Quantikine® ELISA

Human B7-H3 Immunoassay

Catalog Number DB7H30

For the quantitative determination of human B cell antigen #7 homolog 3 (B7-H3) concentrations in cell culture supernates, 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.

TABLE OF CONTENTS

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INTRODUCTION

B7-H3 (B cell antigen #7 homolog 3), also known as PD-L3, B7RP2, and CD276, is a 100-110 kDa monomeric glycoprotein that belongs to Group III of the B7 family, Ig Superfamily of molecules (1-4). It is considered an atypical member of the B7 family in that it is widely expressed and appears not to use the CD28 family as a receptor (1, 2, 5). B7-H3 is expressed by a variety of primary cell types, including osteoblasts (6), fibroblasts (7), ciliated respiratory epithelium (8), anterior pituitary progenitor cells (9), first trimester extravillous trophoblasts (10), and multiple tumor cells (4, 11). It is also known to be induced on dendritic cells (DC) (12), skeletal muscle myocytes (13), and monocytes (2, 14). Notably, mRNA for B7-H3 is widely expressed while protein is restricted in appearance. This suggests tight regulation at the post-transcriptional level (5, 15). Functionally, B7-H3 has been described as a molecule that fine-tunes the immune response. If so, it would appear to be a complex activity. Presumably in a context-dependent manner, B7-H3 has been reported to both co-stimulate and co-inhibit lymphocyte action. As a co-stimulator, B7-H3 appears to augment LPS-induced NFκB activation in macrophages (in a TLR-dependent manner) (14). When combined with anti-CD3, it also reportedly induces $CD4^+$ and $CD8^+$ T cell proliferation plus IFN-γ secretion (3, 5, 16, 17). As a co-inhibitor, the presence of membrane B7-H3 on tumor cells reportedly blocks the cytotoxic activity of NK cells, reduces cytokine production by activated T cells, and imparts an immunosuppressive phenotype to DC as it relates to antigen presentation (2, 5, 12, 18-20). B7-H3 on the surface of fibroblasts has been found to promote IFN-γ and TNF-α production by resting T cells and to decrease cytokine production by activated T cells (7). The contradictory nature of B7-H3 activity is likely related to the B7-H3 receptor(s). One receptor associated with co-stimulatory activity has been identified and found to be TREML2/TLT-2 (21). TREML2 is found on $CD8^+$ and $CD4^+$ T cells, and ligation by B7-H3 induces lymphocyte proliferation and IFN-γ production. In addition, B7-H3 expressing tumor cells have been shown to promote the cytolytic activity of TREML2-expressing $CD8^+$ T cells (22). Others, however, have found no physical association between B7-H3 and TREML2, and no additional receptor has been found to explain B7-H3 co-inhibitory activity (23). B7-H3 does not form homodimers (6).

Human B7-H3 is a type I transmembrane (TM) protein that is synthesized as a 534 amino acid (aa) precursor. The precursor contains a 28 aa signal sequence plus a 506 aa mature region (16, 19). The mature region possesses a 438 aa extracellular domain (ECD), a 21 aa transmembrane (TM) segment (aa #467-487), and a 47 aa cytoplasmic tail. The ECD contains four Ig-like domains that generate a tandem-repeat based on a basic V-type:C2-type structure. This structure is suggested to be the result of exon duplication in the human gene. Notably, mouse B7-H3 ECD also contains one V-type and one C2-type Ig-like domain but is absent a second V-type and C2-type domain due to pseudoexon formation within the B7-H3 gene (20, 24). As noted, full-length human membrane B7-H3 is 100-110 kDa in size. There is also a potential membrane-bound splice variant that is 48-56 kDa in size and, like mouse, contains only one V-type and one C2-type Ig-like domain (7, 16, 17, 20). These human and mouse short forms share 92% aa identity over their ECDs. There are also at least two potential soluble forms of human B7-H3. One is a 16 kDa isoform that is generated by MMP cleavage and circulates at ng/mL concentrations (25, 26). The second is an alternative splice form that shows a 29 aa substitution for aa #465-534. If expressed, this isoform would be expected to run at about 90-95 kDa in SDS-Page (19, 27).

