NCL Method ITA-29

Detection of nanoparticles' ability to stimulate toll-like receptors using HEK-Blue reporter cell lines

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This protocol assumes an intermediate level of scientific competency with regard to techniques, instrumentation, and safety procedures. Rudimentary assay details have been omitted for the sake of brevity.

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Please cite this protocol as:
Cedrone E, Dobrovolskaia MA, NCL Method ITA-29: Detection of nanoparticles' ability to stimulate toll-like receptors using HEK-Blue reporter cell lines. https://ncl.cancer.gov/resources/assay-cascade-protocols. DOI: 10.17917/EYQD-3T61

1. Introduction

This document describes a protocol for analyzing nanoparticles' ability to activate innate pattern recognition receptors of the toll-like receptor (TLR) family. TLRs play a vital role in the innate immune response; activation of these receptors leads to cytokine secretion that guides communication between innate and adaptive immunity [1]. For this reason, TLR agonists are explored as adjuvants for vaccines and immunotherapies [2, 3]. Often, such agonists are formulated using nanoparticles to achieve delivery to a specific subset of immune cells. Via TLRs, the immune cells sense and respond to microbes and their components, such as flagellin, CpG DNA, lipopolysaccharide, beta-glucans, single- and double-stranded RNA, to name a few [3]. Endogenous TLR ligands such as heat shock proteins, fibrinogen and monosodium urate crystals have also been described [4]. Nanoparticles may activate TLRs directly due to either their payloads such as immunostimulatory TLR agonists or the carrier itself. Nanoparticles may also indirectly activate TLRs by inducing immunogenic cell death and the host cells' release of endogenous TLR ligands. The indirect activation could also be due to the nanoparticle carrier or the drug payload. Therefore, understanding nanoparticles' ability to activate TLRs may help verify the activity of TLR agonists formulated using nanoparticles and get an insight into mechanisms of the innate immune responses to nanoparticle formulations, APIs and carriers, should such responses occur. This protocol complements NCL protocols ITA-10, STE-1, and STE-4, used for the detection of nanoparticles' ability to induce cytokine responses, quantification of endotoxins, and quantification of beta-glucans, respectively.

2. Principles

This assay utilizes HEK-Blue hTLR cell lines from Invivogen. These cells are transfected with a reporter construct containing secreted alkaline phosphatase (SEAP) gene under a promoter containing transcription factors such as NFkB and AP-1. Activation of TLRs by test samples or controls leads to the SEAP expression, which is detected using HEK-Blue™ Detection medium. This medium, besides nutrients supporting cell growth, contains SEAP substrate. The hydrolysis of this substrate by SEAP produced by activated cells results in the development of purple/blue color quantified using a spectrophotometer [5].

3. Reagents, Materials, Cell Lines, and Equipment

Note: The NCL does not endorse any of the suppliers listed below; these reagents were used in the development of the protocol, and their inclusion is for informational purposes only. Equivalent supplies from alternate vendors can be substituted. Please note that suppliers may undergo a name change due to a variety of factors. Brands and part numbers typically remain consistent but may also change over time.

3.1 Reagents

3.1.1 Phosphate buffered saline (PBS) (GE Life Sciences, SH30256.01)

3.1.2 LPS-EK UltraPure (*E. coli K12)* or equivalent (Invivogen, tlrl-peklps)

3.1.3 Fetal bovine serum (FBS) (GE Life Sciences, Hyclone, SH30070.03)

3.1.4 DMEM, high glucose (ATCC, 30-2002)

3.1.5 0.25% Trypsin-EDTA (Gibco, 25200-056)
(*Note: Trypsin is not recommended for some cell lines and others use 0.08% Trypsin in PBS. See Appendix 12.3)*

