sCD44std, Soluble (human) ELISA Kit

Manufactured by Bender MedSystems.

ALX-850-053-KI01

96 wells (~80 tests)

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For laboratory use only. Not for human or diagnostic use.

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1. INTENDED USE

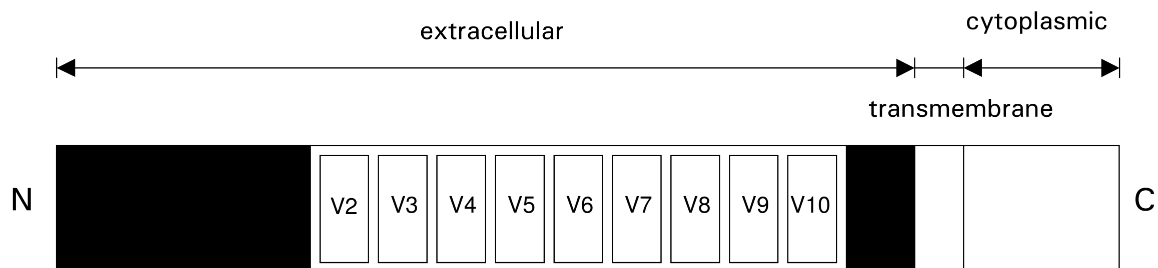
The sCD44std ELISA is an enzyme-linked immunosorbent assay for the quantitative detection of soluble CD44std levels in cell culture supernatants, human serum, EDTA, heparinized plasma, urine or other body fluids. **The sCD44std ELISA is for research use only. Not for use in diagnostic or therapeutic 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 nonhemato-poietic 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).

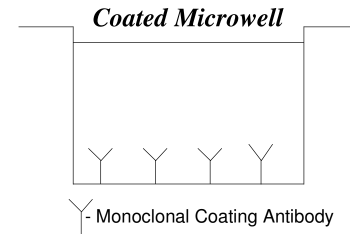
- **lymphoma:** in gastrointestinal lymphoma overexpression of CD44 has been correlated with poor survival and more disseminated disease (6,3).
  Overexpression of CD44 is also found in several aggressive, but not low-grade, non-Hodgkin´s lymphomas (7) as well as in Hodgkin´s and nodal diffuse lymphomas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

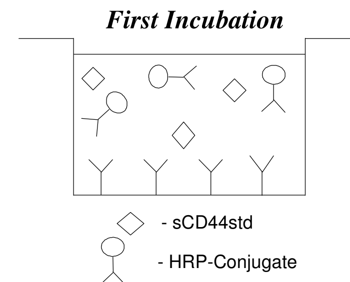
- **HIV:** CD44 is almost completely depleted from the surface of HIV-infected cells (9).

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

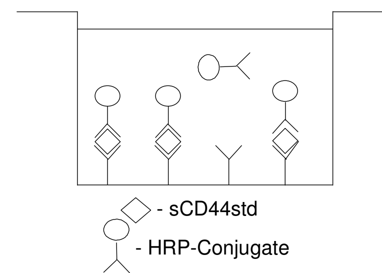
3. PRINCIPLES OF THE TEST



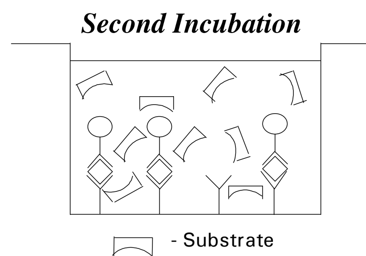
An anti-sCD44std monoclonal coating antibody is adsorbed onto microwells. sCD44std present in the sample or standard binds to antibodies adsorbed to the microwells; a HRP-conjugated monoclonal anti-sCD44std antibody is added and binds to sCD44std captured by the first antibody.

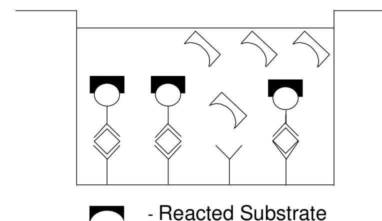


Following incubation unbound enzyme conjugated anti-sCD44std is removed during a wash step and substrate solution reactive with HRP is added to the wells.



