Quantikine® ELISA

Human TIM-3 Immunoassay

Catalog Number DTIM30

For the quantitative determination of human T cell Immunoglobulin and Mucin domain 3 (TIM-3) concentrations in cell culture supernates, cell lysates, serum, plasma, and urine.

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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614 McKinley Place NE, Minneapolis, MN 55413, USA
TEL: (800) 343-7475 (612) 379-2956 FAX: (612) 656-4400
E-MAIL: info@RnDSystems.com

DISTRIBUTED BY:

UK & Europe | R&D Systems Europe, Ltd.
19 Barton Lane, Abingdon Science Park, Abingdon OX14 3NB, UK
TEL: +44 (0)1235 529449 FAX: +44 (0)1235 533420
E-MAIL: info@RnDSystems.co.uk

China | R&D Systems China Co., Ltd.
24A1 Hua Min Empire Plaza, 726 West Yan An Road, Shanghai PRC 200050
TEL: +86 (21) 52380373 FAX: +86 (21) 52371001
E-MAIL: info@RnDSystemsChina.com.cn

INTRODUCTION

TIM-3 (T cell immunoglobulin and mucin domain-3), also known as HAVCR2, is a 60 kDa member of the TIM family of immune regulating molecules. TIMs are type I transmembrane glycoproteins with one Ig-like V-type domain and a Ser/Thr-rich mucin stalk region (1, 2). Mature human TIM-3 consists of a 181 amino acid (aa) extracellular domain (ECD), a 21 aa transmembrane segment, and a 78 aa cytoplasmic tail (3). An alternatively spliced isoform is truncated within the mucin-like stalk. Within the ECD, human TIM-3 shares 58% aa sequence identity with mouse and rat TIM-3. TIM-3 is upregulated on several populations of activated myeloid cells (macrophage, monocyte, dendritic cell, microglia, mast cell) and T cells (Th1, CD8+, NK, Treg) (3-11). It is also upregulated on activated microglia and astrocytes during brain hypoxia (12). Its binding to Galectin-9 induces a range of immunosuppressive functions which enhance immune tolerance and inhibit anti-tumor immunity (13). TIM-3 ligation attenuates CD8+ and Th1 cell responses (13-15) and promotes the activity of Treg and myeloid derived suppressor cells (8, 13, 15, 16). In addition, dendritic cell-expressed TIM-3 dampens inflammation by enabling the phagocytosis of apoptotic cells and the cross-presentation of apoptotic cell antigens (4). TIM-3 also binds the alarmin HMGB1, thereby preventing the activation of TLRs in response to released tumor cell DNA (7). TIM-3 interactions with Galectin-9 can alternatively trigger immune stimulatory effects, such as the coactivation of NK cell cytotoxicity (10). TIM-3 can be shed from the surface of monocytes by ADAM10 or ADAM17-mediated cleavage within the TIM-3 stalk region (17, 18) resulting in a soluble form which is elevated in the plasma of patients with graft-versus-host disease (GVHD) or HIV infection (18, 19). Soluble TIM-3 interferes with the immunosuppressive functions of membrane bound TIM-3 and contributes to the severity of GVHD (20).

The Quantikine Human TIM-3 Immunoassay is a 2.5 hour solid phase ELISA designed to measure TIM-3 levels in cell culture supernates, cell lysates, serum, plasma, and urine. It contains NS0-expressed recombinant human TIM-3 and antibodies raised against the recombinant protein. Results obtained for naturally occurring human TIM-3 showed linear curves that were parallel to the standard curves obtained using the Quantikine Human TIM-3 Immunoassay standards. These results indicate that this kit can be used to determine relative mass values for natural human TIM-3.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human TIM-3 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any TIM-3 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human TIM-3 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 TIM-3 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 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 TIM-3 Microplate	894982	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human TIM-3.	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 TIM-3 Standard	894984	2 vials of recombinant human TIM-3 in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	Discard after use. Use a new standard for each assay.
Human TIM-3 Conjugate	894983	21 mL of a polyclonal antibody specific for human TIM-3 conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1W	895117	12 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5-24	895325	21 mL of a buffered protein base with preservatives. *Use diluted 1:3 in this assay.*	
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.
- 50 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 TIM-3 Controls (optional; R&D Systems, Catalog # QC217).

SUPPLIES REQUIRED FOR CELL LYSATE SAMPLES

- Lysis Buffer 17 (R&D Systems, Catalog # 895943).

PRECAUTIONS

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 Sample Values section.

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.*

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

SAMPLE PREPARATION

Serum and plasma samples require a 5-fold dilution due to high endogenous levels. A suggested 5-fold dilution is 50 µL of sample + 200 µL of Calibrator Diluent RD5-24 (diluted 1:3)*.