3.1.6 Penicillin streptomycin solution (GE Life Sciences, Hyclone, SV30010)

3.1.7 L-glutamine (GE Life Sciences, Hyclone, SH30034.01)

3.1.8 Normocin (Invivogen, ant-nr-1)

3.1.9 HEK-Blue Selection (Invivogen, hb-sel)

3.1.10 HEK-Blue Detection (Invivogen, hb-det2)

3.1.11 Trypan Blue solution (Gibco, 15250-061)

3.1.12 FSL-1 (Invivogen, tlrl-fsl)

3.1.13 Pam3CSK4 (Invivogen, tlrl-pms)

3.1.14 IL-1ß (Invivogen, rcyec-hi1b)

3.1.15 Zeocin (Invivogen, ant-zn-1)

3.1.16 Blasticidin (Invivogen, ant-bl-1)

3.1.17 Recombinant SEAP Protein (Invivogen, rec-hseap)

3.2 Materials

3.2.1 Serological pipets

3.2.2 Multichannel pipettor

3.2.3 Flat bottom 96-well plates

3.2.4 Polypropylene tubes, 50 and 15 mL

3.2.5 0.2 µm Filter Units

3.2.6 Reagent reservoirs

3.3 Cell Lines

3.3.1 HEK-Blue Null and hTLR cell lines (see Table 1)

3.4 Equipment

3.4.1 Centrifuge

3.4.2 Refrigerator, 2-8ºC

3.4.3 Freezer, -20ºC

3.4.4 Cell culture incubator with 5% CO_2 and 95% humidity

3.4.5 Biohazard safety cabinet approved for level II handling of biological material

3.4.6 Inverted microscope

3.4.7 Vortex

3.4.8 Hemocytometer

3.4.9 Plate Shaker

3.4.10 Plate reader capable of operating at 550 nm

4. Reagent and Control Preparation

4.1 Complete DMEM Growth Medium

(See Appendix 12.2 or product data sheets for preparations; each cell line may have different requirements.)

Store at 2-8ºC protected from light for no longer than 1 month. Before use, warm in a water bath.

4.2 HEK-Blue Detection

Pour one pouch of powder into a sterile bottle and solubilize the powder with 50 mL of sterile, endotoxin free water (make up enough medium for the experimental needs). Swirl gently until powder is completely dissolved. Warm at 37°C for 10 minutes. Filter through a 2 µm filter unit.

Store at 4°C for up to 2 weeks.

4.3 Lipopolysaccharide 1 mg/mL (LPS, Stock)

K12 LPS is provided as lyophilized powder. Reconstitute the powder by adding 1 mL of water per 1 mg of LPS to the vial and vortex to mix. Stocks with higher concentration (5-10 mg/mL) can also be prepared. Store daily use aliquots at a nominal temperature of -20ºC. Avoid repeated freezing/thawing.

4.4 Positive Control (LPS)

On the day of experiment thaw a stock aliquot at room temperature, vortex well and dilute this stock LPS solution in cell culture medium to a final concentration of 100 ng/mL. Store at room temperature during the experiment and discard unused portion after use.

4.5 HEK-Blue Cell Line Specific Positive Controls

Each of the HEK-Blue cell lines has specific positive control agonists recommended by Invivogen with suggested concentrations *(refer to Table 1 and the cell line product sheets).*

4.6 Negative Control

Use PBS as a negative control. Process this sample the same way as you do for the study samples. For example, if to prepare nanoparticle test sample the stock nanoparticle was diluted 1:10 in complete culture medium, dilute PBS 1:10 in complete culture medium and use this sample as the negative control.

4.7 Vehicle Control

Vehicle control is the buffer or media used to formulate test nanomaterials. Common excipients used in nanoformulations are trehalose, sucrose, and albumin. However, other reagents and materials are also used alone or in combination. Vehicle control should match formulation buffer of the test-nanomaterial by both composition and concentration. This control can be skipped if nanoparticles are stored in PBS.

4.8 Heat-Inactivated Fetal Bovine Serum

Thaw a bottle of FBS at room temperature, or overnight at 2-8ºC and allow to equilibrate to room temperature. Incubate 30 minutes at 56ºC in a water bath, mixing every 5 minutes. Single use aliquots may be stored at 2-8ºC for up to one month or at a nominal temperature of -20ºC indefinitely.

