distributed in the US/Canada by:
EAGLE BIOSCIENCES, INC.
20A NW Blvd, Suite 112 Nashua, NH 03063
Phone: 617-419-2019 FAX: 617-419-1110
www.EagleBio.com info@eaglebio.com

APPLICATION NOTE

Quantification of Toll-like Receptor 4 (TLR4) inhibitor using *iLite*® TLR4 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

The toll-like receptor (TLR) family consist of receptors responsible for pattern recognition in innate immunity, key in the detection of pathogens and immune responses (1). The TLR4 is the most studied member of this family and induces pro-inflammatory responses upon invasion of pathogens. TLR4 is activated by binding of lipopolysaccharide (LPS, endotoxin) from Gram-negative bacteria (2). An important role of TLR4 is described in many inflammatory diseases including sepsis, asthma, cancer, acute kidney injury, or intestinal inflammation among others (1–4). Briefly, TLR4 signalling is induced upon activation in the plasmatic membrane. The signal transduction extends through TIRAP and MyD88 adaptor proteins, in early endosomes the signal pathway continues via the adaptor proteins TRAM and TRIF (2). Currently, scientist attention had been drawn to identify new molecules that can inhibit/reduce TLR4 signalling for several diseases (1,3,4). An inhibitory molecule that had been described to block TLR4 signalling is the TAK-242, also known as resatorvid (5). TAK-242 inhibits the production of lipopolysaccharide-induced inflammatory mediators by binding to the intracellular domain of TLR4 (6). The binding of TAK-242 interferes with protein-protein interactions between TLR4 and its adaptor molecules TIRAP and TRAM, inhibiting the TIRAP-mediated activation of nuclear factor κB (NF-κB) as well as the TRAM-mediated activation of NF-κB and the interferon-sensitive response element (6). TAK-242 has been shown to inhibit TLR4 activation in both *in vitro* (6,7), and *in vivo* (7).

Principle of the assay

The *iLite*® TLR4 Assay Ready Cells are engineered cells optimized to express Firefly luciferase under the control of a NF-κB responsive promoter. Binding of LPS to membrane bound Toll-like Receptor 4 (TLR4) results in activation of the NF-κB responsive Firefly luciferase reporter gene construct. *iLite*® TLR4 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 LPS 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 addition and incubation of luciferase substrate. The Firefly luciferase signal is proportional to the functional activity of LPS in the sample. In the presence of inhibitory activity against TLR4, the functional activity of the present LPS is reduced, resulting in a decreased stimulation of Firefly luciferase expression.

Thus, the Firefly luciferase signal is inversely proportional to the amount of inhibitory activity against TLR4 in a sample. The *iLite*® TLR4 Assay Ready Cells can therefore be utilized for quantification of TLR4 inhibitor activity in test samples, including human serum.

Material and equipment needed

Material and equipment	Suggested supplier	Reference
iLite® TLR4 Assay Ready Cells	Svar Life Science	BM4025
Diluent (RPMI containing 9% heat inactivated FBS + 1% Penicillin-Streptomycin).	Gibco	61870-044 (RPMI) 26140-079 (FBS) 15140-122 (Penicillin-Streptomycin)
TAK-242	Sigma Aldrich	614316
LPS	Invivogen	Tlrl-3pelps
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 TLR4 inhibitor

TAK-242 from Sigma Aldrich has successfully been used to neutralize functional activity of TRL4 and inhibit the TLR4 regulated Firefly luciferase expression in *iLite*® TRL4 Assay Ready Cells (refer to the table and graph below).

