PRODUCT INFORMATION

Human sCD44std ELISA

IB49608
and
IB49608-10

Enzyme-linked immunosorbent assay for

detection of human sCD44std.

For research use only - Not for use in diagnostic procedures.

Human sCD44std
IB49608
IB49608-10

TABLE OF CONTENTS

1	Intended Use	3
2	Summary	3
3	Principles of the Test	6
4	Reagents Provided	7
5	Storage Instructions – ELISA Kit	9
6	Specimen Collection and Storage Instructions	9
7	Materials Required But Not Provided	10
8	Precautions for Use	11
9	Preparation of Reagents	13
10	Test Protocol	17
11	Calculation of Results	22
12	Limitations	25
13	Performance Characteristics	26
14	Ordering Information	31
15	Reagent Preparation Summary	33
16	Test Protocol Summary	34

1 Intended Use

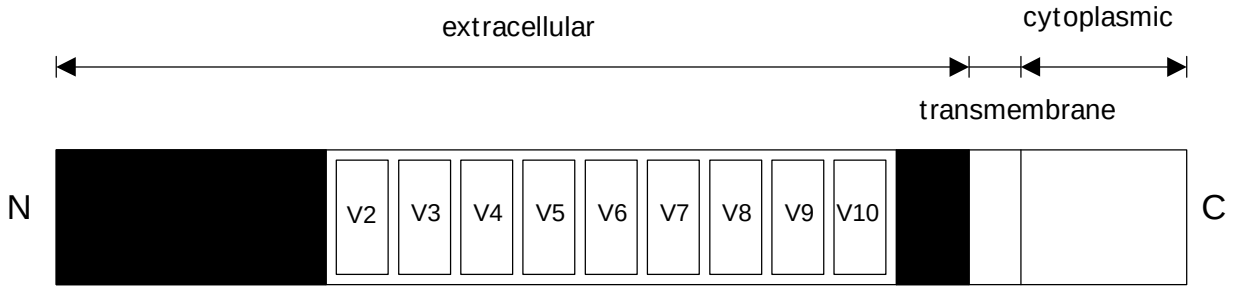
The human sCD44std ELISA is an enzyme-linked immunosorbent assay for the detection of human sCD44std. **The human sCD44std ELISA is for research use only. Not for use in diagnostic procedures.**

2 Summary

CD44 (Pgp-1; Ly-24; ECMR III; F10-44-2; H-CAM; HUTCH-I; In(Lu)-related p80; Hermes antigen; hyaluronan receptor) is a polymorphic glycoprotein with apparent molecular weights ranging from 85 kDa to 250 kDa. This cell membrane associated molecule has a cytoplasmic tail (mediates the interaction with the cytoskeleton), a short hydrophobic transmembrane region and an NH_2-terminal extracellular (binds to hyaluronate) domain (2, 14).
CD44 isoforms participate in a wide variety of cell-cell or cell-matrix interactions including lymphocyte homing, establishment of B and T cell immune responses, tumor metastases formation and inflammation.

Three isoform categories of the CD44 molecule have been identified:
1) an 80-90 kDa isoform, the so-called standard form named CD44std, which is widely distributed on several hematopoietic and nonhematopoietic cells including all subsets of leukocytes, monocytes, erythrocytes, many types of epithelium, mesenchymal elements like fibroblasts, smooth muscle cells and glial cells of the central nervous system,
2) a medium size category of 110-160 kDa which is weakly expressed on epithelial cells and highly expressed in some carcinomas and
3) a category which includes very large isoforms of 250 kDa covalently modified by the addition of chondroitin sulfate (12).
These bigger isoforms of CD44 arise by alternative splicing of one or more "variant" exons (v2-v10) into the extracellular part of the 90kDa constant form molecule. Compared to the standard CD44, all larger isoforms are expressed in a much more restricted fashion, only in a few normal tissues or on the surface of certain tumor cells. Some splice variants of CD44 play important and distinct roles in tumor metastasis (15).

The sCD44std ELISA detects all circulating CD44 isoforms comprising the standard protein sequences (black area).



CD44 protein: - standard protein sequences (black area)
- variant exons (open boxes numbered v2 - v10)

Determination of sCD44std will provide more detailed insight into different pathological modifications during cancer and other diseases.

- **brain tumors:** CD44 is strongly expressed in high-grade gliomas and weakly expressed in meningiomas, medulloblastomas and normal brain (8).

- **colorectal carcinomas:** in human colorectal neoplasia CD44 variant proteins are found on all invasive carcinomas and during carcinoma metastasis. Variants are already expressed at a relatively early stage of colorectal carcinogenesis and tumor progression (4).

- **gastric cancer:** tumors from patients suffering from stomach adenocarcinomas express CD44 variants. Adenocarcinomas of the intestinal type are strongly positive for exon v5 and v6, whereas diffuse type adenocarcinomas predominantly express exon v5 (3).

- **lung, breast cancer:** in malignant tissues there is gross overproduction of alternatively-spliced large molecular variants in all samples, whereas in the control samples only the standard product was routinely detected with occasional minimal quantities of one or two small variants (10).

