KAMIYA BIOMEDICAL COMPANY

Human sCD44var(v6) ELISA

For the quantitative determination of sCD44var(v6) levels in cell culture supernatants, human serum, plasma, urine, amniotic fluid and other body fluids

Cat. No. KT-033

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

PRODUCT INFORMATION

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PRODUCT

The **K-ASSAY®** Human sCD44var(v6) ELISA is an enzyme-linked immunosorbent assay for the quantitative determination of sCD44var(v6) levels in cell culture supernatants, human serum, plasma, urine, amniotic fluid and other body fluids. The **K-ASSAY®** Human sCD44var(v6) ELISA is 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 which participates in a wide variety of cell-cell or cell-matrix interactions including lymphocyte homing, establishment of B and T cell immune responses, tumor metastasis formation and inflammation.

Three isoform categories of the CD44 molecule have been identified:

1) a predominant 80-90 kDa category, the so-called standard form named C44std,
2) an intermediate size category of 110-160 kDa and
3) a category which includes very large isoforms of 250 kDa covalently modified by the addition of chondroitin sulfate.

This CD44-family of transmembrane receptor molecules is derived from a single gene located on chromosome 11. Alternative splicing of the mRNA gives rise to the different isoforms, containing inserts of varying sizes in the extracellular domain of the molecule (exons v2-v10). All CD44 isoforms are variably glycosylated. In contrast to standard CD44 (CD44std) which is almost ubiquitously expressed, the variety of CD44 isoforms (CD44var) have a much more restricted distribution, e.g., on keratinocytes (exons v3-v10), epithelial cells (exons v8-v10), activated lymphocytes and macrophages (exon v6).

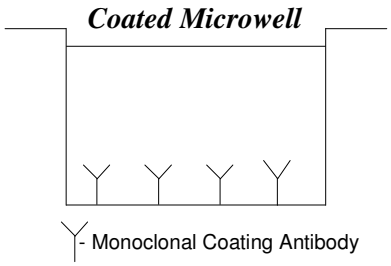
A splice variant of CD44 (exons v4-v7) confers metastatic behavior in a rat carcinoma model; aberrant expression of splice variants has been detected on a variety of human tumor cell lines as well as primary and metastatic human tumors, including lymphomas, carcinomas (colon, thyroid, mamma, bladder), and gliomas.

The **K-ASSAY®** Human sCD44var(v6) ELISA detects all circulating CD44 isoforms containing the sCD44var(v6) sequences.

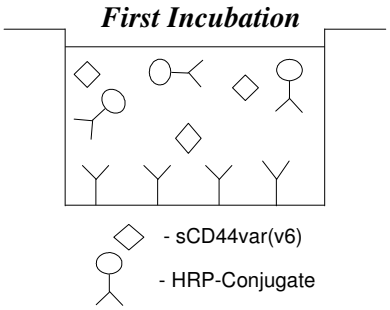
Determination of sCD44var(v6) will provide more detailed insight into different pathological modifications during cancer and other diseases.

PRINCIPLE

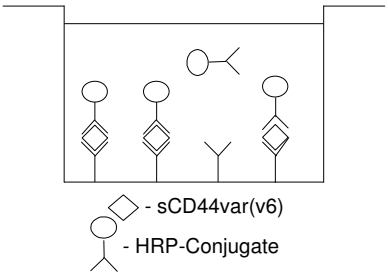
An anti-sCD44var(v6) monoclonal coating antibody is adsorbed onto microwells.



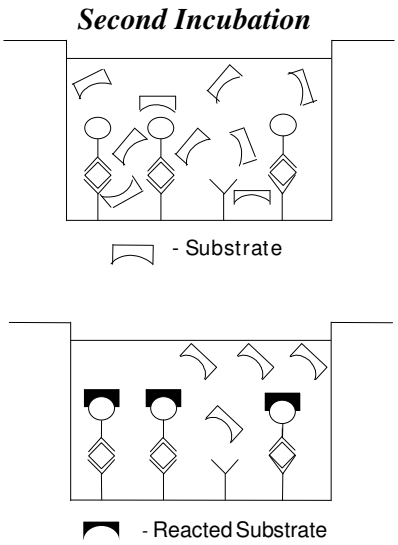
sCD44var(v6) present in the sample or Calibrator binds to antibodies adsorbed to the microwells; a HRP-conjugated monoclonal anti-sCD44var(v6) antibody is added and binds to sCD44var(v6) captured by the first antibody.