5. Preparation of Study Samples

This assay requires ~0.5 mL of nanoparticles/cell line at 5X the highest final test concentration dissolved/resuspended in complete culture medium. The concentration is selected based on the plasma concentration of the nanoparticle at the intended therapeutic dose. For the purposes of this protocol this concentration is called the "theoretical plasma concentration". Considerations for estimating theoretical plasma concentration were reviewed elsewhere [6] and are summarized in Box 1 below.

Box 1. Example Calculation to Determine Nanoparticle Concentration for In Vitro Tests

In this example, we assume a mouse dose of 123 mg/kg. Therefore, the scaled equivalent human dose would be:

$$Human\ dose = \frac{mouse\ dose}{12.3} = \frac{123\ mg/kg}{12.3} = 10\ mg/kg$$

Blood volume constitutes approximately 8% of the body weight. Therefore, an average human of 70 kg body weight has approximately 5.6 L of blood. Assuming all the nanoparticle injected goes into the systemic circulation, this provides a rough approximation of the potential maximum nanoparticle concentration in blood, which is used as the in vitro test concentration.

$$in\ vitro\ concentration_{human\ matrix} = \frac{human\ dose}{human\ blood\ volume} = \frac{70\ kg * 10\ mg/kg}{5.6\ L} = 0.125\ mg/mL$$

The assay will evaluate 4 concentrations: 10X (or when feasible 100X, 30X or 5X) of the theoretical plasma concentration, theoretical plasma concentration and two 1:5 serial dilutions of the theoretical plasma concentration. When the intended therapeutic concentration is unknown, the highest final concentration is 1 mg/mL or the highest reasonably achievable concentration.

For example, if the final theoretical plasma concentration to be tested is 0.2 mg/mL, then a stock of 2 mg/mL will be prepared and diluted 10-fold (0.2 mg/mL), followed by two 1:5 serial dilutions (0.04 and 0.008 mg/mL). Use 500 µL of each of these samples per well. Each nanoparticle concentration is plated 3 times.

6. Cell Preparation

HEK-Blue cell lines are engineered HEK293 cells designed for the investigation of various Toll-Like Receptor (TLR) signaling pathways. The various cell lines have different growth properties and should be maintained according to the specific product sheet provided with each cell line.

Table 1. Summary of cell lines.

N/A = not applicable

Cell Line (Invivogen catalog #)	Control Cells	Positive Control	When to use
HEK-Blue™ Null1 (hkb-null1) control cell line	N/A	N/A	As control with HEK expressing TLR2, TLR3, TLR5, TLR8, or TLR9
HEK-Blue™ Null1-k (hkb-null1k) control cell line	N/A	N/A	As control with HEK expressing TLR7
HEK-Blue™ Null2 (hkb-null2) control cell line	N/A	N/A	As control with HEK expressing TLR4
HEK- Blue™ TLR2 (hkb-htlt2)	HEK- Blue™ Null1	Pam3CSK4 (TLR1/2 ligand)	When activation of TLR2 is of interest
HEK- Blue™ TLR2/TLR1 (hkb-htlr21)	HEK- Blue™ TLR2(TLR1/TLR6_KO)	Pam3CSK4 (TLR1/2 ligand)	When activation of TLR2 and TLR1 is of interest
HEK- Blue™ TLR2/TLR6 (hkb-htlr26)	HEK- Blue™ TLR2(TLR1/TLR6_KO)	FSL-1	When activation of TLR2 and TLR6 is of interest
HEK- Blue™ TLR2(TLR1/TLR6_KO) (hkb-htlr2k16)	N/A	rec hIL-1β	When activation of TLR2 is of interest and a control is wanted to exclude the contribution of TLR1 and TLR6 with which TLR2 may form heterodimers
HEK- Blue™ TLR3 (hkb-htlr3)	HEK- Blue™ Null1	Poly(I:C)HMW/LMW Poly(A:U)	When activation of TLR3 is of interest
HEK- Blue™ TLR4 (hkb-htlr4)	HEK- Blue™ Null2	LPS-EK, ultrapure LPS-EB, ultrapure	When activation of TLR4 is of interest
HEK- Blue™ TLR7 (hkb-htlr7)	HEK- Blue™ Null1-k	CL264	When activation of TLR7 is of interest
HEK- Blue™ TLR8 (hkb-htlr8)	HEK- Blue™ Null1	ssRNA40, ssPoly(U), ORN02, ORN06, CL075, R848	When activation of TLR8 is of interest
HEK- Blue™ TLR9 (hkb-htlr9)	HEK- Blue™ Null1	ODN2006	When activation of TLR9 is of interest