- **lymphoma:** in gastrointestinal lymphoma overexpression of CD44 has been correlated with poor survival and more disseminated disease (6,3).
  Overexpression of CD44 is also found in several aggressive, but not low-grade, non-Hodgkin´s lymphomas (7) as well as in Hodgkin´s and nodal diffuse lymphomas (1).

- **tonsil, skin cancer:** variant CD44 isoform expression can be demonstrated in the plasma membrane of squamous cells of skin and tonsil epithelial and is greatly diminished in malignant squamous epithelial tumors (11,13).

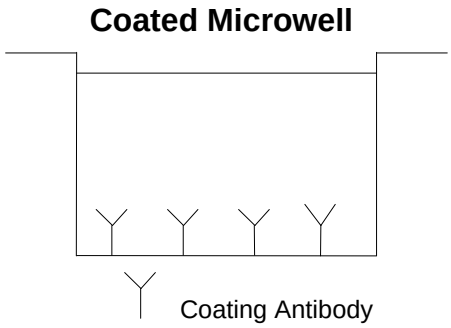
- **HIV:** CD44 is almost completely depleted from the surface of HIV-infected cells (9).

- **inflammatory joint diseases:** CD44 expression was decreased in synovial fluid neutrophils from most patients (5).

3 Principles of the Test

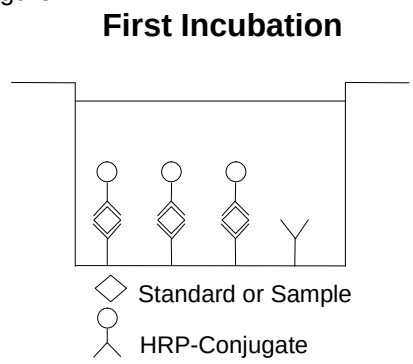
An anti-human sCD44std coating antibody is adsorbed onto microwells.

Figure 1



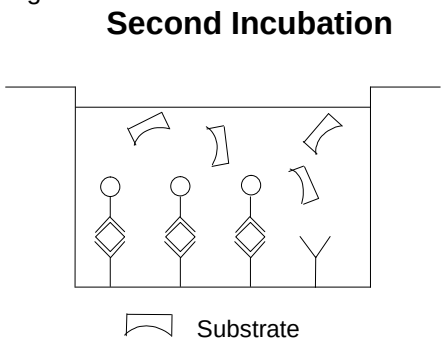
Human sCD44std present in the sample or standard binds to antibodies adsorbed to the microwells. A HRP-conjugated anti-human sCD44std antibody is added and binds to human sCD44std captured by the first antibody.

Figure 2



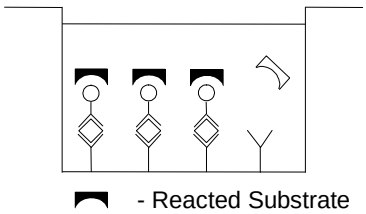
Following incubation unbound HRP-conjugated anti-human sCD44std is removed during a wash step, and substrate solution reactive with HRP is added to the wells.

Figure 3



A coloured product is formed in proportion to the amount of human sCD44std present in the sample or standard. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from 6 human sCD44std standard dilutions and human sCD44std concentration determined.

Figure 4



4 Reagents Provided

4.1 Reagents for human sCD44std ELISA IB49608 (96 tests)

1 aluminium pouch with a **Microwell Plate coated** with monoclonal antibody to human sCD44std

2 vials (10 µl) **HRP-Conjugate** anti-human sCD44std monoclonal antibody

2 vials (300 µl) human sCD44std **Standard**, 8 ng/ml

1 bottle (60 ml) **Sample Diluent**

1 vial (5 ml) **Assay Buffer Concentrate** 20x (PBS with 1% Tween 20 and 10% BSA)

1 bottle (50 ml) **Wash Buffer Concentrate** 20x (PBS with 1% Tween 20)

1 vial (15 ml) **Substrate Solution** (tetramethyl-benzidine)

1 vial (15 ml) **Stop Solution** (1M Phosphoric acid)

1 vial (0.4 ml) **Blue-Dye**

1 vial (0.4 ml) **Green-Dye**

2 **Adhesive Films**

4.2 Reagents for human sCD44std ELISA IB49608-10
(10x96 tests)

10 aluminium pouches with a **Microwell Plate coated** with monoclonal antibody to human sCD44std

10 vials (10 µl) **HRP-Conjugate** anti-human sCD44std monoclonal antibody

10 vials (300 µl) human sCD44std **Standard**, 8 ng/ml

10 bottles (60 ml) **Sample Diluent**

2 vials (5 ml) **Assay Buffer Concentrate** 20x (PBS with 1% Tween 20 and 10% BSA)

3 bottles (50 ml) **Wash Buffer Concentrate** 20x (PBS with 1% Tween 20)

10 vials (15 ml) **Substrate Solution** (tetramethyl-benzidine)

10 vials (15 ml) **Stop Solution** (1M Phosphoric acid)

6 vials (0.4 ml) **Blue-Dye**

6 vials (0.4 ml) **Green-Dye**

10 **Adhesive Films**

5 Storage Instructions – ELISA Kit

Store kit reagents between 2° and 8°C. Immediately after use remaining reagents should be returned to cold storage (2° to 8°C). Expiry of the kit and reagents is stated on labels.
Expiry of the kit components can only be guaranteed if the components are stored properly, and if, in case of repeated use of one component, this reagent is not contaminated by the first handling.