A coloured 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 450nm. A standard curve is prepared from six sCD44std standard dilutions which allows determination of sCD44std concentration in samples.





4. REAGENTS PROVIDED

1 aluminium pouch with **a Microwell Plate coated with Monoclonal Antibody** (murine) to human sCD44std.

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

2 vials (0.3 ml) 8 ng/ml **sCD44std Standard**.

1 bottle (50 ml) **Wash Buffer Concentrate** 20x (phosphate-buffered saline with 1% Tween 20).

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

1 bottle (60 ml) **Sample Diluent** (buffered protein matrix).

1 vial (15 ml) **Substrate Solution**

1 vial (12 ml) **Stop Solution** (1M Phosphoric acid)

1 vial (0.4 ml each) **Blue-Dye** and **Green-Dye**

2 adhesive **Plate Covers**.

Reagent Labels.

5. STORAGE INSTRUCTIONS

Store kit reagents between 2° and 8°C. Immediately after use reagents should be returned to cold storage (2° to 8°C). Expiry of the kit and reagents is stated on labels.

The 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, the reagent is not contaminated by the first handling.

6. SPECIMEN COLLECTION

Cell culture supernatants, human serum, EDTA, or heparinized plasma, amniotic fluid, urine, or other body fluids are suitable for use in the assay. For analysis of **plasma samples** please refer to section 13.H of this manual. 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 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 2° to 8°C. Avoid repeated freeze-thaw cycles. Prior to assay, frozen sera, plasma and urine samples should be brought to room temperature slowly and mixed gently and properly diluted with Sample Diluent according to Test Protocol (see 10.b. page 15).

For sample stability refer to 13. page 23.

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 asoptional reference wave length)
- Glass-distilled or deionized water
- Statistical calculator with program to perform linear 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 statements(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 reagents.

- Exposure to acids will inactivate 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 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.

9. PREPARATION OF REAGENTS

Note: reagents A and B (Wash Buffer, Assay Buffer) may be prepared in advance.
Reagent C (Conjugate) should be prepared immediately before needed.

A. Wash Buffer

If crystals have formed in the Wash Buffer Concentrate, warm it gently until they have completely dissolved.

Pour entire contents (50 ml) of the **Wash Buffer Concentrate** into a clean 1,000 ml graduated cylinder. Bring final volume to 1,000 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 is stable for 30 days. Wash Buffer may be prepared as needed according to the following table:

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

B. Assay Buffer

Mix the contents of the bottle well. Add contents of **Assay Buffer Concentrate** (5.0 ml) to 95 ml distilled or deionized water and mix gently to avoid foaming. Store at 2° to 8°C. Please note that the Assay Buffer is stable for 30 days. Assay Buffer may be prepared as needed according to the following table:

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

C. Preparation of HRP-Conjugate

Dilute the HRP-Conjugate 1 : 50 just prior to use by adding 490μl **Assay Buffer** (reagent B) to the tube containing the HRP-Conjugate concentrate. Mix the contents of the tube well.

Make a further 1:40 dilution with **Assay Buffer** 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:

Prediluted (1:50) HRP-Conjugate	Assay Buffer
150 µl	5.85 ml

D. Addition of colour-giving reagents: Blue-Dye, Green-Dye

In order to help our customers to avoid any mistakes in pipetting the Bender MedSystems ELISAs, Bender MedSystems 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 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 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 Diluent	20 µl ***Blue-Dye***
12 ml Diluent	48 µl ***Blue-Dye***
50 ml Diluent	200 µl ***Blue-Dye***
60 ml Diluent	240 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 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	30 µl ***Green-Dye***
6 ml Assay Buffer	60 µl ***Green-Dye***
12 ml Assay Buffer	120 µl ***Green-Dye***

10. TEST PROTOCOL

a. Mix all reagents thoroughly without foaming before use.

b. Predilute serum, plasma or urine samples 1:60 with **Sample Diluent** according to the following dilution scheme:

   I. 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 standards. Each sample, standard, blank and optional control sample should be assayed in duplicate. Remove extra **Microwell Strips coated with Monoclonal Antibody** (murine) to human sCD44std from holder and store in foil bag with the desiccant provided at 2°-8°C sealed tightly.

d. Wash the microwell strips twice with approximately 300 μl **Wash Buffer** per well with thorough aspiration of microwell contents between washes. Take care not to scratch the surface of the microwells.

