Quantikine® ELISA

Human TLR2 Immunoassay

Catalog Number DTLR20

For the quantitative determination of human Toll-Like Receptor 2 (TLR2) concentrations in cell culture supernates, cell lysates, serum, plasma, saliva, urine, and human milk.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

Toll-Like Receptor 2 (TLR2), also known as CD282, is an approximately 110 kDa transmembrane protein that plays an important role in the inflammatory response to microbial infection. It is one of several TLR family receptors that recognize pathogen-associated molecular patterns (PAMPs) in microbial components (1, 2). Mature human TLR2 consists of a 570 amino acid (aa) extracellular domain (ECD) with 19 leucine rich repeats (LLR) and one C-terminal leucine-rich domain, a 21 aa transmembrane segment, and a 175 aa cytoplasmic domain (3, 4). Within the ECD, human TLR2 shares 67% aa sequence identity with mouse and rat TLR2. TLR2 is expressed on antigen presenting cells (plasmacytoid dendritic cells, B cells, monocytes, and macrophages), T cells (activated $CD4^+$, $CD8^+$, memory, and Treg), renal tubule epithelial cells, trophoblasts, and decidual and amnion epithelial cells (5-9). Soluble forms of TLR2 retain ligand binding capability and function as decoy receptors that suppress TLR2 mediated inflammation (9, 10).

TLR2 associates with multiple proteins in the plasma membrane including TLR1, TLR6, TLR10, CD14, CD36, CXCR4, Dectin-1, and Integrins αVβ3 and α3β1, as well as the ganglioside GD1a (11-21). These molecules are either required as co-receptors or enhance TLR2 responsiveness to various ligands. TLR2-containing receptor complexes are differentially responsive to microbial lipopeptides, peptidoglycans, lipoteichoic acid, enterotoxin, LPS, as well as yeast and parasite glucans (2). TLR2 is also activated by endogenous ligands such as amyloid Aβ peptide oligomers, Serum Amyloid A, HMGB1, beta-Defensin 3, PAUF, Biglycan, and LMW Hyaluronan (22-28). Ligand binding by TLR2 can induce receptor association with CXCR4, resulting in inhibition of CXCR4 and TLR2 signaling (18, 26). TLR2 initiates proinflammatory signaling through its cytoplasmic TIR domain, a domain which is also found in other TLR and IL-1 family receptors and some adaptor proteins (29). It can signal from the cell surface or from intracellular phagosomes following ligand internalization (30). TLR2 activation also regulates adaptive immune responses such as dendritic cell activation, B cell maturation, and T cell mediated tumor regression (6, 24, 31). Following ischemia/reperfusion injury, TLR2 activation contributes to disease pathology in myocardial infarction and the kidney, although it can be neuroprotective following ischemia/reperfusion injury in the brain (8, 32-34).

The Quantikine Human TLR2 Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human TLR2 in cell culture supernates, cell lysates, serum, plasma, saliva, urine, and human milk. It contains NS0-expressed recombinant human TLR2 and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human TLR2 showed linear curves that were parallel to the standard curves obtained using the Quantikine kit standards. These results indicate that this kit can be used to determine relative mass values for naturally occurring human TLR2.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A polyclonal antibody specific for human TLR2 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any TLR2 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human TLR2 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of TLR2 bound in the initial step. The color development is stopped and the intensity of the color is measured.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, further dilute the samples with the appropriate Calibrator Diluent and repeat the assay.
- Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Variations in sample collection, processing, and storage may cause sample value differences.
- This assay is designed to eliminate interference by other factors present in biological samples. Until all factors have been tested in the Quantikine Immunoassay, the possibility of interference cannot be excluded.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- When using an automated plate washer, adding a 30 second soak period following the addition of Wash Buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human TLR2 Microplate	894670	96 well polystyrene microplate (12 strips of 8 wells) coated with a polyclonal antibody specific for human TLR2.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2-8 °C.*
Human TLR2 Conjugate	894671	21 mL of a polyclonal antibody specific for human TLR2 conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Human TLR2 Standard	894672	Recombinant human TLR2 in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	
Assay Diluent RD1-75	895811	11 mL of a buffered animal serum with preservatives. *May contain a precipitate. Mix well before and during use.*	
Calibrator Diluent RD6P	895118	21 mL of animal serum with preservatives. *Used undiluted for serum/ plasma samples. Used diluted 1:5 for cell culture supernate/cell lysate/saliva/urine/ human milk samples.*	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Color Reagent A	895000	12 mL of stabilized hydrogen peroxide.	
Color Reagent B	895001	12 mL of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895032	6 mL of 2 N sulfuric acid.	
Plate Sealers	N/A	4 adhesive strips.	

* Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 20 mL and 500 mL graduated cylinders.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.
- Test tubes for dilution of standards and samples.
- Human TLR2 Controls (optional; available from R&D Systems).

SUPPLIES REQUIRED FOR CELL LYSATE SAMPLES

- Cell Lysis Buffer 3 (R&D Systems, Catalog # 895366)
- PBS

PRECAUTIONS

TLR2 is detectable in saliva. Take precautionary measures to prevent contamination of kit reagents while running this assay.

The Stop Solution provided with this kit is an acid solution.

Some components in this kit contain ProClin® which may cause an allergic skin reaction. Avoid breathing mist.

Color Reagent B may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Please refer to the MSDS on our website prior to use.

SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Cell Lysates - Cells must be lysed prior to assay as directed in the Cell Lysis Procedure.

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Citrate plasma has not been validated for use in this assay.*
Grossly hemolyzed samples are not suitable for use in this assay.

Saliva - Collect saliva in a tube and centrifuge for 5 minutes at 10,000 x g. Collect the aqueous layer, and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Urine - Aseptically collect the first urine of the day (mid-stream), voided directly into a sterile container. Centrifuge to remove particulate matter, and assay immediately or aliquot and store at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Human Milk - Centrifuge for 15 minutes at 1000 x g at 2-8 °C. Collect the aqueous fraction and repeat this process a total of 3 times. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

SAMPLE PREPARATION

Saliva samples require a 3-fold dilution. A suggested 3-fold dilution is 100 µL of sample + 200 µL of Calibrator Diluent RD6P (diluted 1:5).*

Human milk samples require a 30-fold dilution. A suggested 30-fold dilution is 20 µL of sample + 580 µL of Calibrator Diluent RD6P (diluted 1:5).

CELL LYSIS PROCEDURE

Use the following procedure for the preparation of cell lysate samples.

1. Dilute Cell Lysis Buffer 3 concentrate 1:5 in deionized or distilled water.
2. Wash cells three times in cold PBS.
3. Resuspend cells at 1 x 10^7 cells/mL in Cell Lysis Buffer 3 (diluted 1:5).
4. Incubate with gentle agitation for up to 60 minutes at room temperature.
5. Centrifuge at 8000 x g for 10 minutes to remove cell debris.
6. Assay immediately or aliquot the lysis supernates and store at ≤ -70 °C until ready for use.

* See Reagent Preparation section.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Note: *TLR2 is found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

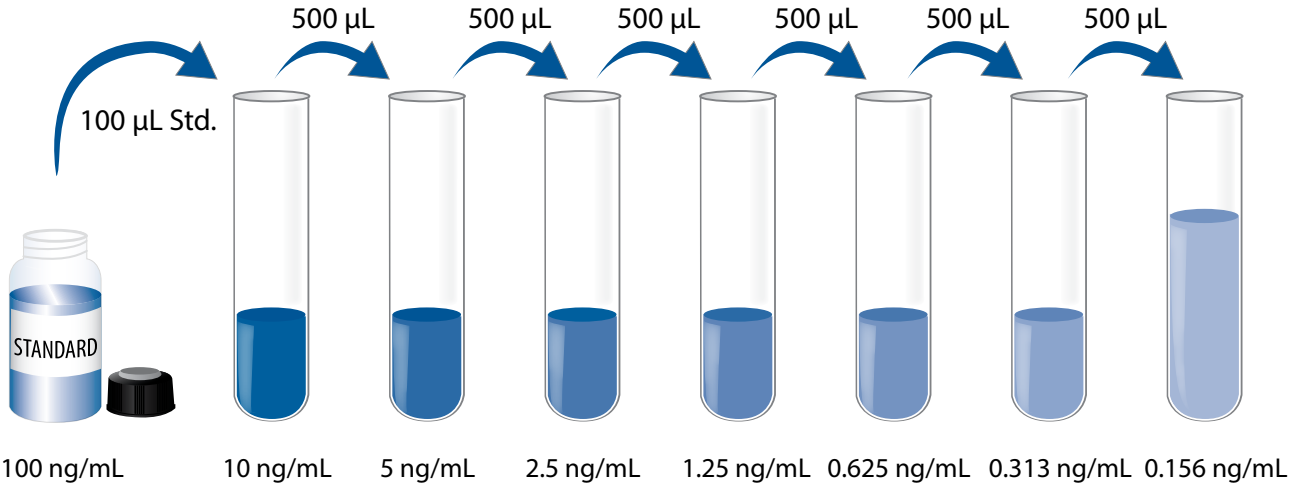
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

Calibrator Diluent RD6P (diluted 1:5) - **For cell culture supernate/cell lysate/saliva/urine/human milk samples only.** Add 4 mL of Calibrator Diluent RD6P to 16 mL of deionized or distilled water to prepare 20 mL of Calibrator Diluent RD6P (diluted 1:5).