Following incubation unbound enzyme conjugated anti-sCD44var(v6) 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 sCD44var(v6) 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 sCD44var(v6) Calibrator dilutions which allows determination of sCD44var(v6) concentration in samples.



COMPONENTS

- 1 aluminum pouch with a Microwell Plate coated with monoclonal antibody (mouse) to human sCD44var(v6)
- 2 vials (10 µL) HRP-Conjugate anti-sCD44var(v6) monoclonal (mouse) antibody
- 2 vials (Lyophilized) sCD44var(v6) Calibrator, 20 ng/mL upon reconstitution
- 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 (50 mL) Sample Diluent (buffered protein matrix)
- 1 vial (7 mL) Substrate Solution I (tetramethylbenzidine)
- 1 vial (7 mL) Substrate Solution II (0.02% buffered hydrogen peroxide)
- 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 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 wavelength)
- Glass-distilled or de-ionized water
- Statistical calculator with program to perform linear regression analysis.

PROTOCOLS

Specimen Collection

Cell culture supernatants, human serum, plasmas, urine, amniotic fluid or other body fluids are suitable for use in the assay. For analysis of plasma samples please refer to comparison of serum and plasma on page 11 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 sCD44var(v6). 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 and properly diluted with Sample Diluent according to Test Protocol (page 6).

For sample stability refer to page 11.

Preparation of Reagents

Note: The Wash Buffer and Assay Buffer may be prepared in advance.
The Calibrator, Conjugate and Substrate should be prepared immediately before needed.

Wash Buffer

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

If you are not using all the microwell strips, a smaller volume of Wash Buffer can be prepared according to the table below. Otherwise 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 de-ionized water. Mix gently to avoid foaming. The pH of the final solution should be 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 be prepared as needed according to the following table:

Number of Strips	Wash Buffer Concentrate (mL)	Distilled Water (mL)
1-6	25	475
7-12	50	950

Assay Buffer

Mix the contents of the bottle well. If you are not using all the microwell strips, a smaller volume of Assay Buffer can be prepared according to the table below. Otherwise add contents of Assay Buffer Concentrate (5.0 mL) to 95 mL distilled or de-ionized water and mix gently to avoid foaming. Store at 4°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
7-12	5.0	95.0

Preparation of sCD44var(v6)-Calibrator
The lyophilized Calibrator must be reconstituted with distilled water. Open vial carefully and add distilled water as indicated on vial label. Swirl vial thoroughly to ensure quantitative solubilization of contents.

Preparation of HRP-Conjugate
Dilute the HRP-Conjugate 1:20 just prior to use by adding 190 µL Assay Buffer to the tube containing the HRP-Conjugate concentrate. Mix the contents of the tube well.
Make a further 1:100 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 (1:100) of the HRP-Conjugate may be prepared as needed according to the following table:

Number of Strips	Prediluted (1:20) HRP-Conjugate (mL)	Assay Buffer (mL)
1-6	0.03	2.97
7-12	0.06	5.94

TMB Substrate Solution
Using clean pipettes and containers known to be metal free, dispense an equal volume of Substrate Solution I into Substrate Solution II and swirl gently to mix. The TMB Substrate Solution may develop a yellow tinge over time. This does not seem to affect product performance. A blue color present in the TMB Substrate Solution, however, indicates that it has been contaminated and must be discarded. The TMB Substrate Solution must be used within a few minutes after mixing. Warm to RT before use. Avoid direct exposure of TMB reagents to intense light and oxidizing agents during storage or incubation.

Number of Strips	Substrate Solution I (mL)	Substrate Solution II (mL)
1-6	3.0	3.0
7-12	6.0	6.0

Addition of color-giving reagents: Blue Dye, Green Dye (OPTIONAL STEP)
In order to help our customers to avoid any mistakes in pipetting the ***K-ASSAY®*** Human sCD44var(v6) ELISA, **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, just following the manual.

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 amount according to the test protocol. After addition of Blue Dye, proceed according to the manual.