The Quantikine Human B7-H3 Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human B7-H3 in cell culture supernates, serum, plasma, and urine. It contains NS0-expressed recombinant human B7-H3 and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human B7-H3 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 B7-H3.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for B7-H3 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any B7-H3 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for B7-H3 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 B7-H3 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, 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.
- This assay is designed to eliminate interference by soluble receptors, binding proteins, and 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
B7-H3 Microplate	894021	96 well polystyrene microplate (12 strips of 8 wells) coated with a mouse monoclonal antibody against B7-H3.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of zip-seal. May be stored for up to 1 month at 2-8 °C.*
B7-H3 Conjugate	894022	21 mL of polyclonal antibody against B7-H3 conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
B7-H3 Standard	894023	500 ng of recombinant human B7-H3 in a buffer with preservatives; lyophilized.	
Assay Diluent RD1-109	895966	11 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD6-41	895840	21 mL of a buffered protein base with preservatives.	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservatives.	
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/vial 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.
- 500 mL graduated cylinder.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.
- Test tubes for dilution of standards.
- Human B7-H3 Controls (optional; available from R&D Systems).

PRECAUTION

The Stop Solution provided with this kit is an acid solution. Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling.

SAMPLE COLLECTION & STORAGE

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

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes 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 heparin or EDTA 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, and assay immediately or aliquot and store at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

REAGENT PREPARATION

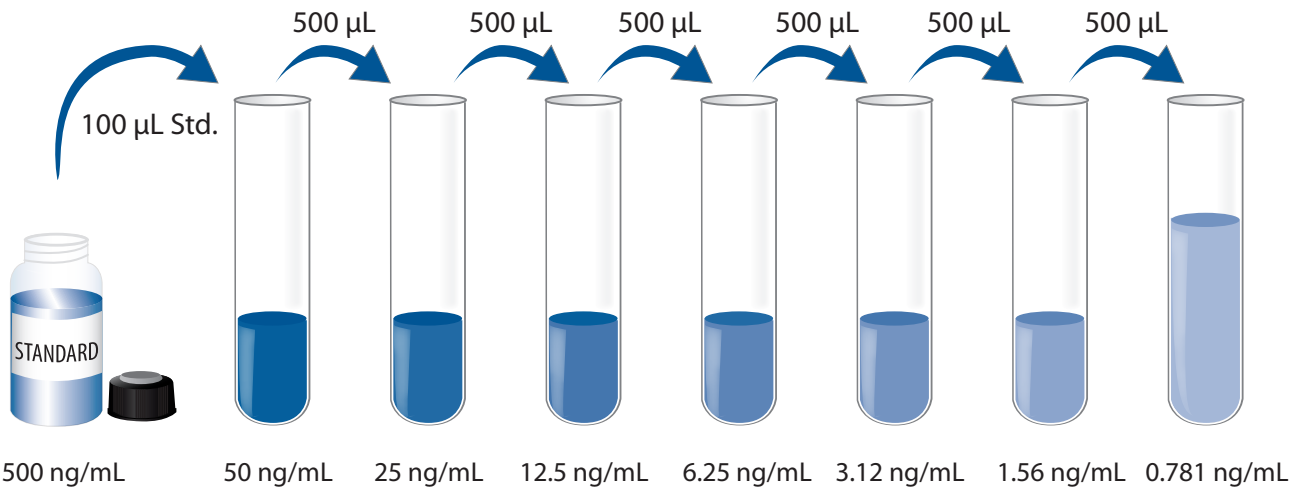
Bring all reagents to room temperature before use.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 mL of Wash Buffer Concentrate into 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.

B7-H3 Standard - Reconstitute the B7-H3 Standard with 1.0 mL of deionized or distilled water. This reconstitution produces a stock solution of 500 ng/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes. Mix well prior to making dilutions.

Pipette 900 µL of Calibrator Diluent RD6-41 into the 50 ng/mL tube. Pipette 500 µL into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 50 ng/mL standard serves as the high standard. Calibrator Diluent RD6-41 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 standards, samples, and controls 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 RD1-109 to each well.
4. Add 50 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.
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 B7-H3 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. **Protect from light.** Incubate for 30 minutes at room temperature **on the benchtop.**
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.

