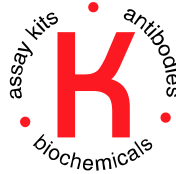


KAMIYA BIOMEDICAL COMPANY

Human sCD44std ELISA

For the quantitative determination of soluble CD44std levels in cell culture supernatants, human serum, EDTA plasma, heparinized plasma, citrate plasma, amniotic fluid, urine and other body fluids

Cat. No. KT-032

For Research Use Only. Not for Use in Diagnostic Procedures.

PRODUCT INFORMATION

Human sCD44std ELISA
Cat. No. KT-032

PRODUCT
The **K-ASSAY®** Human sCD44std ELISA is an enzyme-linked immunosorbent assay for the quantitative determination of soluble CD44std levels in cell culture supernatants, human serum, EDTA plasma, heparinized plasma, citrate plasma, amniotic fluid, urine and other body fluids. For research use only. Not for use in diagnostic or therapeutic procedures.

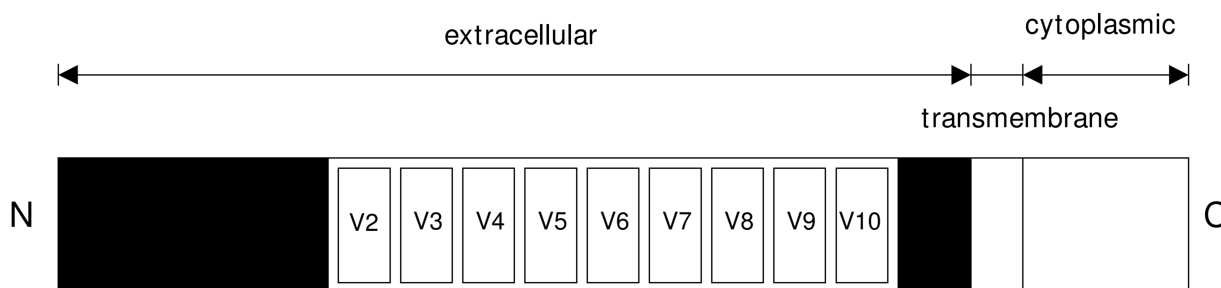
DESCRIPTION
CD44 (Pgp-1; Ly-24; ECMR III; F10-44-2; H-CAM; HUTCH-I; In(Lu)-related p80; Hermes antigen; hyaluronan receptor) is a polymorphic glycoprotein with apparent molecular weights ranging from 85 kDa to 250 kDa. This cell membrane associated molecule has a cytoplasmic tail (mediates the interaction with the cytoskeleton), a short hydrophobic transmembrane region and an NH_2-terminal extracellular (binds to hyaluronate) domain.

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 non-hematopoietic 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.

These larger isoforms of CD44 arise by alternative splicing of one or more "variant" exons (v2-v10) into the extracellular part of the 90 kDa 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.

The **K-ASSAY®** Human sCD44std ELISA detects all circulating CD44 isoforms containing the standard protein sequences (black area).