Human TLR2 Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human TLR2 Standard with deionized or distilled water. This reconstitution produces a stock solution of 100 ng/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 900 µL of Calibrator Diluent RD6P (diluted 1:5) (*for cell culture supernate/cell lysate/saliva/urine/human milk samples*) or Calibrator Diluent RD6P (*for serum/plasma samples*)into the 10 ng/mL tube. Pipette 500 µL of the appropriate Calibrator Diluent into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 10 ng/mL standard serves as the high standard. The appropriate Calibrator Diluent serves as the zero standard (0 ng/mL).



ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all samples, controls, and standards be assayed in duplicate.

Note: *TLR2 is found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 100 µL of Assay Diluent RD1-75 to each well. *Assay Diluent RD1-75 may contain a precipitate. Mix well before and during use.*
4. Add 100 µL of Standard, control, or sample* per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm. A plate layout is provided to record standards and samples assayed.
5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
6. Add 200 µL of Human TLR2 Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature on the shaker.
7. Repeat the aspiration/wash as in step 5.
8. Add 200 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature **on the benchtop. Protect from light.**
9. Add 50 µL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

*Samples may require dilution. See the Sample Preparation section.

CALCULATION OF RESULTS

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

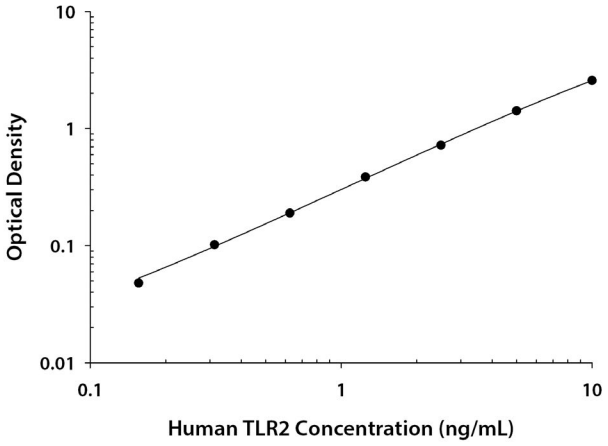
Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the human TLR2 concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

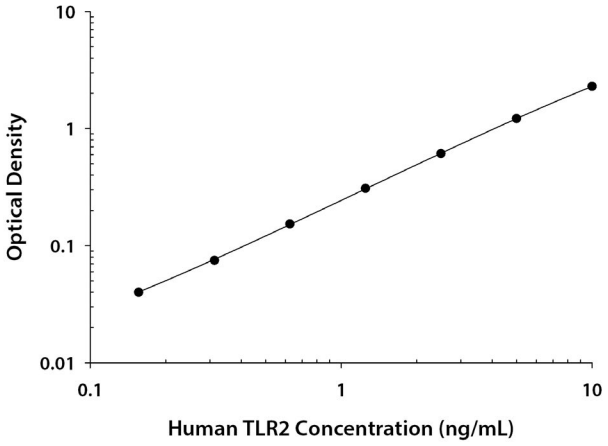
These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.

CELL CULTURE SUPERNATE/CELL LYSATE/ SALIVA/URINE/HUMAN MILK ASSAY



(ng/mL)	O.D.	Average	Corrected
0	0.056 0.056	0.056	—
0.156	0.103 0.104	0.104	0.048
0.313	0.156 0.159	0.158	0.102
0.625	0.244 0.247	0.246	0.190
1.25	0.436 0.447	0.442	0.386
2.5	0.773 0.785	0.779	0.723
5	1.452 1.490	1.471	1.415
10	2.613 2.635	2.624	2.568

SERUM/PLASMA ASSAY



(ng/mL)	O.D.	Average	Corrected
0	0.057 0.063	0.060	—
0.156	0.100 0.100	0.100	0.040
0.313	0.134 0.135	0.135	0.075
0.625	0.207 0.218	0.213	0.153
1.25	0.357 0.379	0.368	0.308
2.5	0.635 0.708	0.672	0.612
5	1.221 1.332	1.277	1.217
10	2.332 2.367	2.350	2.290

PRECISION

Intra-assay Precision (Precision within an assay)
Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays)
Three samples of known concentration were tested in twenty separate assays to assess inter-assay precision. Assays were performed by at least three technicians using two lots of components.

