Quantification of Toll-like Receptor 9 (TLR9) inhibitor using *iLite*® TLR9 Assay Ready Cells

For research and professional use only. Not for use in diagnostic procedures.

This application note contains a suggested protocol and performance data.
Each individual laboratory must set up their own method and perform relevant validations.

Background

Toll-like receptors (TLRs) are pattern-recognition receptors that detect a wide variety of microbial pathogens for the initiation of host defense immunological responses. Toll-like receptor 9 (TLR9) is categorized as an innate immune sensor for DNA and is composed of a leucine-rich repeat (LRR) domain and a Toll/IL-1receptor (TIR) domain. In presence of unmethylated CpG motif, TLR9 dimerizes, and interacts with MyD88, activating the nuclear factor (NF)-κβ pathway and IFN transcription factor 7 (IRF7) nuclear translocation, resulting in inflammatory cytokine and type I IFN expression (1).

As TLR9 participates in the activation of the innate immune system, the development of TLR9 agonists have demonstrated substantial potential as vaccine adjuvants, and as mono- or combination therapies for the treatment of cancer and infectious and allergic diseases. These agonists are mainly CpG oligodeoxynucleotides (ODN) that directly induce the activation of the receptor, resulting in enhanced differentiation of B cells into antibody-secreting plasma cells (2).

On the other hand, the inhibition of TLR9 has been related in the amelioration of the clinical status of patients or animal models with different types of cancer (3–5), lupus (6) and at some points even with COVID-19 (7) despite this relation is being currently under discussion. Several molecules such as hydroxychloroquine or E-6446 (8) had been used to inhibit TLR9 signaling. Both molecules are lysosomotropic compounds that can penetrate lipid membranes at neutral pH but are trapped in low pH compartments.

Principle of the assay

The *iLite*® TLR9 Assay Ready Cells are engineered cells optimized to express Firefly luciferase under the control of an NF-κB responsive promoter. Binding of DNA with unmethylated CpG motif to the Toll-like Receptor 9 results in activation of the NF-κB responsive Firefly luciferase reporter gene construct. The *iLite*® TLR9 Assay Ready Cells also contain the Renilla Luciferase (RL) reporter gene, under the control of a constitutive promoter. The constitutive expression of RL allows normalization of CpG motif induced FL activity and renders assay results independent of variations in cell number or serum matrix effects. The luciferase signal can be measured in a luminometer following the addition and incubation of the luciferase substrate. The Firefly luciferase signal is proportional to the activity induced by CpG oligonucleotides in the sample. In the presence of inhibitory activity against TLR9, the functional activity of the present CpG oligodeoxynucleotides is reduced, resulting in a decreased stimulation of Firefly luciferase expression (Fig.1). Thus, the Firefly luciferase signal is inversely proportional to the amount of inhibitory activity against TLR9 in a sample. The *iLite*® TLR9 Assay Ready Cells can therefore be utilized for quantification of TLR9 inhibitor activity in test samples also when the samples contains human serum.

Material and equipment needed

Material and equipment	Suggested supplier	Reference
iLite® TLR9 Assay Ready Cells	Svar Life Science	BM6069
Diluent (DMEM containing 9% heat inactivated FBS + 1% Penicillin-Streptomycin)	Gibco	31966-021 (DMEM) 26140-079 (FBS) 15140-122 (Penicillin-Streptomycin)
E-6446 Dihydrochloride	Sigma Aldrich	AMBH2D6FF912
Class B CpG oligonucleotide (ODN2006)	Invivogen	Tlrl-2006
Firefly/Renilla luciferase substrate	Promega	E2920, Dual-Glo Luciferase Assay System
Plate; White walled micro well plate suitable for luminescence	PerkinElmer	6005680
Microplate Luminometer with appropriate reading software – no filter on luminometer	Contact Svar Life Science for list of recommended suppliers	NA
Incubator, 37 °C with 5% CO_2	NA	NA
Water bath, 37 °C	NA	NA
Single-channel and multi-channel pipettes with polypropylene disposable tips	NA	NA
Polypropylene tubes or plate for dilution	NA	NA
Single-use polypropylene reservoir	NA	NA
Plate shaker	NA	NA
Timer	NA	NA

Protocol

Preparation of calibrators (ODN2006)

E-6446 from Sigma Aldrich has successfully been used to neutralize functional activity of TLR9 and inhibit the TLR9 regulated Firefly luciferase expression in *iLite*® TRL9 Assay Ready Cells (refer to the table and graph below).