6 Specimen Collection and Storage Instructions

Cell culture supernatant, serum, plasma (EDTA, citrate, heparin), amniotic fluid and urine were tested with this assay. Other biological samples might be suitable for use in the assay. Remove serum or plasma from the clot or cells as soon as possible after clotting and separation.

Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic specimens.

Samples should be aliquoted and must be stored frozen at -20°C to avoid loss of bioactive human sCD44std. If samples are to be run within 24 hours, they may be stored at 2° to 8°C (for sample stability refer to 13.5).
Avoid repeated freeze-thaw cycles. Prior to assay, the frozen sample should be brought to room temperature slowly and mixed gently.

7 Materials Required But Not Provided

- 5 ml and 10 ml graduated pipettes
- 5 µl to 1000 µl adjustable single channel micropipettes with disposable tips
- 50 µl to 300 µl adjustable multichannel micropipette with disposable tips
- Multichannel micropipette reservoir
- Beakers, flasks, cylinders necessary for preparation of reagents
- Device for delivery of wash solution (multichannel wash bottle or automatic wash system)
- Microwell strip reader capable of reading at 450 nm (620 nm as optional reference wave length)
- Glass-distilled or deionized water
- Statistical calculator with program to perform regression analysis

8 Precautions for Use

- All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water. See material safety data sheet(s) and/or safety statement(s) for specific advice.
- Reagents are intended for research use only and are not for use in diagnostic or therapeutic procedures.
- Do not mix or substitute reagents with those from other lots or other sources.
- Do not use kit reagents beyond expiration date on label.
- Do not expose kit reagents to strong light during storage or incubation.
- Do not pipette by mouth.
- Do not eat or smoke in areas where kit reagents or samples are handled.
- Avoid contact of skin or mucous membranes with kit reagents or specimens.
- Rubber or disposable latex gloves should be worn while handling kit reagents or specimens.
- Avoid contact of substrate solution with oxidizing agents and metal.
- Avoid splashing or generation of aerosols.
- In order to avoid microbial contamination or cross-contamination of reagents or specimens which may invalidate the test use disposable pipette tips and/or pipettes.
- Use clean, dedicated reagent trays for dispensing the conjugate and substrate reagent.

- Exposure to acid inactivates the conjugate.
- Glass-distilled water or deionized water must be used for reagent preparation.
- Substrate solution must be at room temperature prior to use.
- Decontaminate and dispose specimens and all potentially contaminated materials as they could contain infectious agents. The preferred method of decontamination is autoclaving for a minimum of 1 hour at 121.5°C.
- Liquid wastes not containing acid and neutralized waste may be mixed with sodium hypochlorite in volumes such that the final mixture contains 1.0% sodium hypochlorite. Allow 30 minutes for effective decontamination. Liquid waste containing acid must be neutralized prior to the addition of sodium hypochlorite.

9 Preparation of Reagents

Buffer Concentrates should be brought to room temperature and should be diluted before starting the test procedure.
If crystals have formed in the **Buffer Concentrates**, warm them gently until they have completely dissolved.

9.1 Wash Buffer (1x)

Pour entire contents (50 ml) of the **Wash Buffer Concentrate** (20x) into a clean 1000 ml graduated cylinder. Bring to final volume of 1000 ml with glass-distilled or deionized water. Mix gently to avoid foaming. The pH of the final solution should adjust to 7.4.

Transfer to a clean wash bottle and store at 2° to 25°C. Please note that Wash Buffer (1x) is stable for 30 days.

Wash Buffer (1x) may also be prepared as needed according to the following table:

Number of Strips	Wash Buffer Concentrate (20x) (ml)	Distilled Water (ml)
1 - 6	25	475
1 - 12	50	950

9.2 Assay Buffer (1x)

Pour the entire contents (5 ml) of the **Assay Buffer Concentrate** (20x) into a clean 100 ml graduated cylinder. Bring to final volume of 100 ml with distilled water. Mix gently to avoid foaming.

Store at 2° to 8°C. Please note that the Assay Buffer (1x) is stable for 30 days.

Assay Buffer (1x) may also be prepared as needed according to the following table:

Number of Strips	Assay Buffer Concentrate (20x) (ml)	Distilled Water (ml)
1 - 6	2.5	47.5
1 - 12	5.0	95.0

9.3 HRP-Conjugate

Please note that the HRP-Conjugate should be used within 30 minutes after dilution.

Dilute the **HRP-Conjugate** 1:50 just prior to use by adding 490 µl Assay Buffer (1x) to the tube containing the HRP-Conjugate concentrate. Mix the contents of the tube well.
Make a further 1:40 dilution with Assay Buffer (1x) in a clean plastic tube or reagent reservoir.

The second dilution (1:40) of the HRP-Conjugate may be prepared as needed according to the following table:

Number of Strips	Prediluted (1:50) HRP-Conjugate (ml)	Assay Buffer (1x) (ml)
1 - 6	0.075	2.925
1 - 12	0.150	5.850

9.4 Human sCD44std Standard

Standard dilutions can be prepared directly on the microwell plate (see 10.d) or alternatively in tubes (see 9.4.1).

