PRODUCT INFORMATION & MANUAL

Human sCD44var (v6) Instant ELISA

BMS210INST

Enzyme-linked immunosorbent assay for quantitative detection of human sCD44var (v6).
For research use only.
Not for diagnostic or therapeutic procedures.
128 Tests

eBioscience®
An Affymetrix Company

Human sCD44var Instant ELISA

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1 Intended Use

The human sCD44var (v6) Instant ELISA is an enzyme-linked immunosorbent assay for the quantitative detection of human sCD44var (v6). **The human sCD44var (v6) Instant ELISA is for research use only. Not for diagnostic or therapeutic procedures**

2 Summary

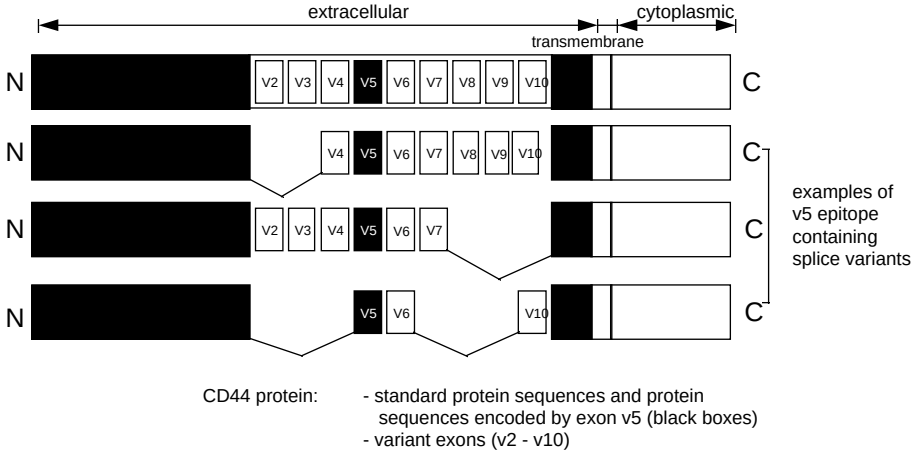
CD44 (Pgp-1; Ly-24; ECMR III; F10-44-2; H-CAM; HUTCH-I; In(Lu)-related p80; Hermes antigen; hyaluronan receptor) is a polymorphic glycoprotein which participates in a wide variety of cell-cell or cell-matrix interactions including lymphocyte homing, establishment of B and T cell immune responses, tumor 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), ephitelial cells (exons v8-v10), activated lymphocytes and macrophages (exon v6).

A splice variant of CD44 (exons v4-v7) confers metastatic behaviour 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. Detection of abnormal regulation of CD44 splicing thus could be helpful in cancer diagnosis and disease evaluation.

The sCD44var(v6) ELISA detects all circulating CD44 isoforms comprising the sCD44var(v6) sequences.



Determination of sCD44var(v6) 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.

- 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.

- 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.

- 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.

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

- 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.
- HIV: CD44 is almost completely depleted from the surface of HIV-infected cells.

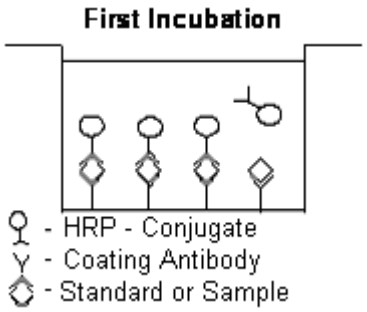
- inflammatory joint diseases: CD44 expression was decreased in synovial fluid neutrophils from most patients.

For literature update refer to **www.eBioscience.com**

3 Principles of the Test

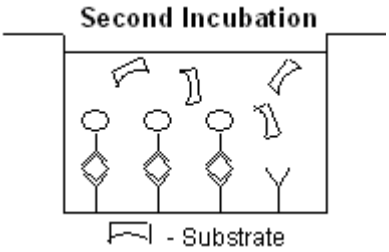
An anti-human sCD44var (v6) coating antibody is adsorbed onto microwells. Human sCD44var (v6) present in the sample or standard binds to antibodies adsorbed to the microwells; an HRP-conjugated anti-human sCD44var (v6) antibody binds to human sCD44var (v6) captured by the first antibody.