5 mL Sample Diluent	20 µL Blue Dye
12 mL Sample Diluent	48 µL Blue Dye
50 mL Sample Diluent	200 µL Blue Dye
60 mL Sample 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 manual, 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

TEST PROTOCOL

a. Mix all reagents thoroughly without foaming before use.

b. Predilute samples 1:20 with Sample Diluent according to the following dilution scheme:
10 µL Sample + 190 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 sCD44var(v6) from holder. Return all unused strips to foil bag with desiccant, seal tightly and store at 4°C.

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 to the duplicate Calibrator wells. Prepare Calibrator dilutions by pipetting 100 µL of sCD44var(v6) Calibrator (20 ng/mL, refer to preparation of reagents on page 5) 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 sCD44var(v6) Calibrator dilutions ranging from 10 to 0.32 ng/mL. Discard 100 µL of the contents from the last microwells (F1, F2) used.

Figure 1. Preparation of sCD44var(v6) Calibrator dilutions:

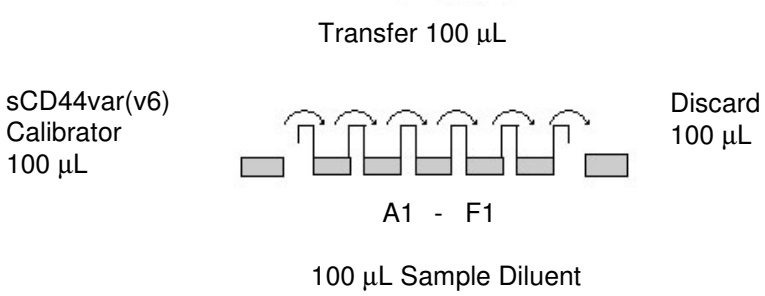


Figure 2. Diagram depicting an example of the arrangement of blanks, Calibrators and samples in the microwell strips:

	1	2	3	4
A	Calibrator 1 (10 ng/mL)	Calibrator 1 (10 ng/mL)	Sample 2	Sample 2
B	Calibrator 2 (5 ng/mL)	Calibrator 2 (5 ng/mL)	Sample 3	Sample 3
C	Calibrator 3 (2.5 ng/mL)	Calibrator 3 (2.5 ng/mL)	Sample 4	Sample 4
D	Calibrator 4 (1.25 ng/mL)	Calibrator 4 (1.25 ng/mL)	Sample 5	Sample 5
E	Calibrator 5 (0.63 ng/mL)	Calibrator 5 (0.63 ng/mL)	Sample 6	Sample 6
F	Calibrator 6 (0.32 ng/mL)	Calibrator 6 (0.32 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 to the duplicate blank wells.

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

h. Add 20 µL of each 1:20 prediluted Sample to the designated duplicate sample wells.

i. Prepare HRP-Conjugate (Refer to preparation of reagents on page 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. Prepare TMB Substrate Solution a few minutes prior to use. (Refer to preparation of reagents on page 5).

m. 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.

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

o. Incubate the microwell strips at RT (18-25°C) for about 10 minutes, if available on a rotator set at 100 rpm. Avoid direct exposure to intense light. **The color development on the plate should be monitored and the substrate reaction stopped (see point 'p' of this protocol) before positive wells are no longer recordable.** 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 an OD of 0.6-0.65 is reached.

p. 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.

q. 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 are 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 sCD44var(v6) 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 sCD44var(v6) concentration on the abscissa. Draw a best fit curve through the points of the graph.

- To determine the concentration of circulating sCD44var(v6) 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 sCD44var(v6) concentration.

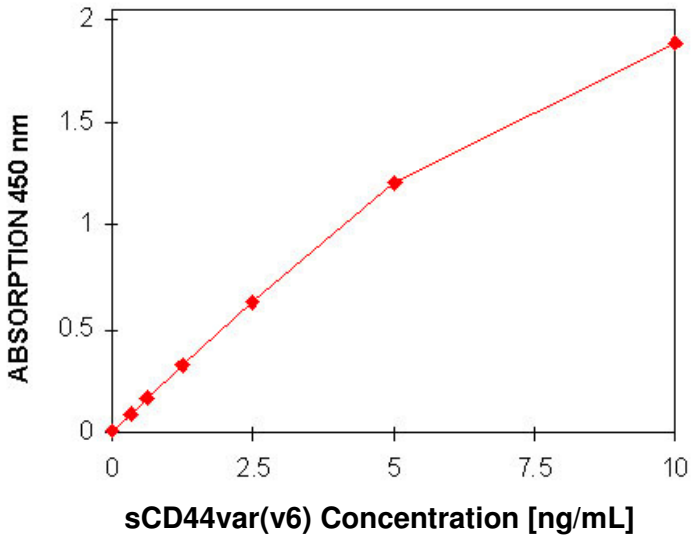
- For samples which have been diluted 1:100 according to the instructions given in this manual the concentration read from the calibration curve must be multiplied by the dilution factor (x 100).