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

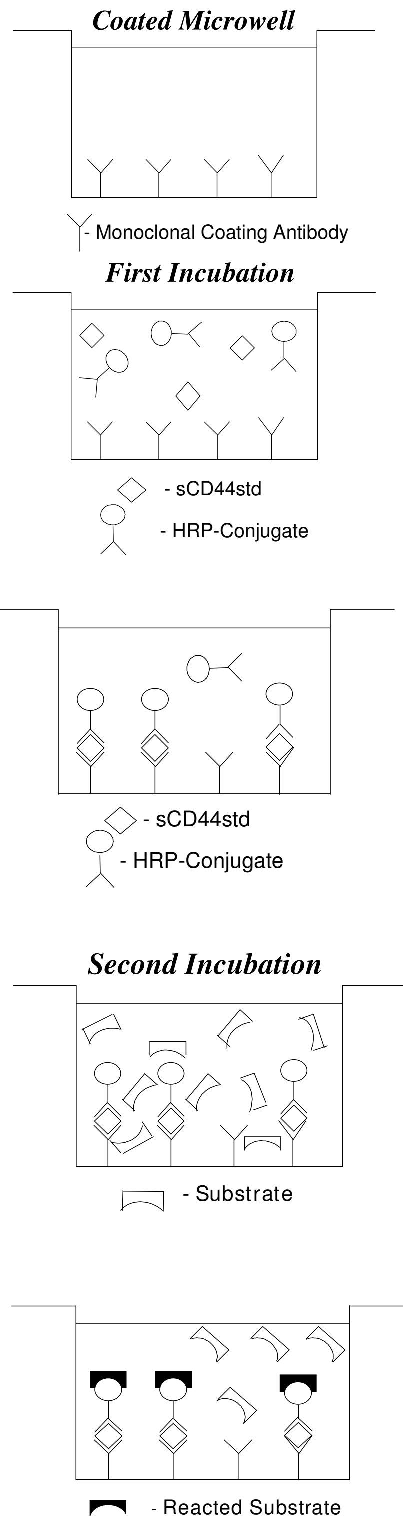
Determination of sCD44std will provide more detailed insight into different pathological modifications during cancer and other diseases.

PRINCIPLES OF THE TEST

An anti-sCD44std monoclonal coating antibody is adsorbed onto microwells. sCD44std present in the sample or calibrator binds to antibodies adsorbed to the microwells; a HRP-conjugated monoclonal anti-sCD44std antibody is added and binds to sCD44std captured by the first antibody.

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

A colored product is formed in proportion to the amount of sCD44std present in the sample. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A calibration curve is prepared from six sCD44std Calibrator dilutions that allows determination of sCD44std concentration in samples.



COMPONENTS

- 1 aluminum pouch with a Microwell Plate coated with monoclonal antibody (mouse) to human sCD44std
- 2 vials (10 µL) HRP-Conjugate anti-sCD44std monoclonal (mouse) antibody
- 2 vials (0.3 mL) 8 ng/mL sCD44std Calibrator
- 1 bottle (50 mL) Wash Buffer Concentrate 20X, phosphate-buffered saline (PBS) with 1% Tween 20
- 1 vial (5 mL) Assay Buffer Concentrate 20X, PBS with 1% Tween 20 and protein stabilizer
- 1 bottle (60 mL) Sample Diluent
- 1 vial (15 mL) Substrate Solution
- 1 vial (12 mL) Stop Solution (1M Phosphoric acid)
- 1 vial (0.4 mL) Blue Dye
- 1 vial (0.4 mL) Green Dye
- 2 adhesive Plate Seals

Materials or Equipment Required but Not Provided

- 5 mL and 10 mL graduated pipettes
- 5 µL to 1,000 µL adjustable single channel micropipettes with disposable tips
- 50 µL to 300 µL adjustable multi-channel micropipette with disposable tips
- Multi-channel micropipette reservoir
- Beakers, flasks, cylinders necessary for preparation of reagents
- Device for delivery of wash solution (multi-channel wash bottle or automatic wash system)
- Microwell strip reader capable of reading at 450 nm (620 nm as optional reference wavelength)
- Glass-distilled or de-ionized water
- Computer or calculator with statistical program to perform linear regression analysis

STORAGE

Store kit reagents at 4°C. Immediately after use reagents should be returned to cold storage (4°C). Expiration date of the kit and reagents is stated on the labels.

The expiration date of the kit components can only be guaranteed if the components are stored properly, and if, in case of repeated use of one component, the reagent is not contaminated by the first handling.

SPECIMEN COLLECTION

Cell culture supernatants, human serum, EDTA, citrate, or heparinized plasma, amniotic fluid, urine, or other body fluids are suitable for use in the assay. Remove the serum or plasma from the clot or red cells, respectively, 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 sCD44std. If samples are to be run within 24 hours, they may be stored at 4°C. Avoid repeated freeze-thaw cycles. Prior to assay, frozen sera, plasma and urine samples should be brought to room temperature (RT) slowly and mixed gently.

