten human TLRs.
The size of the amplified fragments varies from 200 to 700 bp.



Expression of TLR mRNAs in human monocytic cell line THP-1 using the TLR RT-PCR Primer Set.

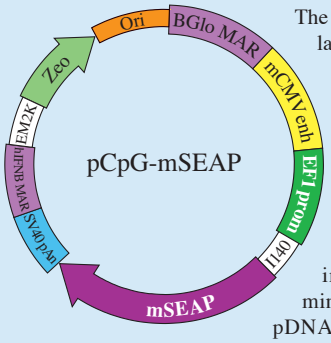
Each **TLR RT-Primer Pair** contains the following:
- 2.5 nmoles of each primer, allowing 50 reactions at 1 μM final primer concentration. 5' sense primer and 3' antisense primer are shipped together in a single vial.
- 150 ng positive control double stranded DNA

The **TLR RT-Primer Set** contains the following:
- 2.5 nmoles of each primer (20 primers total). Each primer pair is provided in a single vial.
- 1 μg positive control double stranded DNA

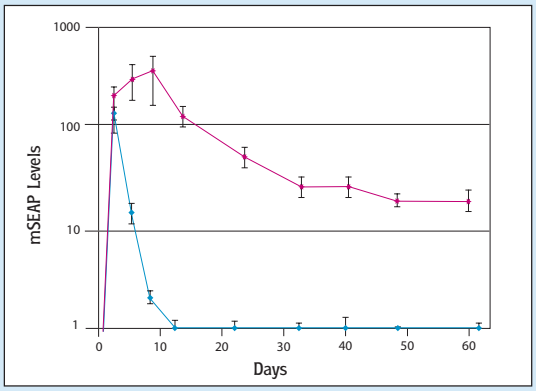
Product	Quantity	Code	Price
TLR RT-Primer Pairs			
- TLR1	2.5 nmol each	rtp-htlr1	$110
- TLR2	2.5 nmol each	rtp-htlr2	$110
- TLR3	2.5 nmol each	rtp-htlr3	$110
- TLR4	2.5 nmol each	rtp-htlr4	$110
- TLR5	2.5 nmol each	rtp-htlr5	$110
- TLR6	2.5 nmol each	rtp-htlr6	$110
- TLR7	2.5 nmol each	rtp-htlr7	$110
- TLR8	2.5 nmol each	rtp-htlr8	$110
- TLR9	2.5 nmol each	rtp-htlr9	$110
- TLR10	2.5 nmol each	rtp-htlr10	$110
TLR RT-Primer Set	2.5 nmol each	rts-htlrs	$480

CpG-free Plasmids

Long lasting expression *in vivo*



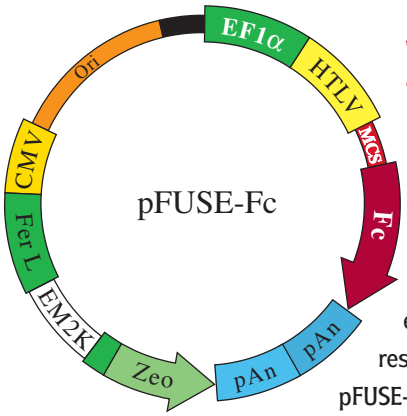
The success of gene therapy will require long lasting expression of the transgene. One of the major limitations to sustained transgene expression is the immunostimulatory effects of the plasmid DNA (pDNA). Indeed, pDNA carry elements of bacterial (origin of replication, resistance gene) and viral (promoter) origins that contain unmethylated CpG motifs known to rapidly induce the innate immune system through TLR9[1]. To minimize the immunostimulatory effects of pDNA, InvivoGen has developed pCpG, a family of revolutionary plasmids that are completely devoid of CpGs. pCpG plasmids were generated by assembling elements that either naturally lack CpG dinucleotides, were modified to remove all CpGs, or chemically synthesized.
pCpG plasmids feature a strong and ubiquitous mammalian promoter that combines CpG-free versions of the mouse CMV enhancer and the human EF1 promoter (mCMV/hEF1). *In vivo* experiments using the hydrodynamic method revealed that the mCMV/hEF1 promoter was as strong as the widely used human CMV promoter. However, in marked contrast to the CMV promoter which expression lasted less than 2 weeks due to extensive methylation[2], the pCpG composite promoter allowed sustained and stable expression of the transgene over 2 months.
In addition to its lack of immunogenicity, the long lasting expression of pCpG plasmids is believed to be due to its episomal status conferred by the presence of two scaffold/matrix attachment regions[3].