9.4.1 External Standard Dilution

Label 6 tubes, one for each standard point.

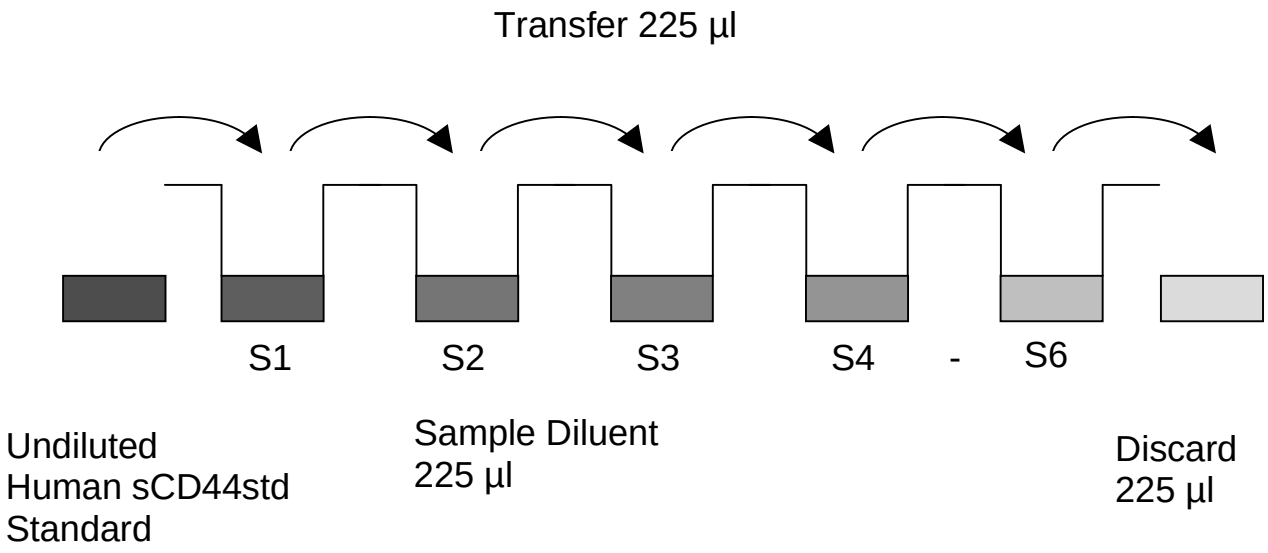
S1, S2, S3, S4, S5, S6

Then prepare 1:2 serial dilutions for the standard curve as follows:
Pipette 225 µl of Sample Diluent into each tube.

Pipette 225 µl of undiluted standard (concentration = 8 ng/ml) into the first tube, labelled S1, and mix (concentration of standard 1 = 4 ng/ml). Pipette 225 µl of this dilution into the second tube, labelled S2, and mix thoroughly before the next transfer.
Repeat serial dilutions 4 more times thus creating the points of the standard curve (see Figure 5).

Sample Diluent serves as blank.

Figure 5



9.5 Addition of Colour-giving Reagents: Blue-Dye, Green-Dye

In order to help our customers to avoid any mistakes in pipetting the IBL-America ELISAs, IBL-America offers a tool that helps to monitor the addition of even very small volumes of a solution to the reaction well by giving distinctive colours to each step of the ELISA procedure.

This procedure is optional, does not in any way interfere with the test results, and is designed to help the customer with the performance of the test, but can also be omitted, just following the instruction booklet.

Alternatively, the dye solutions from the stocks provided (***Blue-Dye, Green-Dye***) can be added to the reagents according to the following guidelines:

1. Diluent:

Before standard and sample dilution add the ***Blue-Dye*** at a dilution of 1:250 (see table below) to the appropriate diluent (1x) according to the test protocol. After addition of ***Blue-Dye***, proceed according to the instruction booklet.

5 ml Sample Diluent	20 µl ***Blue-Dye***
12 ml Sample Diluent	48 µl ***Blue-Dye***
50 ml Sample Diluent	200 µl ***Blue-Dye***

2. HRP-Conjugate:

Before dilution of the concentrated HRP-Conjugate add the ***Green-Dye*** at a dilution of 1:100 (see table below) to the Assay Buffer (1x) used for the final conjugate dilution. Proceed after addition of ***Green-Dye*** according to the instruction booklet: Preparation of HRP-Conjugate.