CELL CULTURE SUPERNATE/CELL LYSATE/SALIVA/URINE/HUMAN MILK ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (ng/mL)	1.32	2.72	5.47	1.40	2.82	5.73
Standard deviation	0.032	0.055	0.109	0.105	0.225	0.427
CV (%)	2.4	2.0	2.0	7.5	8.0	7.5

SERUM/PLASMA ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (ng/mL)	1.43	3.01	5.82	1.48	3.12	6.20
Standard deviation	0.051	0.065	0.155	0.128	0.310	0.460
CV (%)	3.6	2.2	2.7	8.6	9.9	7.4

RECOVERY

The recovery of human TLR2 spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	96	92-98%
Serum (n=4)	99	84-111%
EDTA plasma (n=4)	98	86-107%
Heparin plasma (n=4)	98	88-105%
Urine (n=4)	98	91-108%

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of human TLR2 were diluted with Calibrator Diluent to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=4)	Cell lysates* (n=4)	Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)	Saliva* (n=4)	Urine (n=4)	Human milk* (n=4)
1:2	Average % of Expected	102	102	101	101	102	104	102	104
	Range (%)	101-105	95-112	97-106	101-102	98-107	99-110	99-106	94-114
1:4	Average % of Expected	104	101	101	102	101	104	104	101
	Range (%)	103-106	95-110	97-108	97-107	94-107	98-116	100-109	91-108
1:8	Average % of Expected	107	94	104	102	106	100	111	105
	Range (%)	101-114	92-97	94-116	98-106	100-114	91-113	107-114	91-114
1:16	Average % of Expected	100	97	104	99	104	94	101	102
	Range (%)	94-106	93-101	98-112	95-105	93-113	85-103	95-105	85-114

*Samples were diluted prior to assay.

SENSITIVITY

Fifty-four assays were evaluated and the minimum detectable dose (MDD) of human TLR2 ranged from 0.006-0.109 ng/mL. The mean MDD was 0.024 ng/mL.

The MDD was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

CALIBRATION

This immunoassay is calibrated against a highly purified NS0-expressed recombinant human TLR2 manufactured at R&D Systems.

SAMPLE VALUES

Serum/Plasma/Saliva/Urine/Human Milk - Samples from apparently healthy volunteers were evaluated for the presence of human TLR2 in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean (ng/mL)	Range (ng/mL)	Standard Deviation (ng/mL)
Serum (n=36)	0.575	0.296-0.880	0.140
EDTA plasma (n=36)	0.484	0.246-0.744	0.117
Heparin plasma (n=36)	0.509	0.232-0.830	0.121
Saliva (n=10)	6.82	2.37-13.5	3.47
Urine (n=10)	0.533	0.226-0.791	0.200
Human milk (n=10)	70.9	19.1-260	71.5

Cell Culture Supernates/Cell Lysates:
Human peripheral blood leukocytes PBLs were cultured in RPMI 1640 supplemented with 10% fetal bovine serum. Cells were cultured unstimulated for 1 and 6 days. Aliquots of the cell culture supernates were removed, assayed for human TLR2, and measured 0.779 ng/mL and 1.13 ng/mL respectively.

Human Aortic Endothelial Cells (HAEC) were cultured in EGM2 and grown until confluent. Cells were cultured stimulated with 40 ng/mL recombinant human TNF-α and 50 ng/mL recombinant human IFN-α. Aliquots were removed and assayed for human TLR2.

Cell Culture Supernate Value (ng/mL)	Cell Lysate Value (ng/mg)
ND	1.69

ND=Non-detectable

SPECIFICITY

This assay recognizes natural and recombinant human TLR2.

The factors listed below were prepared at 100 ng/mL in Calibrator Diluent and assayed for cross-reactivity. Preparations of the following factors at 100 ng/mL in a mid-range recombinant human TLR2 control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

CD14
MD-2
MyD88
PRAT4A
TLR1
TLR3
TLR4
TLR5
TLR6
TLR7
TLR9
TLR10

Recombinant mouse TLR2 cross-reacts approximately 0.7% in this assay.

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PLATE LAYOUT

Use this plate layout to record standards and samples assayed.