Product Information & ELISA Manual

Human B7-H2/ICOSLG ELISA Kit (Colorimetric)
NBP3-43462

Enzyme-linked Immunosorbent Assay
for quantitative detection.

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Novus kits are guaranteed for 6 months from date of receipt.

For research use only. Not for diagnostic or therapeutic procedures.

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1. Intended Use

The Human B7-H2/ICOSLG ELISA Kit (Colorimetric) is to be used for the *in vitro* quantitative determination of human ICOSL in cell culture supernatants, serum and plasma. This ELISA Kit is for research use only.

2. Introduction

Inducible T-cell costimulatory protein (ICOS, also called CD278, AILIM, H4), a member of the CD28 family of costimulatory receptors, has a role in the generation and maintenance of germinal centers (GCs) in lymphatic organs, induction of thymus-dependent (TD) antibody (Ab) responses, and antibody class switching (1, 2). ICOS has a low expression on naïve T cells but is rapidly induced on activated T cells (1, 2, 3). ICOS binds to the inducible co-stimulator ligand (ICOSL, also called CD275, B7-H2, B7h, B7RP-1) that is found in professional APCs such as dendritic cells (DCs), B lymphocytes, various non-hematopoietic cells such as endothelial cells (ECs), as well as some cancer cells (2, 4). ICOS activated by ICOSL induces T cell proliferation, survival and differentiation and co-induces the secretion of IL-4, IL-5, IL-6, IL-10, IL-21, TNF-α, and interferon gamma (IFN-γ) (whereas CD28 induces IL-2 production) (5). Therefore, ICOS enhances Th1, Th2, and Th17 function largely through augmented production of these effector cytokines. ICOSL is upregulated by TNF-α and other inflammatory mediators and has an important co-stimulation role in EC (endothelial cells)-mediated T-cell activation, especially in reactivation of effector/memory T cells on the endothelium, which promote the homing of immune cells into inflamed tissue (5, 6). As seen in diverse types of costimulatory molecules in the CD28 family in T cells, ICOS is able to deliver a reverse signal through ICOSL that has a direct effect on dendritic cells (7). ICOS/ICOSL is involved in some hematologic malignancies such as myeloma or lymphoma. ICOS and its ligand ICOSL have been shown to play diverse roles in mediating autoimmunity as well as enhancing the development/activity of regulatory T cells (8). ICOS has a dual role in oncogenesis: i) the costimulatory signal of ICOS/ICOSL clearly participates in an antitumor T-cell response; ii) the ICOS signaling also exhibits pro-tumoral features, which are related to the induction of Treg immunosuppressive effects (9, 10, 11). In humans, homozygous ICOS deficiency results in common variable immunodeficiency (CVID), a condition characterized by aberrantly low serum gammaglobulin concentration (12). ICOS/ICOSL pathway is necessary for the optimal therapeutic effect of anti-CTLA-4, thus implicating this pathway as a target for future combinatorial strategies to improve the efficacy of anti-CTLA-4 therapy (13). ICOSL sheds from the cell membrane and the soluble form of ICOSL (sICOSL), found in plasma and sera, can be a potential biomarker for autoimmune diseases and some cancers (8).

3. General References

(1) The past, present, and future of costimulation blockade in organ transplantation: P.M. Schroder, et al.; Curr. Opin. Organ Transplant. **(Epub ahead of print)** (2019)

(2) Characterization of H4: a mouse T lymphocyte activation molecule functionally associated with the CD3/T cell receptor: V. Redoglia, et al.; Eur. J. Immunol. **26**, 2781 (1996)

(3) ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28: A. Hutloff, et al.; Nature **397**, 263 (1999)

(4) Inducible Co-Stimulator (ICOS) as a potential therapeutic target for anti-cancer therapy: F. Amatore, et al.; Expert Opin. Ther. Targets **22**, 343 (2018)