Figure 1



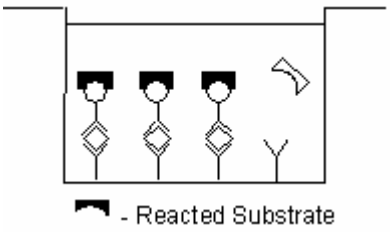
Following incubation unbound enzyme conjugated anti-human sCD44var (v6) is removed during a wash step and substrate solution reactive with HRP is added to the wells.

Figure 2



A coloured product is formed in proportion to the amount of human sCD44var (v6) present in the sample. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from 7 human sCD44var (v6) standard dilutions and human sCD44var (v6) sample concentration determined.

Figure 3



4 Reagents Provided

1 aluminium pouch with a **Microwell Plate coated** with monoclonal antibody to human sCD44var (v6), **HRP-Conjugate** (human sCD44var (v6) monoclonal antibody) and Sample Diluent, lyophilized

2 aluminium pouches with a human sCD44var (v6) **Standard curve** (coloured)

1 bottle (25 ml) **Wash Buffer Concentrate** 20x (phosphate-buffered saline with 1% Tween 20)

1 vial (50 ml) **Sample Diluent**

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

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

2 **Adhesive Films**

5 Storage Instructions

Store ELISA plate and Standard curves or whole kit at -20°C. The plate and the standard curves can also be removed, stored at -20°C, remaining kit reagents can be stored between 2° and 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 supernatant, serum, plasma (EDTA, citrate, heparin) 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.

Pay attention to a possible **“Hook Effect”** due to high sample concentrations (see chapter 11).

Samples must be stored frozen at -20°C to avoid loss of bioactive human sCD44var (v6). If samples are to be run within 24 hours, they may be stored at 2° to 8°C (for sample stability refer to 13).
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
- adjustable multichannel micropipettes (for volumes between 50 µl and 500 µl) 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 linear regression analysis

8 Precautions for Use

- All reagents 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 substrate reagent.

- 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 and Samples

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

9.1 Wash Buffer (1x)

Pour entire contents (25 ml) of the Wash Buffer Concentrate (20x) into a clean 500 ml graduated cylinder. Bring to final volume to 500 ml with glass-distilled or deionized water. Mix gently to avoid foaming.

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

10 Test Protocol

- **Use plate immediately after removal from -20°C!**
- **Do not wait until pellets have completely dissolved before applying samples - the binding reaction in the standard strips starts immediately after addition of water!**
- **Do not try to dissolve pellets by pipetting up and down in the wells - some parts of the pellet could stick to the tip creating high variation of results.**
- **Perform the washing step with at least 400 µl of washing buffer as stated in the manual or fill the wells completely - otherwise any pellet residues sticking to the rim of the well will not be removed and create high variation of results.**
- **Allow the washing buffer to sit in the wells for a few seconds before aspiration.**
- **Remove covers of the standard strips carefully so that all the lyophilized pellets remain in the wells.**

a. Prepare your samples before starting with the test procedure. Dilute serum or urine samples 1:20 with **Sample Diluent** according to the following dilution scheme:
10 µl sample + 190 µl Sample Diluent

b. Determine the number of microwell strips required to test the desired number of samples plus microwell strips for blanks and standards (coloured). Each sample, standard and blank should be assayed in duplicate. Remove extra microwell strips from holder and store in foil bag with the desiccant provided at -20°C sealed tightly. Place microwell strips containing the standard curve in position A1/A2 to H1/H2 (see Table 1).

c. Add **distilled water** to all **standard and blank wells** as indicated on the label of the standard strips (A1, A2 to H1, H2).

d. Add 130 µl of **distilled water** to the **sample wells**.