CALCULATION OF RESULTS

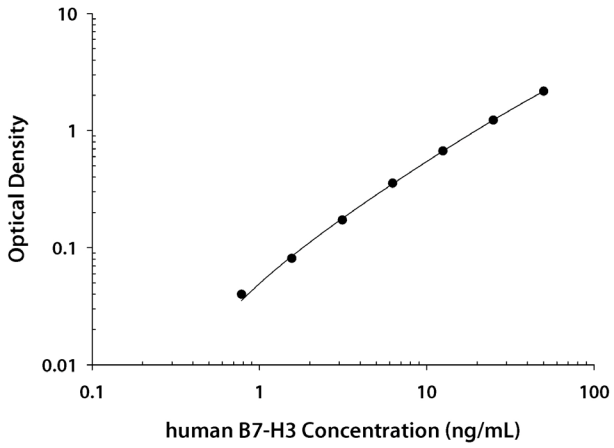
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 B7-H3 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.



(ng/mL)	O.D.	Average	Corrected
0	0.040 0.040	0.040	—
0.781	0.079 0.081	0.080	0.040
1.56	0.118 0.123	0.121	0.081
3.12	0.210 0.213	0.212	0.172
6.25	0.393 0.397	0.395	0.355
12.5	0.709 0.709	0.709	0.669
25	1.266 1.268	1.267	1.227
50	2.201 2.208	2.205	2.165

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.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (ng/mL)	5.89	19.0	37.5	5.88	17.6	35.1
Standard deviation	0.2	0.6	0.9	0.3	0.6	1.7
CV (%)	3.4	3.3	2.5	5.1	3.5	5.0

RECOVERY

The recovery of B7-H3 spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	105	99-112%
Serum (n=4)	103	94-117%
EDTA plasma (n=4)	104	91-115%
Heparin plasma (n=4)	102	93-114%

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of B7-H3 were serially diluted with Calibrator Diluent RD6-41 to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=5)	Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)	Urine (n=4)
1:2	Average % of Expected	97	97	98	94	102
	Range (%)	96-99	92-98	96-102	88-97	94-114
1:4	Average % of Expected	93	99	96	93	96
	Range (%)	89-97	92-103	92-100	89-101	88-112
1:8	Average % of Expected	93	96	95	94	89
	Range (%)	89-100	93-98	85-105	89-105	81-102
1:16	Average % of Expected	92	92	98	92	———
	Range (%)	88-95	88-100	83-109	82-106	———

SENSITIVITY

Thirty-two assays were evaluated and the minimum detectable dose (MDD) of B7-H3 ranged from 0.037-0.274 ng/mL. The mean MDD was 0.160 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 B7-H3 produced at R&D Systems.

SAMPLE VALUES

Serum/Plasma/Urine - Samples from apparently healthy volunteers were evaluated for the presence of B7-H3 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=35)	17.7	12.2-36.0	4.1
EDTA plasma (n=35)	17.1	12.0-35.7	4.1
Heparin plasma (n=35)	17.2	11.9-34.8	4.0
Urine (n=15)	8.76	2.61-19.7	5.2

Cell Culture Supernates -
G361 human melanoma cells were cultured in McCoy's medium supplemented with 10% fetal bovine serum, and 2 mM L-glutamine. An aliquot of the cell culture supernate was removed, assayed for levels of natural B7-H3, and measured 10.3 ng/mL.

Capan-1 human pancreatic adenocarcinoma cells were cultured in IMDM supplemented with 20% fetal bovine serum, and 4 mM L-glutamine. An aliquot of the cell culture supernate was removed, assayed for levels of natural B7-H3, and measured 0.786 ng/mL.

SPECIFICITY

This assay recognizes recombinant and natural human B7-H3. The factors listed below were prepared at 500 ng/mL in Calibrator Diluent and assayed for cross-reactivity. Preparations of the following factors at 500 ng/mL in a mid-range recombinant human B7-H3 control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

B7-1
B7-2
B7-H1
B7-H2
B7-H4
B7-H6
PD-L2

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