3 ml Assay Buffer (1x)	30 µl ***Green-Dye***
6 ml Assay Buffer (1x)	60 µl ***Green-Dye***

10 Test Protocol

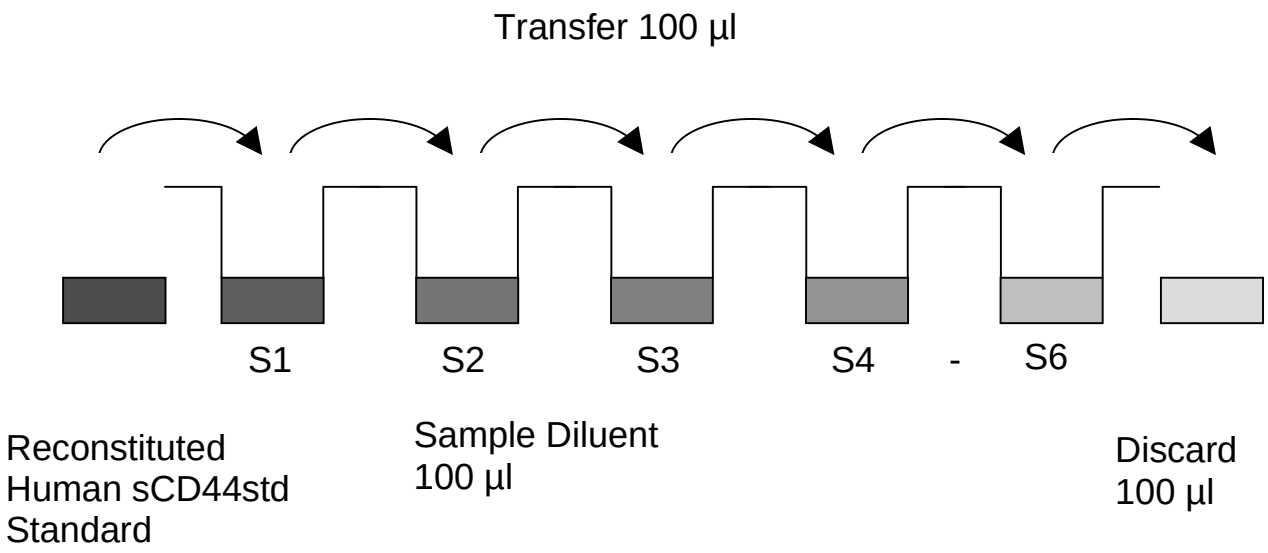
a. Predilute your serum, plasma and urine samples before starting with the test procedure.
   Dilute these samples 1:60 with Sample Diluent according to the following scheme:
   10 µl sample + 590 µl Sample Diluent

b. Determine the number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running blanks and standards. Each sample, standard, blank and optional control sample should be assayed in duplicate. Remove extra microwell strips from holder and store in foil bag with the desiccant provided at 2°-8°C sealed tightly.

c. Wash the microwell strips twice with approximately 400 µl **Wash Buffer** per well with thorough aspiration of microwell contents between washes. Allow the Wash Buffer to sit in the wells for about **10 – 15 seconds** before aspiration. Take care not to scratch the surface of the microwells.
   After the last wash step, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. Use the microwell strips immediately after washing. Alternatively microwell strips can be placed upside down on a wet absorbent paper for not longer than 15 minutes. **Do not allow wells to dry**.

d. **Standard dilution on the microwell plate** (Alternatively the standard dilution can be prepared in tubes - see 9.4.1):
   Add 100 µl of Sample Diluent in duplicate to all **standard wells**. Pipette 100 µl of undiluted **standard** (see Preparation of Standard 9.4, concentration = 8 ng/ml) in duplicate into well A1 and A2 (see Table 1). Mix the contents of wells A1 and A2 by repeated aspiration and ejection (concentration of standard 1, S1 = 4 ng/ml), and transfer 100 µl to wells B1 and B2, respectively (see Figure 6). Take care not to scratch the inner surface of the microwells. Continue this procedure 4 times, creating two rows of human sCD44std standard dilutions ranging from 4.00 to 0.13 ng/ml. Discard 100 µl of the contents from the last microwells (F1, F2) used.

Figure 6


Transfer 100 µl
S1
S2
S3
S4
-
S6
Reconstituted
Human sCD44std
Standard
Sample Diluent
100 µl
Discard
100 µl

In case of an **external standard dilution** (see 9.4.1), pipette 100 µl of these standard dilutions (S1 - S6) in the standard wells according to Table 1.

Table 1
Table depicting an example of the arrangement of blanks, standards and samples in the microwell strips:

	1	**2**	**3**	**4**
A	Standard 1 (4.00 ng/ml)	Standard 1 (4.00 ng/ml)	Sample 2	Sample 2
B	Standard 2 (2.00 ng/ml)	Standard 2 (2.00 ng/ml)	Sample 3	Sample 3
C	Standard 3 (1.00 ng/ml)	Standard 3 (1.00 ng/ml)	Sample 4	Sample 4
D	Standard 4 (0.50 ng/ml)	Standard 4 (0.50 ng/ml)	Sample 5	Sample 5
E	Standard 5 (0.25 ng/ml)	Standard 5 (0.25 ng/ml)	Sample 6	Sample 6
F	Standard 6 (0.13 ng/ml)	Standard 6 (0.13 ng/ml)	Sample 7	Sample 7
G	Blank	Blank	Sample 8	Sample 8
H	Sample 1	Sample 1	Sample 9	Sample 9

e. Add 100 µl of **Sample Diluent** in duplicate to the **blank wells**.

f. Add 80 µl of **Sample Diluent** to the **sample wells**.

g. Add 20 µl of each **sample** in duplicate to the **sample wells**.

h. Prepare **HRP-Conjugate** (see Preparation of HRP-Conjugate 9.3).

i. Add 50 µl of **HRP-Conjugate** to all wells.

j. Cover with an adhesive film and incubate at room temperature (18 to 25°C) for 3 hours, if available on a microplate shaker set at 100 rpm.

k. Remove adhesive film and empty wells. **Wash** microwell strips 3 times according to point c. of the test protocol. Proceed immediately to the next step.

l. Pipette 100 µl of **TMB Substrate Solution** to all wells.

m. Incubate the microwell strips at room temperature (18° to 25°C) for about 10 min. Avoid direct exposure to intense light.