Expression levels of pCpG and CMV-based plasmids - Nine week old mice (5 mice/group) were injected with 30 µg pCpG-mSEAP or pcDNA3-mSEAP, a CMV-based plasmid (Invitrogen), using the hydrodynamic method[4]. Both plasmids express the mouse secreted embryonic alkaline phosphatase (mSEAP). The activity of mSEAP present in the sera was measured over 60 days with the SEAP Detection Kit (cat. code #rep-sap).

NEW **pCpG-mSEAP:** Catalog code: pcpg-mseap

1. Sawamura D. *et al*., 2005. Direct injection of plasmid DNA into the skin induces dermatitis by activation of monocytes through toll-like receptor 9. J Gene Med. 7(5):664-71.
2. Hodges BL *et al*., 2004. Long-term transgene expression from plasmid DNA gene therapy vectors is negatively affected by CpG dinucleotides. Mol Ther. 10(2):269-278
3. Conese M. *et al*., 2004. Gene therapy progress and prospects: episomally maintained self-replicating systems. Gene Ther. 11(24):1735-41.
4. Zhang G *et al*, 1999. High levels of foreign gene expression in hepatocytes after tail vein injections of naked plasmid DNA. Hum Gene Ther. 10(10):1735-7



pFUSE-Fc

Fc Fusions Made Easy

Fc-fusion proteins are widely used to promote long-lasting expression of a protein of interest *in vivo*. They are generated by fusing the Fc region of an immunoglobulin to their C terminus. These soluble chimera retain the activity of the native protein and present, in addition to a long half-life in the circulatory system, the advantages of efficient mammalian expression and ease of purification. Fc-fusion proteins are useful research tools for many applications and hold promise as therapeutics. InvivoGen provides pFUSE-Fc, a new expression plasmid, designed to facilitate the generation of Fc-fusion proteins.

pFUSE-Fc plasmids yield high levels of Fc-Fusion proteins. The level of expression is usually in the μg/mL range.

pFUSE-Fc plasmids feature several Fc regions from various species:

- human Fc from IgG1
- mouse Fc from IgG2a
- rabbit Fc from IgG

pFUSE-Fc plasmids can be transfected in a variety of mammalian cells commonly used in protein purification systems, including CHO, COS, HEK293 and myeloma cell lines.

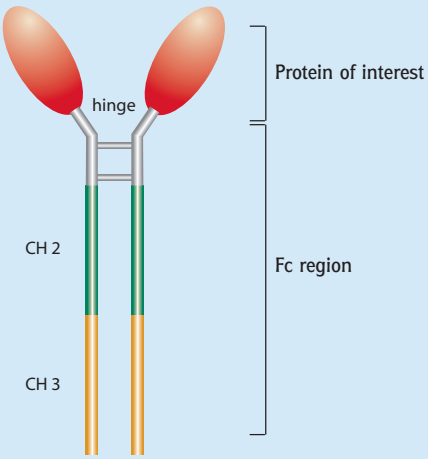
pFUSE-Fc plasmids are selectable with Zeocin™ in *E. coli* as well as in mammalian cells. Thus, they can be used to transfect mammalian cells transiently or stably.

pFUSE-Fc plasmids allow the secretion of Fc-Fusion proteins.

- pFUSE-Fc1 is recommended when the protein of interest contains a native signal sequence.
- pFUSE-Fc2 contains an IL2 signal sequence (IL2ss) for the generation of Fc-Fusion proteins derived from proteins that are not naturally secreted.

Fc-Fusion proteins can be easily detected in the supernatant of pFUSE-Fc-transfected cells by SDS-PAGE. Their functional domains can be identified by immunoblotting and ligand blotting.

Fc-Fusion proteins can be easily purified by single-step protein A or protein G affinity chromatography.



Schematic representation of an Fc fusion protein: The Fc region comprises the CH2 and CH3 domains of the IgG heavy chain and the hinge region. The hinge serves as a flexible spacer between the two parts of the Fc-Fusion protein, allowing each part of the molecule to function independently.

Product	Quantity	Code	Price
pFUSE-hFc1	20 μg	pfuse-hfc1	$435
pFUSE-hFc2 (IL2ss)	20 μg	pfuse-hfc2	$435
pFUSE-mFc1	20 μg	pfuse-mfc1	$435
pFUSE-mFc2 (IL2ss)	20 μg	pfuse-mfc2	$435
pFUSE-rFc1	20 μg	pfuse-rfc1	$435
pFUSE-rFc2 (IL2ss)	20 μg	pfuse-rfc2	$435

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