   After the last wash, 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 standard wells. Prepare standard dilutions by pipetting 100 μl of **sCD44std Standard** (8 ng /ml), in duplicate, into well A1 and A2 (see Figure 1 and 2). Mix the contents 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 standard dilutions ranging from 4 to 0.12 ng/ml. Discard 100 μl of the contents from the last microwells (F1, F2) used.

Figure 1. Preparation of sCD44std standard dilutions:

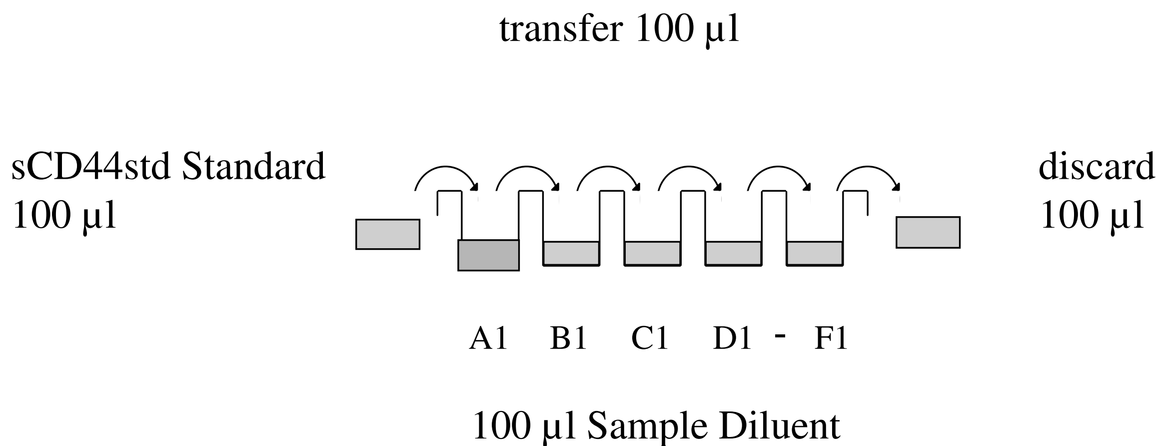


Figure 2. Diagram depicting an example of the arrangement of blanks, standards and samples in the microwell strips:

	1	**2**	**3**	**4**
A	Standard 1 (4 ng/ml)	Standard 1 (4 ng/ml)	Sample 2	Sample 2
B	Standard 2 (2 ng/ml)	Standard 2 (2 ng/ml)	Sample 3	Sample 3
C	Standard 3 (1 ng/ml)	Standard 3 (1 ng/ml)	Sample 4	Sample 4
D	Standard 4 (0.5 ng/ml)	Standard 4 (0.5ng/ml)	Sample 5	Sample 5
E	Standard 5 (0.25 ng/ml)	Standard 5 (0.25ng/ml)	Sample 6	Sample 6
F	Standard 6 (0.12 ng/ml)	Standard 6 (0.12 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 1:60 prediluted **Sample**, in duplicate, to the designated sample wells.

i. Prepare HRP-Conjugate. (Refer to preparation of reagents 9.C.)

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

k. Cover with a **Plate Cover** and incubate at room temperature (18° to 25°C) for 3 hours on a rotator set at 100 rpm.

l. Remove Plate Cover 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 room temperature (18° to 25°C) for about 10 minutes. Avoid direct exposure to intense light.
The colour 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.
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 620nm. The substrate reaction should be stopped as soon as an OD of 0.6 – 0.65 is reached.

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 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.

p. 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 sCD44std 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.