For sample stability refer to page 11.

PROTOCOLS

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.

Wash Buffer

Pour entire contents (50 mL) of the **Wash Buffer Concentrate** (20X) into a clean 1,000 mL graduated cylinder. Bring final volume to 1,000 mL with glass-distilled or de-ionized 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 2-25°C. Please note that Wash Buffer is stable for 30 days.
Wash Buffer may also be prepared as needed according to the following table:

Number of Strips	Wash Buffer Conc. (20X) (mL)	Distilled Water (mL)
1-6	25	475
1-12	50	950

Assay Buffer

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 4 °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 Conc. (20X) (mL)	Distilled Water (mL)
1-6	2.5	47.5
1-12	5.0	95.0

Preparation of HRP-Conjugate

Dilute the HRP-Conjugate 1:50 just prior to use by adding 490 µL of (1X) Assay Buffer 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.

Please note that the HRP-Conjugate should be used within 30 minutes after dilution. The second dilution (1:40) of the HRP-Conjugate may be prepared 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

Addition of color-giving reagents: Blue Dye, Green Dye (OPTIONAL STEP)

In order to help our customers to avoid any mistakes in pipetting, KAMIYA BIOMEDICAL COMPANY now offers a new tool that helps to monitor the addition of even very small volumes of a solution to the reaction well by giving distinctive colors 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.

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

1. Sample Diluent: Before sample dilution add the *Blue Dye* at a dilution of 1:250 (see table below) to the appropriate Sample Diluent (1X) amount 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 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 package insert, preparation of HRP-conjugate.

3 mL Assay Buffer	30 µL *Green Dye*
6 mL Assay Buffer	60 µL *Green Dye*

TEST PROTOCOL

a. Mix all reagents thoroughly without foaming before use.

b. Predilute serum, plasma or urine samples before starting with test procedure.
Dilute these samples 1:60 with Sample Diluent according to the following dilution scheme:
10 µL Sample + 590 µL Sample Diluent

c. Determine the number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running blanks and Calibrators. Each sample, Calibrator, blank and optional control sample should be assayed in duplicate. Remove extra Microwell Strips coated with Monoclonal Antibody (mouse) to human sCD44std from holder and store in the foil bag with the desiccant provided at 4°C sealed tightly.

d. 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, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. Use the microwell strips immediately after washing or place upside down on a wet absorbent paper for not longer than 15 minutes. Do not allow wells to dry.

e. Add 100 µL of Sample Diluent, in duplicate, to the Calibrator wells. Prepare Calibrator dilutions by pipetting 100 µL of undiluted sCD44std Calibrator (8 ng/mL), in duplicate, into well A1 and A2 (see Figure 1 and 2). Mix the contents of the wells A1 and A2 by repeated aspiration and ejection and transfer 100 µL to well B1 and B2, respectively. Take care not to scratch the inner surface of the microwells. Repeat this procedure four times, creating two parallel rows of sCD44std Calibrator dilutions ranging from 4 to 0.13 ng/mL. Discard 100 µL of the contents from the last microwells (F1, F2) used.

Figure 1. Preparation of sCD44std Calibrator dilutions:

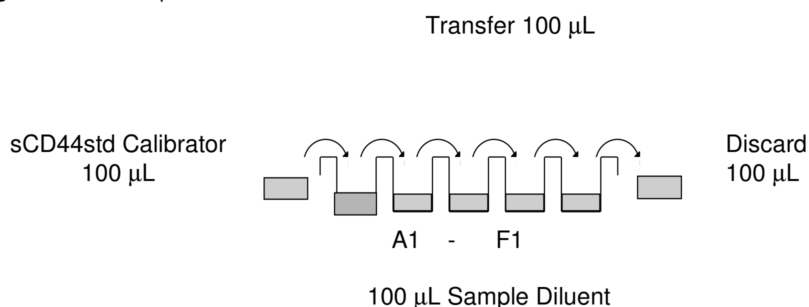


Figure 2. Diagram depicting an example of the arrangement of blanks, Calibrators and samples in the microwell strips:

	1	2	3	4
A	Calibrator 1 (4 ng/mL)	Calibrator 1 (4 ng/mL)	Sample 2	Sample 2
B	Calibrator 2 (2 ng/mL)	Calibrator 2 (2 ng/mL)	Sample 3	Sample 3
C	Calibrator 3 (1 ng/mL)	Calibrator 3 (1 ng/mL)	Sample 4	Sample 4
D	Calibrator 4 (0.5 ng/mL)	Calibrator 4 (0.5 ng/mL)	Sample 5	Sample 5
E	Calibrator 5 (0.25 ng/mL)	Calibrator 5 (0.25 ng/mL)	Sample 6	Sample 6
F	Calibrator 6 (0.13 ng/mL)	Calibrator 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

f. Add 100 µL of Sample Diluent in duplicate to the blank wells.

g. Add 80 µL of Sample Diluent to all sample wells.

h. Add 20 µL of each Sample in duplicate to the sample wells.

i. Prepare HRP-Conjugate. (Refer to preparation of HRP-Conjugate, pg. 5)

j. Add 50 µL of diluted HRP-Conjugate to all wells, including the blank wells.

k. Cover with a Plate Seal and incubate at RT (18-25°C) for 3 hours on a rotator set at 100 rpm.

l. Remove Plate Seal and empty wells. Wash microwell strips 3 times according to point 'd' of the test protocol. Proceed immediately to the next step.

m. Pipette 100 µL of TMB Substrate Solution to all wells, including the blank wells.

n. Incubate the microwell strips at RT (18-25°C) for about 10 minutes. Avoid direct exposure to intense light. **The color development on the plate should be monitored and the substrate reaction stopped (see point 'o' of this protocol) before positive wells are no longer properly recordable. Determination of the ideal time period for color development has to be done individually for each assay.**
It is recommended to add the Stop Solution when the highest calibrator has developed a dark blue color. Alternatively the color development can be monitored by the ELISA reader at 620 nm. The substrate reaction should be stopped as soon as Calibrator 1 has reached an OD of 0.6-0.65.

o. Stop the enzyme reaction by quickly pipetting 100 µL of Stop Solution into each well, including the blank wells. It is important that the Stop Solution is dispensed 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 4°C in the dark.

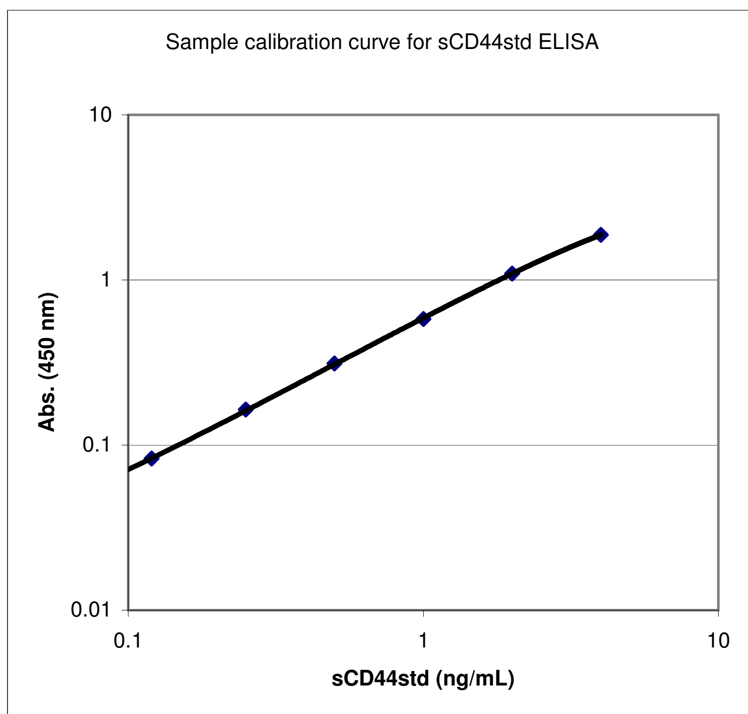
p. Read absorbance of each microwell on a spectrophotometer using 450 nm as the primary wavelength (optionally 620 nm as the reference wavelength; 610 nm to 650 nm is acceptable). Blank the plate reader according to the manufacturer's instructions by using the blank wells. Determine the absorbance of both the samples and the sCD44std Calibrators.