  Note: Calculation of samples with an O.D. exceeding 2.0 may result in incorrect low sCD44var(v6) levels. Such samples require further dilution of 1:200-1:400 with Sample Diluent in order to precisely quantitate their actual sCD44var(v6) levels. You can conclude from the OD how to further predilute your sample.

- It is suggested that each testing facility establishes a control sample of known sCD44var(v6) 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.

Figure 3. Representative calibration curve for sCD44var(v6) ELISA. Recombinant sCD44var(v6) was diluted in serial two-fold steps in Sample Diluent; symbols represent the mean of three parallel titrations. Do not use this calibration curve to derive test results. A new calibration curve must be generated for each assay.



Typical Data Using the sCD44var(v6) ELISA

Measuring wavelength: 450 nm Reference wavelength: 620 nm

Calibrator	sCD44var(v6) Concentration (ng/mL)	O.D. (450 nm)	O.D. Mean	C.V. (%)
1	10	1.749	1.884	7.2
	10	2.019		
2	5	1.246	1.203	3.6
	5	1.159		
3	2.5	0.638	0.628	1.7
	2.5	0.617		
4	1.25	0.334	0.323	3.6
	1.25	0.311		
5	0.625	0.168	0.164	2.8
	0.625	0.159		
6	0.32	0.087	0.085	3.0
	0.32	0.082		
Blank	0	0.010	0.013	16.5
	0	0.015		
	0	0.011		
	0	0.018		

The OD values of the calibration 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 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 Diluent.

PERFORMANCE CHARACTERISTICS

Sensitivity

The limit of detection for sCD44var(v6), 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 0.05 ng/mL (mean of 10 independent assays).

Reproducibility

Intra-assay

Reproducibility within the assay was evaluated in three independent experiments. Each assay was carried out with 6 replicates of 8 serum samples containing different concentrations of sCD44var(v6). Two calibration curves were run on each plate. Data below show the mean sCD44var(v6) concentration and the coefficient of variation for each sample. The overall intra-assay coefficient of variation has been calculated to be 3.0%.

Positive Sample	Experiment	sCD44var(v6) Concentration (ng/mL)	Coefficient of Variation (%)
1	1	143	2.7
	2	157	5.0
	3	143	3.7
2	1	30	1.6
	2	37	4.7
	3	33	2.6
3	1	555	1.1
	2	564	4.3
	3	582	2.3
4	1	367	3.7
	2	391	1.9
	3	405	2.2
5	1	247	1.5
	2	268	2.3
	3	266	3.1
6	1	223	5.0
	2	238	3.4
	3	244	3.1
7	1	298	1.8
	2	309	2.7
	3	324	4.9
8	1	720	1.8
	2	714	3.0
	3	781	1.8

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 sCD44var(v6).

Two calibration curves were run on each plate. Data below show the mean sCD44var(v6) 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 4.2%.

Sample	sCD44var(v6) Concentration (ng/mL)	Coefficient of Variation (%)
1	148	4.3
2	33	8.8
3	567	2.0
4	388	4.0
5	260	3.6
6	235	3.6
7	310	3.5
8	739	4.1

Recovery Studies

Spiked samples were prepared by adding four different levels of sCD44var(v6) to 2 human serum samples. As shown below, recoveries were determined in two independent experiments ranging from 89% to 115% with an overall mean recovery of 105%.

Experiment 1

sCD44var(v6) Base level (ng/mL)	Recovery (%) sCD44var(v6) Spike			
	400 ng	200 ng	100 ng	50 ng
217	114	101	92	89

Experiment 2

sCD44var(v6) Base level (ng/mL)	Recovery (%) sCD44var(v6) Spike			
	400 ng	200 ng	100 ng	50 ng
53	115	112	109	105

Dilution Parallelism

Four serum samples with different levels of sCD44var(v6) were assayed at four serial two-fold dilutions (1:100-1:800 covering the working range of the calibration curve). In the table below the percent recovery of expected values is listed. Recoveries ranged from 98% to 106% with an overall mean recovery of 102%.