- Create a standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the sCD44std concentration on the abscissa. Draw a best fit curve through the points of the graph.

- 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 standard curve. At the point of intersection, extend a vertical line to the abscissa and read the corresponding sCD44std concentration.

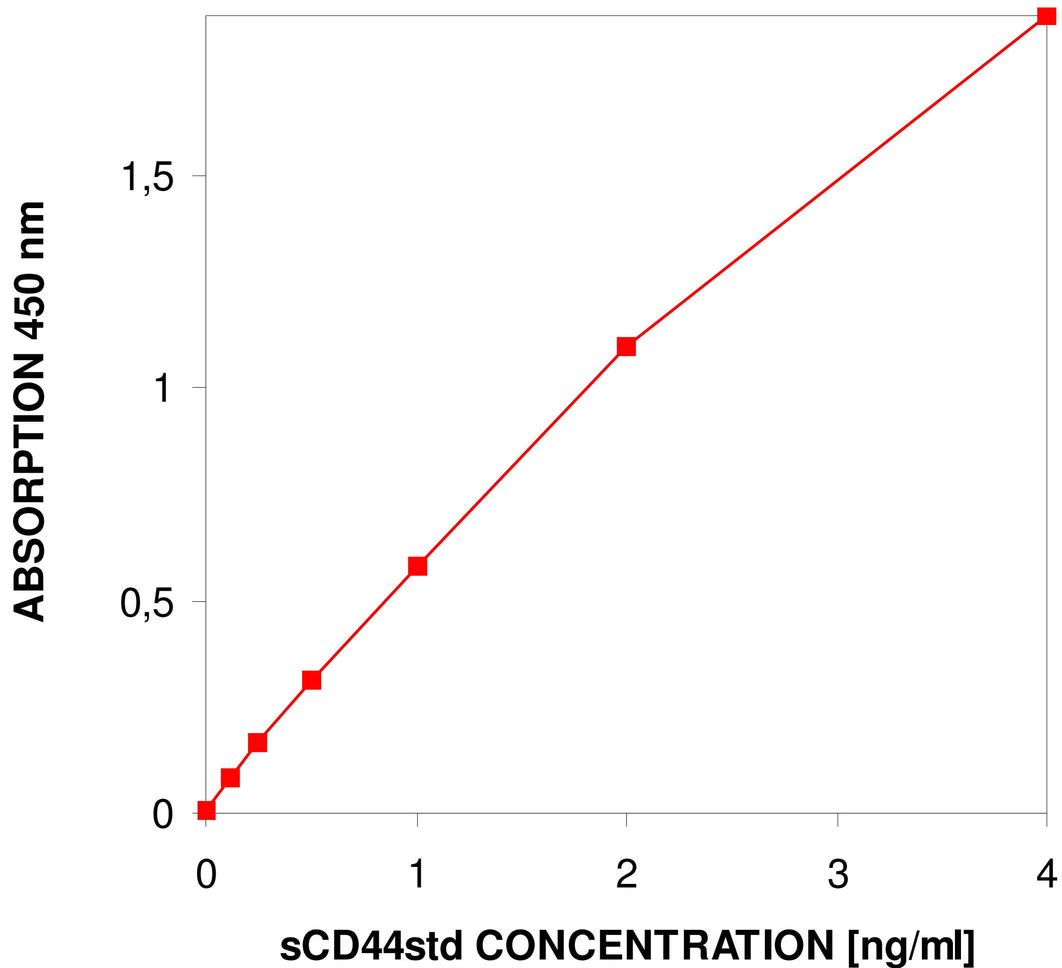
- **For samples which have been diluted according to the instructions given in this manual 1 : 300 the concentration read from the standard curve must be multiplied by the dilution factor (x 300).**

  Note: Calculation of samples with an O.D. exceeding 2.0 may result in incorrect low sCD44std levels. Such samples require further dilution of 1:600 - 1:1200 with Sample Diluent in order to precisely quantitate their actual sCD44std levels.