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

CALCULATION OF RESULTS

- Calculate the average absorbance values for each set of duplicate Calibrators and samples. Duplicates should be within 20 percent of the mean.
- Create a calibration curve by plotting the mean absorbance for each Calibrator concentration on the ordinate against the 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 sCD44std for each sample, first find the mean absorbance value on the ordinate and extend a horizontal line to the calibration curve. At the point of intersection, extend a vertical line to the abscissa and read the corresponding sCD44std concentration.
- For samples which have been diluted according to the instructions given in this manual, 1:300, the concentration read from the calibration curve must be multiplied by the dilution factor (x 300).
- Calculation of samples with a concentration exceeding calibrator 1 may result in incorrect, low human sCD44std levels (Hook Effect). 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 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 calibration curve is shown in Figure 3. This curve cannot be used to derive test results. A new calibration curve must be generated every time an assay is done.

Figure 3. Representative calibration curve for sCD44std ELISA. Human sCD44std was diluted in serial two-fold steps in Sample Diluent. Do not use this calibration curve to derive test results. A new calibration curve must be generated for each assay.



Typical Data Using the sCD44std ELISA

Measuring wavelength: 450 nm Reference wavelength: 620 nm

Calibrator	sCD44std Conc. (ng/mL)	O.D. (450 nm)	O.D. Mean	C.V. (%)
1	4	1.901	1.876	1.9
	4	1.851		
2	2	1.080	1.095	1.9
	2	1.109		
3	1	0.608	0.581	6.7
	1	0.553		
4	0.5	0.330	0.312	8.4
	0.5	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 calibration 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 effect enzymatic activity and thus color intensity. Values measured are still valid.

LIMITATIONS

- Since exact conditions may vary from assay to assay, a calibration 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 detergent before use.
- Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Completely empty wells before dispensing fresh Wash Buffer, 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 antibody (HAMA). HAMA may interfere with assays utilizing mouse monoclonal antibodies leading to both false positive and false negative results. Serum samples containing antibodies to mouse immunoglobulins can still be analyzed in such assays when mouse immunoglobulins (serum, ascitic fluid, or monoclonal antibodies of irrelevant specificity) are added to the sample.

PERFORMANCE CHARACTERISTICS

Sensitivity

The limit of detection for sCD44std, defined as the analyte concentration resulting in an absorption significantly higher than the absorption of the dilution medium (mean plus two standard deviations), was determined to be 0.02 ng/mL (mean of 6 independent assays).

Reproducibility

a. Intra-assay

Reproducibility within the assay was evaluated in two independent experiments. Each assay was carried out with 6 replicates of 8 serum samples containing different concentrations of sCD44std. Two calibration curves were run on each plate. Data below show the mean sCD44std concentration and the coefficient of variation for each sample. The overall intra-assay coefficient of variation has been calculated to be 4.8%.

Sample	Experiment	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

b. Inter-Assay

Assay to assay reproducibility within one laboratory was evaluated in three independent experiments. Each assay was carried out with 6 replicates of 8 serum samples containing different concentrations of sCD44std. Two calibration curves were run on each plate. Data below show the mean sCD44std concentration and the coefficient of variation calculated on 18 determinations of each sample. The overall inter-assay coefficient of variation has been calculated to be 4.1%.

Sample	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

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%.