7. Experimental Procedure

7.1 *(Optional for calibration curve)* Prepare SEAP at 5X working concentrations for running a calibration curve *(suggested range of 0.1 ng/mL to 1 µg/mL final concentration).*

7.2 Adjust cell concentration to ~1.6- 5 x 10^5 cells/mL using HEK-Blue Detection. Consult Table 2 for details on recommended seeding density of individual cell lines.

7.3 Add 40 µL of study samples, controls, or medium to the appropriate wells.

7.4 Plate 160 µL of cell suspension per well in a 96 well plate. Prepare triplicate wells for each sample. Prepare one cell-free well per nanoparticle concentration per plate. These wells will be used to assay potential nanoparticle interference with the assay.

7.5 Incubate for 24 hours in a humidified 37°C, 5% CO_2 incubator.

7.6 Check the culture plate for color development, image *(see Appendix 12.4)* the plate and then read the optical density (OD) at 620-655 nm on a plate reader.

Table 2. Recommended seeding density of individual reporter cell lines.

Cell line	Number of cells per well	Number of cells per mL
Null 1	50,000	3.2 x 105
Null1-K	50,000	3.2 x 105
Null2	50,000	3.2 x 105
TLR2	50,000	3.2 x 105
TLR3	50,000	3.2 x 105
TLR4	25,000	1.6 x 105
TLR7	40,000	2.5 x 105
TLR8	40,000	2.5 x 105
TLR2/TLR1	50,000	3.2 x 105
TLR2/TLR6	50,000	3.2 x 105
TLR2KO/TLR1/TLR6	50,000	3.2 x 105
TLR9	80,000	5 x 105

8. Calculations

8.1 A percent coefficient of variation is used to control precision and is calculated for each control or test sample according to the following formula:

$$\%CV = \frac{standard\ deviation}{mean} * 100\%$$

9. Acceptance Criteria

9.1 %CV for each control and test sample should be within 30%.

9.2 The response is considered positive when SEAP levels in the test sample are ≥2-fold above that in the baseline, negative control sample.

10. References

1. Pasare C, Medzhitov R. Toll-like receptors: linking innate and adaptive immunity. Microbes Infect. 2004 Dec;6(15):1382-7.
2. Gnjatic, S., Sawhney, N. B., & Bhardwaj, N. (2010). Toll-like receptor agonists: are they good adjuvants? *Cancer journal (Sudbury, Mass.)*, *16*(4), 382–391.
3. Luchner M, Reinke S, Milicic A. TLR Agonists as Vaccine Adjuvants Targeting Cancer and Infectious Diseases. Pharmaceutics. 2021 Jan 22;13(2):142.
4. Yu L, Wang L, Chen S. Endogenous toll-like receptor ligands and their biological significance. J Cell Mol Med. 2010 Nov;14(11):2592-603.
5. Invivogen product sheets for HEK Blue TLR cell lines, HEK Blue Detection, SEAP Reporter Assay Kit and SEAP Protein. (https://www.invivogen.com/hek-blue-detection).
6. Dobrovolskaia MA, McNeil SE. Understanding the correlation between in vitro and in vivo immunotoxicity tests for nanomedicines. J Control Release. 2013 Dec 10;172(2):456-66.