   The colour development on the plate should be monitored and the substrate reaction stopped (see next point of this protocol) before positive wells are no longer properly recordable. Determination of the ideal time period for colour development has to be done individually for each assay.

   It is recommended to add the stop solution when the highest standard has developed a dark blue colour. Alternatively the colour development can be monitored by the ELISA reader at 620 nm. The substrate reaction should be stopped as soon as Standard 1 has reached an OD of 0.9 – 0.95.

n. Stop the enzyme reaction by quickly pipetting 100 µl of **Stop Solution** into each well. It is important that the Stop Solution is spread quickly and uniformly throughout the microwells to completely inactivate the enzyme. Results must be read immediately after the Stop Solution is added or within one hour if the microwell strips are stored at 2 - 8°C in the dark.

o. Read absorbance of each microwell on a spectro-photometer using 450 nm as the primary wave length (optionally 620 nm as the reference wave length; 610 nm to 650 nm is acceptable). Blank the

plate reader according to the manufacturer's instructions by using the blank wells. Determine the absorbance of both the samples and the standards.

Note: In case of incubation without shaking the obtained O.D. values may be lower than indicated below. Nevertheless the results are still valid.

11 Calculation of Results

- Calculate the average absorbance values for each set of duplicate standards and samples. Duplicates should be within 20 per cent of the mean value.
- Create a standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the human sCD44std concentration on the abscissa. Draw a best fit curve through the points of the graph (a 5-parameter curve fit is recommended).
- To determine the concentration of circulating human sCD44std for each sample, first find the mean absorbance value on the ordinate and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the abscissa and read the corresponding human sCD44std concentration.
- **If instructions in this protocol have been followed samples have been diluted 1:300 (1:60 predilution, 1:5 dilution on the plate: 20 µl sample + 80 µl Sample Diluent), the concentration read from the standard curve must be multiplied by the dilution factor (x 300).**
- **Calculation of samples with a concentration exceeding standard 1 may result in incorrect, low human sCD44std levels. Such samples require further external predilution according to expected human sCD44std values with Sample Diluent in order to precisely quantitate the actual human sCD44std level.**
- It is suggested that each testing facility establishes a control sample of known human sCD44std concentration and runs this additional control with each assay. If the values obtained are not within the expected range of the control, the assay results may be invalid.
- A representative standard curve is shown in Figure 7. This curve cannot be used to derive test results. Each laboratory must prepare a standard curve for each group of microwell strips assayed.

Figure 7

Representative standard curve for human sCD44std ELISA. Human sCD44std was diluted in serial 2-fold steps in Sample Diluent. Do not use this standard curve to derive test results. A standard curve must be run for each group of microwell strips assayed.