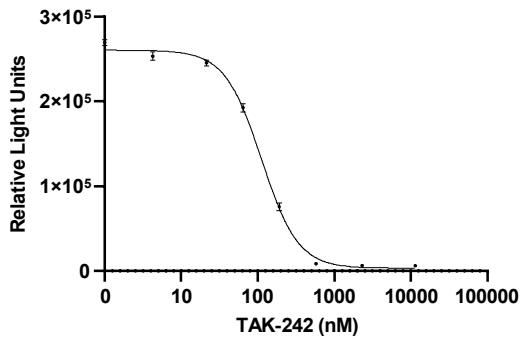

Figure 1. Example of TLR4 inhibitory curve

Final 1.25 EU/ml LPS	TAK-242 Suggested calibrator solution concentrations, nM
A	46063
B	9213
C	2303
D	768
E	256
F	85
G	17
H	0

Table 1. Suggested calibrator **solution concentrations** for anti-TLR4 TAK-242

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 TAK-242. Ensure matrix consistency between reference inhibitor solutions, control solutions, and sample solutions.
3. Add 20 µL of the reference TLR4 inhibitor dilutions, controls and samples to assigned wells (final concentration will be a quater of the solution concentration).
4. Thaw the vial of *iLite*® TLR4 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 2.75 mL Diluent.
6. Add 20 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 40 µL of 2.5 EU/ml LPS to all wells (final concentration will be 1.25 EU/ml LPS).
9. Place the lid on the plate, mix and incubate for 5 hours 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 suppliers instructions and add 80 µL per well. Mix and protect the plate from light. After 10 minutes incubation at room temperature read in a luminometer.
12. If appropriate, prepare the **Renilla luciferase** substrate according to the suppliers instructions and add 80 µL per well. Mix and protect the plate from light. After 10 minutes 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.

Propriety 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 TLR4 inhibitor using *iLite*® TLR4 Assay Ready Cells

1 Sample dilution plus cell addiiton

- Equilibrate reagents and samples to room temperature – **do not thaw cells and substrate reagents at this stage**
- Dilute reference TLR4 inhibitor, controls and samples.
- Add 20 µL of ref. TLR4 inhibitor solutions, controls and diluted samples to pre-assigned wells.
- Thaw the cell vial in a 37°C water bath. Mix cell suspension with a pipette in order to ensure a uniform cell suspension. Dilute the cells.
- Add 20 µL diluted cells to each well.

2 Incubation 30 min

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

3 Add LPS

- Add 40 µL of LPS to each well.

4 Incubation 5 h

- Incubate at 37 °C with 5% CO_2 for 5 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 80 µL per well. Mix. Protect 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

Svar Life Science AB

Lundavägen 151
SE-212 24 Malmö
Sweden

P.O. Box 50117
SE-202 11 Malmö
Sweden

info@svarlifescience.com
+46 40 53 76 00

References

1. Kashani B, Zandi Z, Pourbagheri-Sigaroodi A, Bashash D, Ghaffari SH. The role of toll-like receptor 4 (TLR4) in cancer progression: A possible therapeutic target? J Cell Physiol. 2021;236(6):4121–37.

2. Ciesielska A, Matyjek M, Kwiatkowska K. TLR4 and CD14 trafficking and its influence on LPS-induced pro-inflammatory signaling. Cell Mol Life Sci. 2021;78(4):1233–61.

3. Jha AK, Gairola S, Kundu S, Doye P, Syed AM, Ram C, et al. Toll-like receptor 4: An attractive therapeutic target for acute kidney injury. Life Sci. 2021;271:119155.

4. Tam JSY, Coller JK, Hughes PA, Prestidge CA, Bowen JM. Toll-like receptor 4 (TLR4) antagonists as potential therapeutics for intestinal inflammation. Indian J Gastroenterology. 2021;1–17.

5. Kawamoto T, Ii M, Kitazaki T, Iizawa Y, Kimura H. TAK-242 selectively suppresses Toll-like receptor 4-signaling mediated by the intracellular domain. Eur J Pharmacol. 2008;584(1):40–8.

6. Matsunaga N, Tsuchimori N, Matsumoto T, Ii M. TAK-242 (Resatorvid), a Small-Molecule Inhibitor of Toll-Like Receptor (TLR) 4 Signaling, Binds Selectively to TLR4 and Interferes with Interactions between TLR4 and Its Adaptor Molecules. Mol Pharmacol. 2011;79(1):34–41.

7. Wang H, Li X, Dong G, Yan F, Zhang J, Shi H, et al. Toll-like Receptor 4 Inhibitor TAK-242 Improves Fulminant Hepatitis by Regulating Accumulation of Myeloid-Derived Suppressor Cell. Inflammation. 2021;44(2):671–81.