(5) ICOS co-stimulation: friend or foe? D.J. Wikenheiser & J.S. Stumhofer; Front. Immunol. **7**, 304 (2016)

(6) B7h, a novel costimulatory homolog of B7.1 and B7.2, is induced by TNFalpha: M.M. Swallow, et al.; Immunity **11**, 423 (1999)

(7) Reverse signaling using an inducible costimulator to enhance immunogenic function of dendritic cells: G. Tang, et al.; Cell Mol. Life Sci. **66**, 3067 (2009)

(8) Increased expression of soluble inducible costimulator ligand (ICOSL) in patients with systemic lupus erythematosus: M. Her, et al.; Lupus **18**, 501 (2009)

(9) Melanoma cells express ICOS ligand to promote the activation and expansion of T-regulatory cells: N. Martin-Orozco, et al.; Cancer Res. **70**, 9581 (2010)

(10) The inducible costimulator augments Tc17 cell responses to self and tumor tissue: M.H. Nelson, et al.; J. Immunol. **194**, 1737 (2015)

(11) Follicular B lymphomas generate regulatory T cells via the ICOS/ICOSL pathway and are susceptible to treatment by anti-ICOS/ICOSL therapy: K.-S. Le, et al.; Cancer Res. **76**, 4648 (2016)

(12) Homozygous loss of ICOS is associated with adult-onset common variable immunodeficiency: B. Grimbacher, et al.; Nat. Immunol. **4**, 261 (2003)

(13) The ICOS/ICOSL pathway is required for optimal antitumor responses mediated by anti-CTLA-4 therapy: T. Fu, et al.; Cancer Res. **71**, 5445 (2011)

4. Assay Principle

This assay is a sandwich Enzyme Linked-Immunosorbent Assay (ELISA) for quantitative determination of human ICOSL in cell culture supernatants, serum and plasma. A monoclonal antibody specific for ICOSL has been precoated onto the 96-well microtiter plate. Standards (STD) and samples are pipetted into the wells for binding to the coated antibody. After extensive washing to remove unbound compounds, ICOSL is recognized by the addition of a biotinylated monoclonal antibody specific for ICOSL (DET). After removal of excess biotinylated antibody, streptavidin-peroxidase (STREP-HRP) is added. Following a final washing, peroxidase activity is quantified using the substrate 3,3',5,5'-tetramethylbenzidine (TMB). The intensity of the color reaction is measured at 450nm after acidification and is directly proportional to the concentration of ICOSL in the samples.

5. Handling & Storage

- Reagent must be stored at 2-8°C when not in use
- Plate and reagents should be at room temperature before use.
- Do not expose reagents to temperatures greater than 25°C.

6. Kit Components

• 1 vial human ICOSL Standard (lyophilized)	(100 ng)	(STD)
• 1 vial Detection Antibody	(30 µl)	(DET)
• 1 vial HRP Labeled Streptavidin (lyophilized)	(2 µg)	(STREP-HRP)
• 2 bottles Wash Buffer 10X	(2 x 30 ml)	(Wash Buffer 10X)
• 2 bottles ELISA Buffer 10X	(2 x 30 ml)	(ELISA Buffer 10X)
• 1 bottle TMB Substrate Solution	(12 ml)	(TMB)
• 1 bottle Stop Solution	(12 ml)	(STOP)
• 1 plate coated with ICOSL Antibody	(6 x 16-well strips)	
• 2 plate covers (plastic film)		
• 2 silica gel minibags		

7. Materials Required but *Not* Supplied

- Microtiterplate reader at 450nm
- Calibrated precision pipettes. Disposable pipette tips
- Deionized water
- Microtubes or equivalent for preparing dilutions
- Disposable plastic containers for preparing working buffers
- Plate washer: automated or manual
- Glass or plastic tubes for diluting and aliquoting standard

8. General ELISA Protocol

8.1. Preparation and Storage of Reagents

NOTE: *Prepare just the appropriate amount of the buffers necessary for the assay.*