Urine samples require a 50-fold dilution due to high endogenous levels. A suggested 50-fold dilution is 10 µL of sample + 490 µL of Calibrator Diluent RD5-24 (diluted 1:3).

*See Reagent Preparation section.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

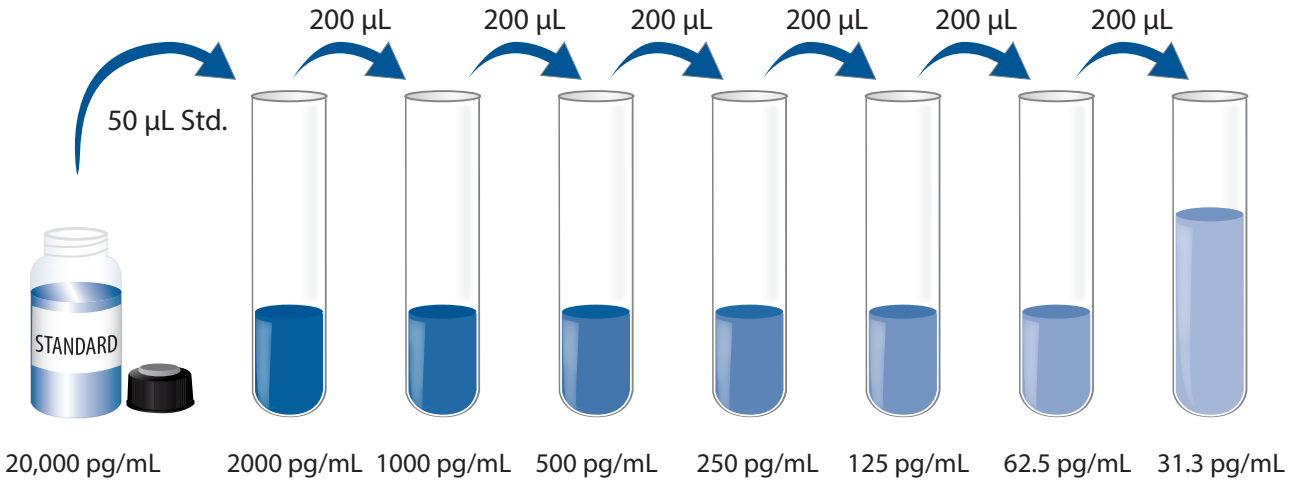
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

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 RD5-24 (diluted 1:3) - Add 10 mL of Calibrator Diluent RD5-24 to 20 mL of deionized or distilled water to prepare 30 mL of Calibrator Diluent RD5-24 (diluted 1:3).

Human TIM-3 Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human TIM-3 Standard with deionized or distilled water. This reconstitution produces a stock solution of 20,000 pg/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 450 µL of Calibrator Diluent RD5-24 (diluted 1:3) into the 2000 pg/mL tube. Pipette 200 µL into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 2000 pg/mL standard serves as the high standard. Calibrator Diluent RD5-24 (diluted 1:3) serves as the zero standard (0 pg/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.

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 RD1W to each well.
4. Add 50 µL of Standard, control, or sample* per well. Cover with the adhesive strip provided. Incubate for 1 hour 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 TIM-3 Conjugate to each well. Cover with a new adhesive strip. Incubate for 1 hour 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

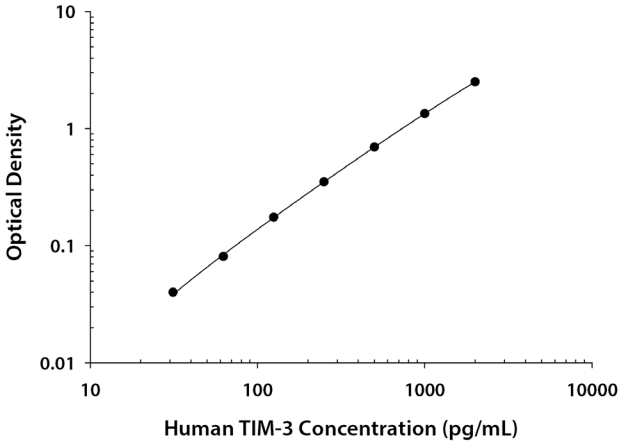
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 TIM-3 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

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



(pg/mL)	O.D.	Average	Corrected
0	0.013 0.013	0.013	—
31.3	0.052 0.054	0.053	0.040
62.5	0.094 0.094	0.094	0.081
125	0.187 0.188	0.188	0.175
250	0.363 0.364	0.364	0.351
500	0.704 0.710	0.707	0.694
1000	1.348 1.361	1.355	1.342
2000	2.514 2.529	2.522	2.509

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.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pg/mL)	182	561	1132	184	549	1103
Standard deviation	4.05	11.7	29.0	9.69	21.9	42.7
CV (%)	2.2	2.1	2.6	5.3	4.0	3.9

RECOVERY

The recovery of human TIM-3 spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	103	94-108%
Cell Lysis Buffer (n=1)	102	96-107%
Serum* (n=4)	102	99-106%
EDTA plasma* (n=4)	100	93-105%
Heparin plasma* (n=4)	97	92-100%

*Samples were diluted prior to assay.