11. Abbreviations

Cal	calibration standards
CV	coefficient of variation
FBS	fetal bovine serum

HEK	human embryonic kidney
LPS	lipopolysaccharide
NC	negative control
PBS	phosphate buffered saline
PC	positive control
SEAP	secreted embryonic alkaline phosphatase
TLR	toll-like receptor
VC	vehicle control
w/v	weight to volume ratio

12. Appendix

12.1 Example Culture Plate Maps

	1	2	3	4	5	6	7	8	9	10	11	12
A	Medium	NC	PC1	PC2	PC3				TS1 (No cells)	TS2 (No cells)	TS3 (No cells)	TS4 (No cells)
B	Medium	NC	PC1	PC2	PC3				TS1 (No cells)	TS2 (No cells)	TS3 (No cells)	TS4 (No cells)
C	TS1	TS1	TS1	TS2	TS2	TS2	TS3	TS3	TS3	TS4	TS4	TS4
D	TS1	TS1	TS1	TS2	TS2	TS2	TS3	TS3	TS3	TS4	TS4	TS4
E	Medium	NC	PC1	PC2	PC3							
F	Medium	NC	PC1	PC2	PC3							
G	TS1	TS1	TS1	TS2	TS2	TS2	TS3	TS3	TS3	TS4	TS4	TS4
H	TS1	TS1	TS1	TS2	TS2	TS2	TS3	TS3	TS3	TS4	TS4	TS4

Rows A through D: HEK-Blue cell line 1

Rows E through H: HEK-Blue cell line 2

PC1 through PCx: Various cell line - specific positive controls/concentrations

	1	2	3	4	5	6	7	8	9	10	11	12
A	Medium	NC	PC1	PC2	PC3				TS1 (No cells)	TS2 (No cells)	TS3 (No cells)	TS4 (No cells)
B	TS1	TS1	TS1	TS2	TS2	TS2	TS3	TS3	TS3	TS4	TS4	TS4
C	Medium	NC	PC1	PC2	PC3							
D	TS1	TS1	TS1	TS2	TS2	TS2	TS3	TS3	TS3	TS4	TS4	TS4
E	Medium	NC	PC1	PC2	PC3							
F	TS1	TS1	TS1	TS2	TS2	TS2	TS3	TS3	TS3	TS4	TS4	TS4
G	Medium	NC	PC1	PC2	PC3							
H	TS1	TS1	TS1	TS2	TS2	TS2	TS3	TS3	TS3	TS4	TS4	TS4

Rows A, B: HEK-Blue cell line 1

Rows C, D: HEK-Blue cell line 2

Rows E, F: HEK-Blue cell line 3

Rows G, H: HEK-Blue cell line 4

PC1 through PCx: Various cell line - specific positive controls/concentrations

12.2 Media Formulations:

(See product sheets specific to each cell line at https://www.invivogen.com/hek-blue-tlr)

HEK-Blue™ Cells, Base Medium

DMEM, high glucose (4.5g/L)

10% FBS (heat inactivated)

2 mM L-glutamine

100 µg/mL normocin

Additives [cell line specific concentrations]

50U/mL penicillin (Null1, Null1-k, Null2, hTLR2, hTLR3, hTLR4, hTLR7, hTLR8, hTLR9)

100 U/mL penicillin (hTLR2-TLR1, hTLR-TLR6, hTLR2 KO-TLR1/6)

50 µg/mL streptomycin sulfate (Null1, Null1-k, Null2, hTLR2, hTLR3, hTLR4, hTLR7, hTLR8, hTLR9)

100 µg/mL streptomycin sulfate (hTLR2-TLR1, hTLR-TLR6, hTLR2, KO-TLR1/6)

Selection additives [cell line specific]

(*to be added after 2 passages from thaw*)

100 µg/mL Zeocin (Null1, Null1-k, Null2, hTLR3, hTLR7, hTLR8, hTLR9)

10 µg/mL Blasticidin (hTLR7, hTLR9)

30 µg/mL Blasticidin (hTLR3, hTLR8)

1x HEK-Blue Selection (hTLR2, hTLR2-TLR1, hTLR2-hTLR6, hTLR2 KO-TLR1/6, hTLR4)

12.3 Trypsin [cell line specific]

No Trypsin, use warm PBS only, avoid centrifugation

(Null1, Null1-K, Null2, hTLR2, hTLR3, hTLR4, hTLR7, hTLR8, hTLR9)

0.08% Trypsin (dilute 0.25% trypsin with 1xPBS), Ok to centrifuge

(hTLR2-TLR1, hTLR2-hTLR6, hTLR2 KO-TLR1/6)

12.4 Example plate images after treatment and incubation of cells with TLR agonists