- **Wash Buffer 10X** has to be diluted with deionized water 1:10 before use (e.g. 30 ml Wash Buffer 10X + 270 ml water) to obtain Wash Buffer 1X.
- **ELISA Buffer 10X** has to be diluted with deionized water 1:10 before use (e.g. 10 ml ELISA Buffer 10X + 90 ml water) to obtain ELISA Buffer 1X.
- **Detection Antibody (DET)** has to be diluted to 1:500 in ELISA Buffer 1X (20 µl DET + 10 ml ELISA Buffer 1X).

  NOTE: *The diluted Detection Antibody is not stable and cannot be stored!*

- **HRP Labeled Streptavidin (STREP-HRP)** has to be reconstituted with 100 µl of ELISA Buffer 1X.

  - After reconstitution of STREP-HRP, prepare aliquots and store them at -20°C. **Avoid freeze/thaw cycles**.
  - Dilute the reconstituted STREP-HRP to the working concentration by adding 50 µl in 10 ml of ELISA Buffer 1X (1:200).

  NOTE: *The diluted STREP-HRP is not stable and cannot be stored!*

- **Human ICOSL Standard (STD)** has to be reconstituted with 100 µl of ELISA Buffer 1X.

  - This reconstitution produces a stock solution of 1 µg/ml. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes **at room temperature.** Mix well prior to making dilutions.

  NOTE: *The reconstituted standard is aliquoted and stored at -20°C!*

  - Dilute the standard protein concentrate (STD) (**1 µg/ml**) in ELISA Buffer 1X. A seven-point standard curve using 2-fold serial dilutions in ELISA Buffer 1X is recommended.

  - Suggested standard points are:
    4, 2, 1, 0.5, 0.25, 0.125, 0.0625 and 0 ng/ml.

Start with the dilution of the concentrate (STD):

To obtain	Add	Into
10 ng/ml	10µl of ICOSL (STD) (1 µg/ml)	990 µl of ELISA Buffer 1X

Dilute further for the standard curve:

To obtain	Add	Into
4 ng/ml	400 µl of ICOSL (10 ng/ml)	600 µl of ELISA Buffer 1X
2 ng/ml	300 µl of ICOSL (4 ng/ml)	300 µl of ELISA Buffer 1X
1 ng/ml	300 µl of ICOSL (2 ng/ml)	300 µl of ELISA Buffer 1X
0.5 ng/ml	300 µl of ICOSL (1 ng/ml)	300 µl of ELISA Buffer 1X
0.250 ng/ml	300 µl of ICOSL (0.5 ng/ml)	300 µl of ELISA Buffer 1X
0.125 ng/ml	300 µl of ICOSL (0.25 ng/ml)	300 µl of ELISA Buffer 1X
0.0625 ng/ml	300 µl of ICOSL (0.125 ng/ml)	300 µl of ELISA Buffer 1X
0 ng/ml	300 µl of ELISA Buffer 1X	Empty tube

8.2. Sample collection, storage and dilution

Serum: Use a serum separator tube. Let samples clot at room temperature for 30 minutes before centrifugation for 20 minutes at 1,000xg. Assay freshly prepared serum or store serum in aliquot at ≤ -20°C for later use. Avoid repeated freeze/thaw cycles.

Plasma: Collect plasma using heparin, citrate or EDTA as an anticoagulant. Centrifuge for 15 minutes at 1000xg within 30 minutes of collection. Assay freshly prepared plasma or store plasma sample in aliquot at ≤ -80°C for later use. Avoid repeated freeze/ thaw cycles.

Serum, Plasma and Cell Culture Supernatant have to be diluted in ELISA Buffer 1X. Samples containing visible precipitates must be clarified before use.