- 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 standard curve is shown in Figure 3. This curve cannot be used to derive test results. A new standard curve must be generated everytime an assay is done.

Figure 3. Representative standard curve for sCD44std ELISA. Recombinant sCD44std was diluted in serial two-fold steps in Sample Diluent; symbols represent the mean of three parallel titrations. Do not use this standard curve to derive test results. A new standard curve must be generated for each assay.



Typical data using the sCD44std ELISA

Measuring wavelength: 450 nm
Reference wavelength: 620 nm

Standard	sCD44std Concentration (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	0.12	0.081	0.083	2.6
	0.12	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. temperature effects). Furthermore shelf life of the kit may effect 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 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 murine monoclonal antibodies leading to both false positive and false negative results. Serum samples containing antibodies to murine immunoglobulins can still be analyzed in such assays when murine immunoglobulins (serum, ascitic fluid, or monoclonal antibodies of irrelevant specificity) are added to the Sample Diluent.

13. PERFORMANCE CHARACTERISTICS

A. 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 0.015 ng/ml (mean of 6 independent assays).

B. 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 standard 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 %.

Positive 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 by three technicians. Each assay was carried out with 6 replicates of 8 serum samples containing different concentrations of sCD44std. Two standard 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

C. Recovery Studies

Spiked samples were prepared by adding four different levels of sCD44std to human serum. As shown below, recoveries were determined ranging from 76 % to 101 % with an overall mean recovery of 89 %.

sCD44std Base level (ng/ml)	Recovery (%) sCD44std Spike			
	900 ng	450 ng	180 ng	60 ng
130	76	95	101	84

D. Dilution Parallelism

Four serum samples with different levels of sCD44std were assayed at four serial two-fold dilutions (1:300 - 1:2400) covering the working range of the standard 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 %.

		sCD44std Concentration (ng/ml)		
Sample	Dilution	Expected Value	Observed Value	% Recovery of Exp. Value
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

E. Expected Values

A panel of 22 sera from healthy blood donors (male and female) was tested for sCD44std. The detected 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. Normal sCD44std levels may vary depending on the serum collective used.

F. Sample Freeze-Thaw Stability

Aliquots of serum samples were stored at -20°C and thawed several times, and the sCD44std level determined. There was no significant loss of sCD44std concentrations between 0 and 5 freeze-thaw cycles.

G. Sample Storage Stability

Aliquots of a serum sample were stored at -20°C, 2-8°C, room temperature and at 37°C and the sCD44std level determined after 24 hours. There was no significant loss of sCD44std immunoreactivity caused by storage under above conditions.

H. Comparison of Serum and Plasma

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

I. 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.

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15. ORDERING INFORMATION

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16. REAGENT PREPARATION SUMMARY

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

B. Assay Buffer

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

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

1:50 Prediluted HRP-Conjugate (µl)	Assay Buffer (ml)
150 µl	5.85 ml

17. 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 standard wells
- Pipette 100 µl **sCD44std Standard** into the first standard wells and create standard dilutions ranging from 4 to 0.12 ng/ml by transferring 100 µl from well to well; Discard 100µl from the last wells.
- Add 100 µl **Sample Diluent**, in duplicate, to the blank wells
- Add 80 µl **Sample Diluent** to all sample wells
- Add 20 µl prediluted **Sample**, in duplicate, to designated wells

  (= 1:300 final dilution)
- Prepare HRP-Conjugate
- Add 50 µl diluted **HRP-Conjugate** to all wells
- Cover microwell strips and incubate 3 hours at room temperature (18° to 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 about 15 minutes at room temperature (18° to 25°C)
- Add 100 µl **Stop Solution** to all wells including blank wells
- Blank microwell reader and measure colour intensity at 450 nm

Note: For samples which have been diluted according to the instructions given in this manual 1:300 the concen-tration read from the standard curve must be multiplied by the dilution factor (x300). Calculation of samples with an O.D. exceeding 2.0 may result in incorrect low sCD44std levels. Such samples require further dilution of 1:600 - 1:1200 with Sample Diluent in order to precisely quantitate the actual sCD44std level.