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 (10.00 ng/ml)	Standard 1 (10.00 ng/ml)	Sample 1	Sample 1
B	Standard 2 (5.00 ng/ml)	Standard 2 (5.00 ng/ml)	Sample 2	Sample 2
C	Standard 3 (2.50 ng/ml)	Standard 3 (2.50 ng/ml)	Sample 3	Sample 3
D	Standard 4 (1.25 ng/ml)	Standard 4 (1.25 ng/ml)	Sample 4	Sample 4
E	Standard 5 (0.63 ng/ml)	Standard 5 (0.63 ng/ml)	Sample 5	Sample 5
F	Standard 6 (0.32 ng/ml)	Standard 6 (0.32 ng/ml)	Sample 6	Sample 6
G	Standard 7 (0.16 ng/ml)	Standard 7 (0.16 ng/ml)	Sample 7	Sample 7
H	Blank	Blank	Sample 8	Sample 8

e. Add 20 µl of each 1:20 prediluted **sample**, in duplicate, to the **designated wells** and mix the contents.

f. Cover with an adhesive film and incubate at room temperature (18ºC to 25ºC) for 3 hours, if available on a microplate shaker at 400 rpm.

g. Remove adhesive film and empty wells. **Wash** the microwell strips 3 times 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, 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 no longer than 15 minutes. Do not allow wells to dry.

h. Pipette 100 µl of **TMB Substrate Solution** to all wells, including the blank wells.

i. 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.

j. 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.

k. 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 human sCD44var (v6) 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 human sCD44var (v6) 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 sCD44var (v6) 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 sCD44var (v6) concentration.

- ***Samples have been diluted 1:100 (1:20 external predilution, 1:5 dilution on the plate: 20 µl sample + 80 µl Sample Diluent), thus the concentration read from the standard curve must be multiplied by the dilution factor (x 100).**

- **Calculation of samples with a concentration exceeding standard 1 may result in incorrect, low human sCD44var (v6) levels (Hook Effect). Such samples require further external predilution according to expected human sCD44var (v6) values with Sample Diluent in order to precisely quantitate the actual human sCD44var (v6) level.**

- It is suggested that each testing facility establishes a control sample of known human 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 standard curve is shown in Figure 4. This curve cannot be used to derive test results. Every laboratory must prepare a standard curve for each group of microwell strips assayed.

* N.B: There is a common dilution factor for samples due to the conjugate which must then be included in the calculation. The samples contribute 100 µl to the final volume per well. These 100 µl are composed of 80 µl of Sample Diluent plus 20 µl of the 1:20 prediluted sample. This is a 1:100 dilution.
The remaining 50 µl to give 150 µl are due to the addition of 50 µl conjugate to all wells.
80 µl Sample Diluent and 50 µl conjugate results in 130 µl reconstitution volume, addition of 20 µl sample (80 µl + 20 µl of 1:20 prediluted sample= 1:100 dilution)

Figure 4

Representative standard curve for human sCD44var (v6) Instant ELISA. Human sCD44var (v6) was diluted in serial 2-fold steps in Sample Diluent. Each symbol represents the mean of 3 parallel titrations. Do not use this standard curve to derive test results. A standard curve must be run for each group of microwell strips assayed.