Dilution Parallelism

Four serum samples with different levels of sCD44std were assayed at four serial two-fold dilutions (1:300-1:2,400) covering the working range of the calibration curve. In the table below, the percent recovery of expected values is listed. Recoveries ranged from 91% to 99% with an overall mean recovery of 94%.

Sample	Dilution	sCD44std Concentration (ng/mL) Expected Value	Observed Value	% Recovery of Exp. Value
1	1:300	--	347	--
	1:600	173	160	92
	1:1,200	87	80	92
	1:2,400	43	42	96
2	1:300	--	402	--
	1:600	201	186	92
	1:1,200	100	94	94
	1:2,400	50	46	91
3	1:300	--	292	--
	1:600	146	145	99
	1:1,200	73	72	99
	1:2,400	37	34	92
4	1:300	--	368	--
	1:600	184	173	94
	1:1,200	92	89	96
	1:2,400	46	43	94

Sample Stability

Freeze-Thaw Stability

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

Storage Stability

Aliquots of a serum sample were stored at -20°C, 4°C, RT and at 37°C and the sCD44std level determined after 24 hours. There was no significant loss of sCD44std immunoreactivity detected during storage under above conditions.

Comparison of Serum and Plasma

Sera, as well as EDTA, citrate and heparin plasmas from 22 individuals were obtained at the same time. All these blood preparations were found suitable for sCD44std determinations, although 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!

Specificity

The interference of circulating factors of the immune system was evaluated by spiking various proteins (listed below) at physiologically relevant concentrations into a sCD44std positive serum. There was no detectable cross reactivity.
TNF-α, TNF-β, TNF-R, IFN-α2c, IFN-γ, IL-8, annexin, sELAM-1, sL-selectin, sICAM-1, HER-2.

WARNINGS AND PRECAUTIONS

- All chemicals should be considered as potentially hazardous. We recommend that only those persons who have been trained in laboratory techniques handle this product. It should be 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.
- 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 reagents.
- Exposure to acids will inactivate the conjugate.
- Glass-distilled water or de-ionized water must be used for reagent preparation.
- Substrate solution must be at RT prior to use.
- Decontaminate and dispose specimens and all potentially contaminated materials as if 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.

REAGENT PREPARATION SUMMARY

A. Wash Buffer

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

B. Assay Buffer

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

C. HRP Conjugate

Predilution: Add 490 µL Assay Buffer to tube containing HRP-Conjugate concentrate. Mix. Make further dilution according to table.

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

TEST PROTOCOL SUMMARY

- Predilute serum, plasma or urine samples with Sample Diluent 1:60.
- Wash Microwell Strips twice with Wash Buffer.
- Add 100 µL Sample Diluent, in duplicate, to Calibrator wells.
- Pipette 100 µL sCD44std Calibrator into the first Calibrator wells and create Calibrator dilutions ranging from 4 to 0.13 ng/mL by transferring 100 µL from well to well; Discard 100 µL from the last wells.
- Add 100 µL Sample Diluent to the duplicate blank wells.
- Add 80 µL Sample Diluent to all sample wells.
- Add 20 µL prediluted Sample to designated duplicate sample wells (= 1:300 final dilution).
- Prepare HRP-Conjugate.
- Add 50 µL diluted HRP-Conjugate to all wells, including the blank wells.
- Cover microwell strips and incubate 3 hours at RT (18-25°C).
- Empty and wash microwell strips 3 times with Wash Buffer.
- Add 100 µL of TMB Substrate Solution to all wells, including blank wells.

- Incubate the microwell strips for approximately 10 minutes at RT (18-25°C) (See detailed protocol, pg. 7, for time determination guidelines).
- Add 100 µL Stop Solution to all wells including blank wells.
- Blank microwell reader and measure color intensity at 450 nm.

Note: For samples which have been diluted according to the instructions given in this manual, 1:300, the concentration read from the calibration curve must be multiplied by the dilution factor (x300).

FOR RESEARCH USE ONLY

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