Sample	Dilution	sCD44var(v6) Concentration (ng/mL) Expected Value	Observed Value	% Recovery of Exp. Value
1	1:100	---	603	---
	1:200	302	302	100
	1:400	151	153	101
	1:800	77	75	97
2	1:100	---	408	---
	1:200	204	211	103
	1:400	105	108	103
	1:800	54	57	105
3	1:100	---	265	---
	1:200	133	130	98
	1:400	65	66	101
	1:800	33	33	101
4	1:100	---	242	---
	1:200	121	120	99
	1:400	60	61	102
	1:800	30	32	106

Sample Stability

Freeze-Thaw Stability

Aliquots of serum samples were stored at -20°C and thawed several times and the sCD44var(v6) level determined. There was no significant loss of sCD44var(v6) concentrations between 0 and 5 freeze-thaw cycles.

Storage Stability

Aliquots of a serum sample were stored at -20°C, 4°C, RT and at 37°C and the sCD44var(v6) level determined after 24 hours. There was no significant loss of sCD44var(v6) immunoreactivity caused by 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 sCD44var(v6) determinations, although sCD44var(v6) levels in citrate and EDTA plasmas were slightly lower than serum levels. It is, therefore, highly recommended to assure the uniformity of sample preparations!

Specificity

This assay recognizes both natural and recombinant forms of the sCD44var(v6) molecule. To define the specificity of this ELISA, several structurally related and non-related polypeptides were tested for cross reactivity. Notably, there was no detectable cross reactivity with CD44-polypeptides lacking the protein sequence encoded by exon 6.

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 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.

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.
- Reagents containing thimerosal as preservative may be toxic if ingested.
- Avoid contact of substrate solutions 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 solutions 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
7-12	50	950

B. Assay Buffer

Number of Strips	Assay Buffer Concentrate (mL)	Distilled Water (mL)
1-6	2.5	47.5
7-12	5.0	95.0

C. Calibrator

Add distilled water as indicated on vial label to vial containing lyophilized Calibrator

D. HRP Conjugate

Add 190 µL Assay Buffer to tube containing HRP-Conjugate concentrate. Mix. Make further dilution according to table.

Number of Strips	1:20 Prediluted HRP-Conjugate (mL)	Assay Buffer (mL)
1-6	0.03	2.97
7-12	0.06	5.94

E. TMB Substrate Solution

Number of Strips	Substrate Solution I (mL)	Substrate Solution II (mL)
1-6	3.0	3.0
7-12	6.0	6.0

TEST PROTOCOL SUMMARY

- Predilute serum, plasma or urine samples with Sample Diluent 1:20 (10 µL serum + 190 µL Sample Diluent).
- Wash Microwell Strips twice with Wash buffer.
- Reconstitue lyophilized sCD44var(v6) Calibrator.
- Add 100 µL Sample Diluent to duplicate Calibrator wells.
- Pipette 100 µL sCD44var(v6) Calibrator into the first Calibrator wells and create Calibrator dilutions ranging from 10 to 0.32 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 (=1:100 final dilution).
- Prepare HRP-Conjugate.
- Add 50 µL diluted HRP-Conjugate to all wells, including blank wells.
- Cover microwell strips and incubate 3 hours at RT (18-25°C) on a rotator platform.
- Prepare TMB Substrate Solution a few minutes before use.
- Empty and wash microwell strips 3 times with Wash Buffer.
- Add 100 µL of mixed TMB Substrate Solution to all wells, including blank wells.
- Incubate the microwell strips for about 15 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, 1:100, the concentration read from the calibration curve must be multiplied by the dilution factor (x100). Calculation of samples with an O.D. exceeding 2.0 may result in incorrect low sCD44var(v6) levels. Such samples require further dilution of 1:200-1:400 with Sample Diluent in order to precisely quantitate their actual sCD44var(v6) levels. You can conclude from the OD how to further predilute your sample.

FOR RESEARCH USE ONLY

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