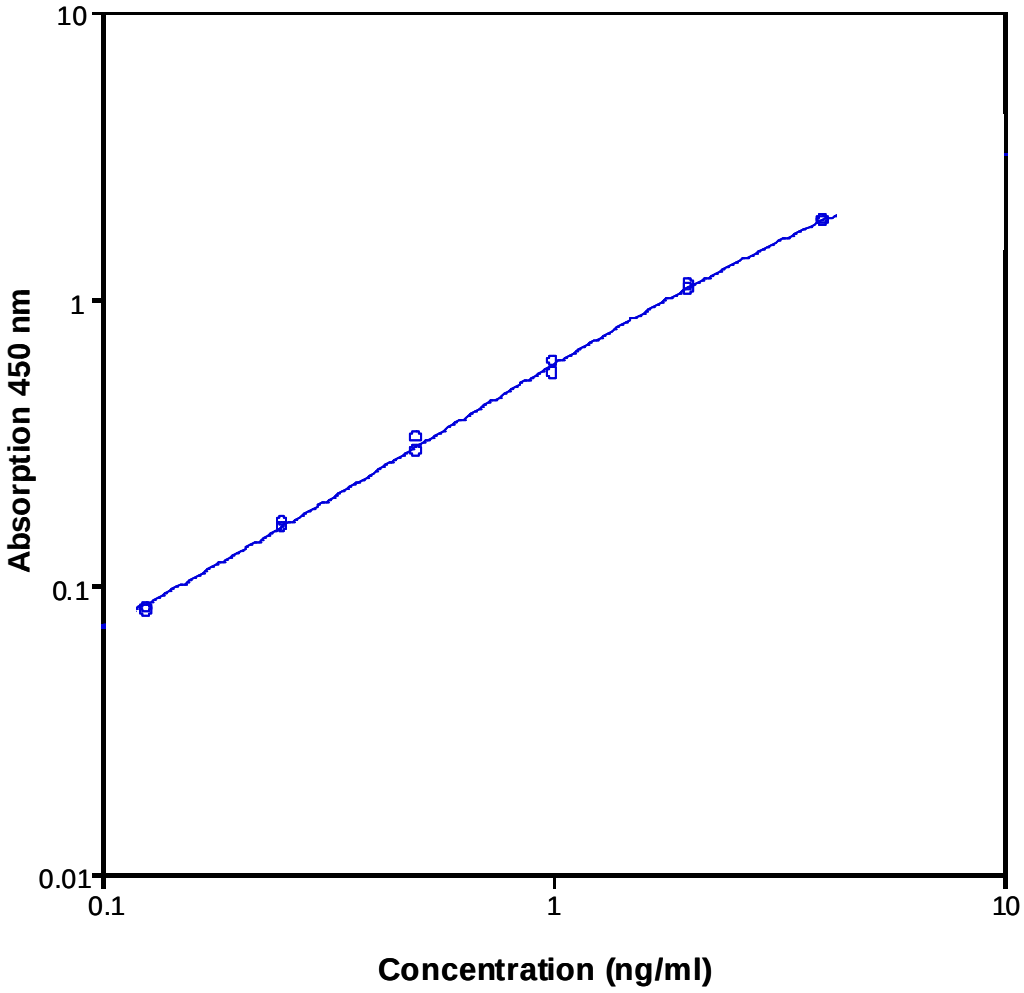


Table 2
Typical data using the human sCD44std ELISA
Measuring wavelength: 450 nm
Reference wavelength: 620 nm

Standard	Human sCD44std Concentration (ng/ml)	O.D. at 450 nm	Mean O.D. at 450 nm	C.V. (%)
1	4.00	1.901	1.876	1.9
	4.00	1.851		
2	2.00	1.080	1.095	1.9
	2.00	1.109		
3	1.00	0.608	0.581	6.7
	1.00	0.553		
4	0.50	0.330	0.312	8.4
	0.50	0.293		
5	0.25	0.160	0.164	3.4
	0.25	0.168		
6	0.13	0.081	0.083	2.6
	0.13	0.084		
Blank	0	0.008	0.008	
	0	0.007		

The OD values of the standard curve may vary according to the conditions of assay performance (e.g. operator, pipetting technique, washing technique or temperature effects). Furthermore shelf life of the kit may affect enzymatic activity and thus colour intensity. Values measured are still valid.

12 Limitations

- Since exact conditions may vary from assay to assay, a standard curve must be established for every run.
- Bacterial or fungal contamination of either screen samples or reagents or cross-contamination between reagents may cause erroneous results.
- Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.
- Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Empty wells completely before dispensing fresh wash solution, fill with Wash Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.
- The use of radioimmunotherapy has significantly increased the number of patients with human anti-mouse IgG antibodies (HAMA). HAMA may interfere with assays utilizing murine monoclonal antibodies leading to both false positive and false negative results. Serum samples containing antibodies to murine immunoglobulins can still be analysed in such assays when murine immunoglobulins (serum, ascitic fluid, or monoclonal antibodies of irrelevant specificity) are added to the sample.

13 Performance Characteristics

13.1 Sensitivity

The limit of detection of human sCD44std defined as the analyte concentration resulting in an absorbance significantly higher than that of the dilution medium (mean plus 2 standard deviations) was determined to be 0.02 ng/ml (mean of 6 independent assays).

13.2 Reproducibility

13.2.1 Intra-assay

Reproducibility within the assay was evaluated in 2 independent experiments. Each assay was carried out with 6 replicates of 8 serum samples containing different concentrations of human sCD44std. 2 standard curves were run on each plate. Data below show the mean human sCD44std concentration and the coefficient of variation for each sample (see Table 3). The calculated overall intra-assay coefficient of variation was 4.8%.

Table 3

The mean human sCD44std concentration and the coefficient of variation for each sample

Sample	Experiment	Mean Human sCD44std Concentration (ng/ml)	Coefficient of Variation (%)
1	1	292	4.4
	2	343	3.8
2	1	332	5.9
	2	361	1.4
3	1	295	2.4
	2	291	8.3
4	1	318	9.6
	2	345	3.7
5	1	177	3.7
	2	173	1.1
6	1	437	4.1
	2	427	4.1
7	1	370	8.1
	2	370	3.3
8	1	297	11.3
	2	278	1.2

13.2.2 Inter-assay

Assay to assay reproducibility within one laboratory was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 8 serum samples containing different concentrations of human sCD44std. 2 standard curves were run on each plate. Data below show the mean human sCD44std concentration and the coefficient of variation calculated on 18 determinations of each sample (see Table 4). The calculated overall inter-assay coefficient of variation was 4.1%.

Table 4
The mean human sCD44std concentration and the coefficient of variation of each sample

Sample	Mean Human sCD44std Concentration (ng/ml)	Coefficient of Variation (%)
1	318	11.3
2	297	6.9
3	293	1.1
4	332	5.8
5	175	1.4
6	432	1.5
7	370	0.1
8	288	4.5

13.3 Spike Recovery

The spike recovery was evaluated by spiking 4 levels of human sCD44std into pooled normal human serum. Recoveries were determined in 3 independent experiments with 6 replicates each.
The amount of endogenous human sCD44std in unspiked serum was subtracted from the spike values.
The recovery ranged from 76% to 101% with an overall mean recovery of 89%.