NOTE: *As a starting point, 1/400-1/800 dilutions of serum or plasma is recommended! If sample values fall outside the detection range of the assay, a lower or higher dilution may be required!*

8.3. Assay Procedure (Checklist)

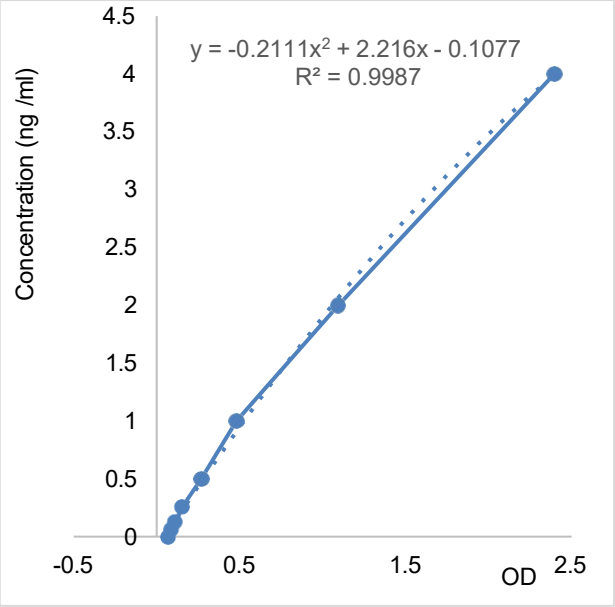
☐	1. Determine the number of 16-well strips needed for the assay and insert them in the frame for current use. The extra strips are left in the bag with 2 silica gel minibags and stored at 4°C. **NOTE:** *Remaining 16-well strips coated with ICOSL antibody when opened can be stored in the presence of 2 silica gel minibags at 4°C for up to 1 month.*
☐	2. Add 100 µl of the different standards into the appropriate wells in duplicate! At the same time, add 100 µl of diluted plasma, serum or cell culture supernatant samples in duplicate to the wells **(see 8.1. Preparation and Storage of Reagents and 8.2 Preparation of Samples).**
☐	3. Cover the plate with plastic film and incubate for **2 hours at Room Temperature.**
☐	4. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multichannel pipette or auto-washer. Repeat the process for a total of five washes. After the last wash, complete removal of liquid is essential for good performance.
☐	5. Add 100 µl to each well of the diluted Detection Antibody **(DET) (see 8.1 Preparation and Storage of Reagents)**.
☐	6. Cover the plate with plastic film and incubate for **1 hour at Room Temperature.**
☐	7. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multichannel pipette or auto-washer. Repeat the process for a total of five washes. After the last wash, complete removal of liquid is essential for good performance.
☐	8. Add 100 µl to each well of the diluted HRP Labeled Streptavidin **(STREP-HRP) (see 8.1. Preparation and Storage of Reagents)**.
☐	9. Cover the plate with plastic film and incubate for **30 min at Room Temperature**.
☐	10. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multichannel pipette or auto-washer. Repeat the process for a total of five washes. After the last wash, complete removal of liquid is essential for good performance.
☐	11. Add 100 µl to each well of TMB substrate solution **(TMB)**.
☐	12. Allow the color reaction to develop **at Room Temperature in the dark for 10-15 minutes**. Do not cover the plate.
☐	13. Stop the reaction by adding 100 µl of Stop Solution (**STOP**). Tap the plate gently to ensure thorough mixing. The substrate reaction yields a blue solution that turns yellow when Stop Solution (**STOP**) is added.
	! CAUTION: CORROSIVE SOLUTION !
☐	14. Measure the OD at 450 nm in an ELISA reader.

9. Calculation of Results

- Average the duplicate readings for each standard and sample and subtract the average blank value (obtained with the 0 ng/ml point).
- Generate the standard curve by plotting the average absorbance obtained for each standard concentration on the horizontal (X) axis vs. the corresponding ICOSL concentration (ng/ml) on the vertical axis (see **10.** TYPICAL DATA).
- Calculate the ICOSL concentrations of samples by interpolation of the regression curve formula in a form of a quadratic equation.
- If the test sample was diluted, multiply the interpolated value by the dilution factor to calculate the concentration of human ICOSL in the sample.