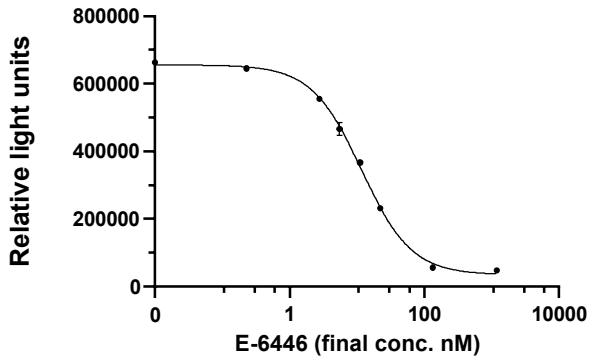

Figure 1. Example of TLR9 inhibitory curve.

Final 1µM ODN2006	E-6446 Suggested calibrator **solution** conc. (nM)
A	1196
B	132
C	22
D	11
E	5.5
F	2.8
G	0.23
H	0

Table 1. Suggested calibrator **solution** concentrations for TLR9 inhibitor E-6446

Assay preparation and incubation

1. Design a plate layout. It is recommended to perform the test at least in duplicates.
2. Perform a serial dilution of the inhibitor E-6446. Ensure matrix consistency between calibrator inhibitor solutions, control solutions, and sample solutions.

3. Add 20 µL calibrators, controls, and samples to assigned wells (final concentration will be a quarter of the solution concentration).
4. Thaw the vial of *iLite*® TLR9 Assay Ready Cells in a 37°C water bath with gentle agitation. The cell suspension is mixed very carefully ten times with pipette in order to ensure a homogeneous distribution of cells.
5. Dilute 250 µL cell suspension with 5.75 mL of diluent.
6. Add 40 µL diluted cells to each well.
7. Place the lid on the plate, mix and incubate the plate for 30 minutes at 37 °C with 5% CO_2.
8. Add 20 µL 4 µM ODN2006 to all wells (final concentration will be 1 µM ODN2006).
9. Place the lid on the plate, mix and incubate the plate for 6 h at 37 °C with 5% CO_2.

Adding substrate solutions

10. Equilibrate the plate and the substrate solution to room temperature.
11. Prepare the **Firefly luciferase** substrate according to the supplier's instructions and add 80 µL per well. Mix and protect the plate from light. After 10 minutes of incubation at room temperature read in a luminometer.
12. If appropriate, prepare the **Renilla luciferase** substrate according to the supplier's instructions and add 80 µL per well. Mix and protect the plate from light. After 10 minutes of incubation at room temperature read in a luminometer.

Precautions

- This application note is intended for professional laboratory research use only. The data and results originating from following the Application Note should not be used either in diagnostic procedures or in human therapeutic applications.
- Use and handle the material and instruments referenced according to the supplier's/manufacturer's instructions or product specifications accompanying the individual material and instruments.
- Dispose of all sample specimens, infected or potentially infected material in accordance with good microbiological practice. All such materials should be handled and disposed as though potentially infectious.
- Residues of chemicals and preparations are generally considered as biohazardous waste and should be inactivated prior to disposal by autoclaving or using bleach. All such materials should be disposed of in accordance with established safety procedures.

Proprietary Information

In accepting delivery of *iLite*® Assay Ready Cells the recipient agrees not to sub-culture these cells, attempt to sub-culture them or to give them to a third-party recipient, and only to use them directly in assays. *iLite*® cell-based products are covered by patents which are the property of Svar Life Science AB and any attempt to reproduce the delivered *iLite*® Assay Ready Cells is an infringement of these patents.

QUICK GUIDE

Quantification of Toll-like Receptor 9 (TLR9) inhibitor using *iLite*® TLR9 Assay Ready Cells

1 Sample dilution

- Equilibrate reagents and samples to room temperature – **do not thaw cells and substrate reagents at this stage**
- Dilute reference TLR9 inhibitor, controls, and samples.
- Add 20 µL of calibrators, controls, and diluted samples to pre-assigned wells.
- Thaw the cell vial in a 37°C water bath. Mix the cell suspensions with a pipette in order to ensure a uniform solution. Dilute the cells.
- Add 40 µL diluted cells to each well.

2 Incubation 30 min

- Incubate at 37 °C with 5% CO_2 for 30 min.

3 Add ODN2006

- Add 20 µl of 4 µM ODN2006 to each well.

4 Incubation 6 h

- Incubate at 37°C with 5% CO_2 for 6 hours.

5 Read plate

- Equilibrate the plate to room temperature
- Prepare the **Firefly luciferase** substrate according to the supplier's instructions and add 80 µL per well. Mix. Protect the plate from light. After 10 min incubation read in a luminometer.
- If appropriate, prepare the **Renilla luciferase** substrate according to the supplier's instructions and add 80µl per well. Mix. Protext the plate from light. After 10 min incubation read in a luminometer.

Troubleshooting and FAQ

Please consult the Svar Life Science website www.svarlifescience.com

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