LINEARITY

To assess the linearity of the assay, samples containing high concentrations of human TIM-3 were diluted with Calibrator Diluent to produce samples with values within the dynamic range of the assay.

		Cell culture supernates* (n=4)	Cell lysate (n=2)	Serum* (n=4)	EDTA plasma* (n=4)	Heparin plasma* (n=4)	Urine* (n=4)
1:2	Average % of Expected	96	98	98	101	99	94
	Range (%)	94-99	94-102	97-100	99-106	96-102	90-96
1:4	Average % of Expected	93	107	98	103	100	93
	Range (%)	89-96	106-107	96-100	98-106	97-104	88-95
1:8	Average % of Expected	92	112	96	103	99	93
	Range (%)	87-98	110-113	95-99	98-106	96-101	90-95
1:16	Average % of Expected	91	116	100	100	101	91
	Range (%)	88-94	113-118	92-110	91-108	97-106	89-93

*Samples were diluted prior to assay.

SENSITIVITY

Twenty-one assays were evaluated and the minimum detectable dose (MDD) of human TIM-3 ranged from 0.554-8.75 pg/mL. The mean MDD was 2.17 pg/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 highly purified NS0-derived recombinant human TIM-3 produced at R&D Systems.

SAMPLE VALUES

Serum/Plasma/Urine - Samples from apparently healthy volunteers were evaluated for the presence of human TIM-3 in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean (pg/mL)	Range (pg/mL)	Standard Deviation (pg/mL)
Serum (n=36)	1726	951-2788	484
EDTA plasma (n=36)	1565	810-2637	427
Heparin plasma (n=36)	1431	828-2397	416

Sample Type	Mean (µg/g Creatinine)	Range (pg/mL)	Standard Deviation (µg/g Creatinine)
Urine (n=10)	14.4	10.1-17.6	2.64

Cell Culture Supernates - Human peripheral blood leukocytes (PBL) were cultured in RPMI 1640 and supplemented with 10% fetal bovine serum. Cells were then cultured unstimulated or stimulated with 10 µg/mL PHA for 1 or 5 days. Aliquots of the cell culture supernates were removed and assayed for levels of human TIM-3.

Condition	Day 1 (pg/mL)	Day 5 (pg/mL)
Unstimulated	ND	45.7
Stimulated	ND	363

Cell Culture Supernates/Cell Lysates:
Human PBMC-derived CD4+ T cells were cultured in RPMI supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin sulfate, and 2 mM L-glutamine. Cells were cultured unstimulated or stimulated with 5 µg/mL anti-human CD28 and of immobilized anti-human CD3 for 6 days. On the 6th day, 10 ng/mL PMA and 500 ng/mL ionomycin were added and allowed to incubate overnight. Cell culture supernate and cell lysate samples were collected on the 7th day.

Human PBMC-derived Th2 cells were cultured in RPMI supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin sulfate, and 2 mM L-glutamine. Cells were stimulated with 10 ng/mL of recombinant human IL-2 and 5 µg/mL PHA for 6 days. On the 6th day, 10 ng/mL PMA and 500 ng/mL ionomycin were added and allowed to incubate overnight. Cell culture supernate and cell lysate samples were collected on the 7th day.

Note: *Quantitation of sample protein concentration using a total protein assay is recommended. The suggested range for total cell lysate protein added is 10-50 µg/well.*

Cell Line	Cell Culture Supernates (pg/mL)	Cell Lysates (pg/mL)
$CD4^+$ T cells, unstimulated	36.4	677
$CD4^+$ T cells, stimulated	2642	938
Th2 cells, stimulated	2454	1217

SPECIFICITY

This assay recognizes natural and recombinant human TIM-3.

The factors listed below were prepared at 50 ng/mL in Calibrator Diluent and assayed for cross-reactivity. Preparations of the following factors at 50 ng/mL in a mid-range recombinant human TIM-3 control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

Galectin-9
HMG-1
TIM-1/KIM-1
TIM-4

Other recombinants:

mouse TIM-3

Cynomolgus monkey TIM-3 cross-reacts approximately 0.26% in this assay.

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PLATE LAYOUT

Use this plate layout to record standards and samples assayed.