10. Typical Data

The following data are obtained using the different concentrations of standard as described in this protocol:



Standard ICOSL (ng/ml)	Optical Density (mean)
4	2.397
2	1.095
1	0.480
0.5	0.269
0.25	0.152
0.125	0.111
0.0625	0.090
0	0.073

Figure: Standard curve

11. Performance Characteristics

A. Sensitivity (Limit of detection):

The lowest level of human ICOSL that can be detected by this assay is **60 pg/ml**.

NOTE: *The Limit of detection was measured by adding three standard deviations to the mean value of 50 zero standard.*

B. Assay range: 0.0625 ng/ml – 4 ng/ml

C. Specificity:

This ELISA is specific for the measurement of natural and recombinant human ICOSL [CD274].

D. Intra-assay precision:

Four samples of known concentrations of human ICOSL were assayed in replicates 6 times to test precision within an assay.

Samples	Means (ng/ml)	SD	CV (%)	n
A1	299.13	5.93	1.98	6
A2	288.58	8.99	3.11	6
A3	361.86	9.07	2.50	6
A4	159.80	9.34	5.84	6

E. Inter-assay precision:

Four samples of known concentrations of human ICOSL were assayed in 5 separate assays to test precision between assays.

Samples	Means (ng/ml)	SD	CV (%)	n
B1	319.28	6.95	2.17	5
B2	371.84	21.19	5.70	5
B3	234.15	5.34	2.28	5
B4	197.86	5.41	2.73	5

F. Recovery:

When samples are spiked with known concentrations of human ICOSL, the recovery averages range from 88% to 113%.

G. Linearity:

Different samples containing human ICOSL were diluted several fold (1/400 to 1/1'600 for sera and plasma) and the measured recoveries ranged from 88% to 115%.

H. Expected values:

Human ICOSL levels range in serum and plasma from **150 ng/ml to >500ng/ml.**

12. Technical Hints and Limitations

- It is recommended that all standards and samples be run in duplicate.
- Do not combine leftover reagents with those reserved for additional wells.
- Reagents from the kit with a volume less than 100µl should be centrifuged.
- Residual wash liquid should be drained from the wells after last wash by tapping the plate on absorbent paper.
- Crystals could appear in the 10X solution due to high salt concentration in the stock solutions. Crystals are readily dissolved at room temperature or at 37°C before dilution of the buffer solutions.
- Once reagents have been added to the 16-well strips, DO NOT let the strips DRY at any time during the assay.
- Keep TMB Solution protected from light.
- The Stop Solution (STOP) consists of sulfuric acid. Although diluted, the Stop Solution should be handled with gloves, eye protection and protective clothing.

13. Troubleshooting

PROBLEM	POSSIBLE CAUSES	SOLUTIONS
No signal or weak signal	Omission of key reagent	Check that all reagents have been added in the correct order.
	Washes too stringent	Use an automated plate washer if possible.
	Incubation times inadequate	Incubation times should be followed as indicated in the manual.
	Plate reader settings not optimal	Verify the wavelength and filter setting in the plate reader.
	Incorrect assay temperature	Use recommended incubation temperature. Bring substrates to room temperature before use.
High background	Concentration of STREP-HRP too high	Use recommended dilution factor.
	Inadequate washing	Ensure all wells are filling wash buffer and are aspirated completely.
Poor standard curve	Wells not completely aspirated	Completely aspirate wells between steps.
	Reagents poorly mixed	Be sure that reagents are thoroughly mixed.
Unexpected results	Omission of reagents	Be sure that reagents were prepared correctly and added in the correct order.
	Dilution error	Check pipetting technique and double-check calculations.

14. Notes