13.4 Dilution Parallelism

4 serum samples with different levels of human sCD44std were analysed at serial 2 fold dilutions with 4 replicates each.
The recovery ranged from 91% to 99% with an overall recovery of 94% (see Table 5).

Table 5

Sample	Dilution	Expected Human sCD44std Concentration (ng/ml)	Observed Human sCD44std Concentration (ng/ml)	Recovery of Expected Human sCD44std Concentration (%)
1	1:300	--	347	--
	1:600	173	160	92
	1:1200	87	80	92
	1:2400	43	42	96
2	1:300	--	402	--
	1:600	201	186	92
	1:1200	100	94	94
	1:2400	50	46	91
3	1:300	--	292	--
	1:600	146	145	99
	1:1200	73	72	99
	1:2400	37	34	92
4	1:300	--	368	--
	1:600	184	173	94
	1:1200	92	89	96
	1:2400	46	43	94

13.5 Sample Stability

13.5.1 Freeze-Thaw Stability

Aliquots of serum samples were stored at -20°C and thawed 5 times, and the human sCD44std levels determined. There was no significant loss of human sCD44std immunoreactivity detected by freezing and thawing.

13.5.2 Storage Stability

Aliquots of serum samples were stored at -20°C, 2-8°C, room temperature (RT) and at 37°C, and the human sCD44std level determined after 24 h. There was no significant loss of human sCD44std immunoreactivity detected during storage under above conditions.

13.6 Comparison of Serum and Plasma

Serum, as well as EDTA, citrate and heparin plasma, from 22 individuals was obtained at the same time point. All these blood preparations were found suitable for human sCD44std determinations, although human sCD44std levels in citrate and EDTA plasma were slightly lower than serum levels. It is, therefore, highly recommended to assure the uniformity of sample preparations.

13.7 Specificity

The interference of circulating factors of the immune systeme was evaluated by spiking these proteins at physiologically relevant concentrations into a human sCD44std positive serum.
There was no crossreactivity detected, namely not with TNF-α, TNF-β, TNF-R, IFN-α2c, IFN-γ, IL-8, Annexin, sELAM-1, sL-selectin, sICAM-1 and HER-2.

13.8 Expected Values

A panel of 22 sera samples from randomly selected apparently healthy donors (males and females) was tested for human sCD44std.

The detected human sCD44std levels ranged between 251 and 925 ng/ml with a mean level of 443 ng/ml and a standard deviation of 125 ng/ml.
The levels measured may vary with the sample collection used.

For literature update refer to **www.ibl-america.com**

14 Ordering Information

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15 Reagent Preparation Summary

15.1 Wash Buffer (1x)

Add **Wash Buffer Concentrate** 20x (50 ml) to 950 ml distilled water.

Number of Strips	Wash Buffer Concentrate (ml)	Distilled Water (ml)
1 - 6	25	475
1 - 12	50	950

15.2 Assay Buffer (1x)

Add **Assay Buffer Concentrate** 20x (5 ml) to 95 ml distilled water.

Number of Strips	Assay Buffer Concentrate (ml)	Distilled Water (ml)
1 - 6	2.5	47.5
1 - 12	5.0	95.0

15.3 HRP-Conjugate

Add 490 µl Assay Buffer (1x) to the vial containing the **HRP-Conjugate** concentrate (1:50 predilution).
Make a further 1:40 dilution in Assay Buffer (1x):

Number of Strips	Prediluted (1:50) HRP-Conjugate (ml)	Assay Buffer (1x) (ml)
1 - 6	0.075	2.925
1 - 12	0.150	5.850

16 Test Protocol Summary

1. Predilute serum, plasma and urine samples with Sample Diluent 1:60.
2. Determine the number of microwell strips required.
3. Wash microwell strips twice with Wash Buffer.
4. Standard dilution on the microwell plate: Add 100 µl Sample Diluent, in duplicate, to all standard wells. Pipette 100 µl undiluted standard into the first wells and create standard dilutions by transferring 100 µl from well to well. Discard 100 µl from the last wells.
   Alternatively external standard dilution in tubes (see 9.4.1): Pipette 100 µl of these standard dilutions in the microwell strips.
5. Add 100 µl Sample Diluent, in duplicate, to the blank wells.
6. Add 80 µl Sample Diluent to sample wells.
7. Add 20 µl prediluted sample in duplicate, to designated sample wells.
8. Prepare HRP-Conjugate.
9. Add 50 µl HRP-Conjugate to all wells.
10. Cover microwell strips and incubate 3 hours at room temperature (18° to 25°C).
11. Empty and wash microwell strips 3 times with Wash Buffer.
12. Add 100 µl of TMB Substrate Solution to all wells.
13. Incubate the microwell strips for about 10 minutes at room temperature (18° to 25°C).
14. Add 100 µl Stop Solution to all wells.
15. Blank microwell reader and measure colour intensity at 450 nm.

Note: If instructions in this protocol have been followed samples have been diluted 1:300 (1:60 predilution, 1:5 dilution on the plate: 20 µl sample + 80 µl Sample Diluent), the concentration read from the standard curve must be multiplied by the dilution factor (x 300).