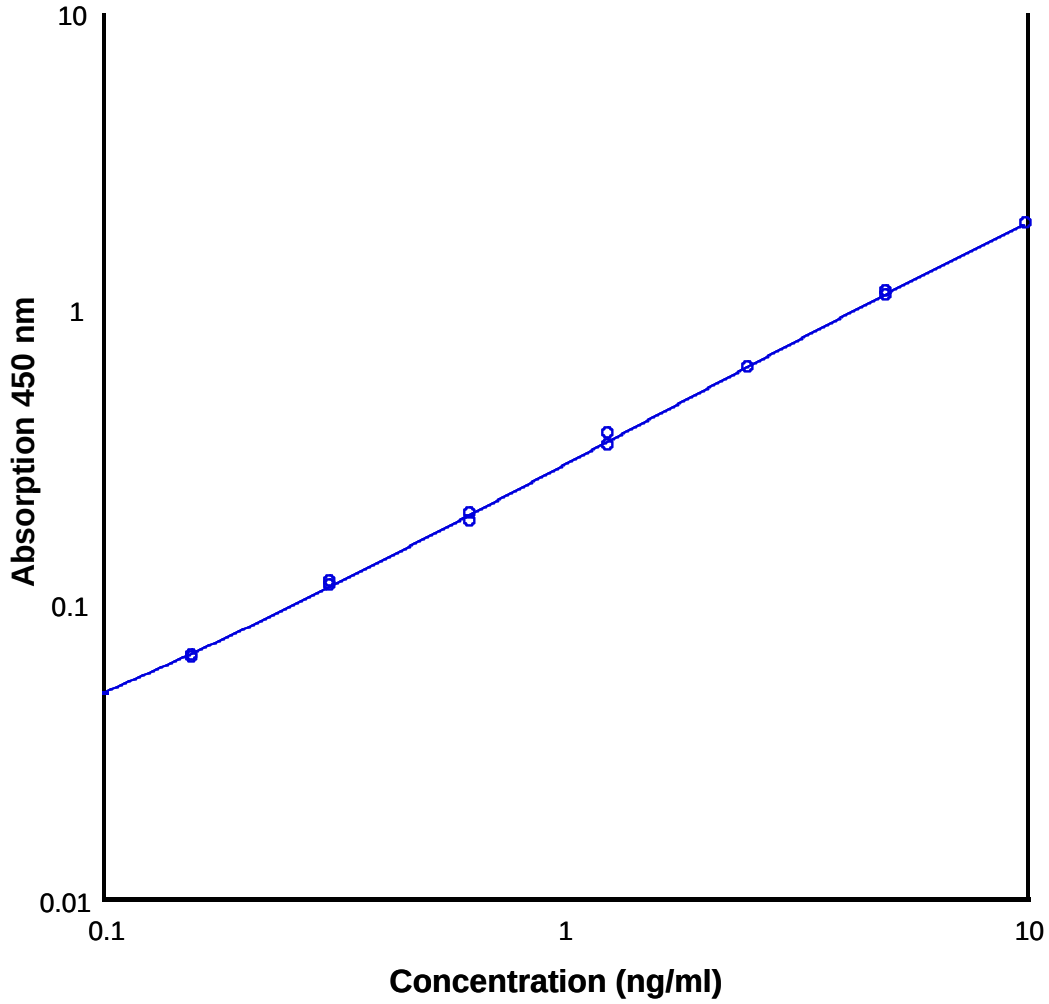


Table 2
Typical data using the human sCD44var (v6) INSTANT ELISA
Measuring wavelength: 450 nm
Reference wavelength: 620 nm

Standard	Human sCD44var (v6) Concentration (ng/ml)	O.D. (450 nm)	O.D. Mean	C.V. (%)
1	10.00	1.910	1.913	0.2
		1.915		
2	5.00	1.135	1.110	3.2
		1.085		
3	2.50	0.625	0.625	0.1
		0.624		
4	1.25	0.339	0.354	6.2
		0.370		
5	0.63	0.201	0.194	4.7
		0.188		
6	0.32	0.114	0.116	2.4
		0.118		
7	0.16	0.066	0.066	1.1
		0.065		
Blank	0.00	0.022	0.023	
		0.025		

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 sCD44var (v6) 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 0.06 ng/ml (mean of 6 independent assays).

13.2 Reproducibility

13.2.1 Intra-assay

Reproducibility within the assay was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 8 serum samples containing different concentrations of human sCD44var (v6). 2 standard curves were run on each plate. Data below show the mean human sCD44var (v6) concentration and the coefficient of variation for each sample (see Table 3). The calculated overall intra-assay coefficient of variation was 8.5%.

Table 3

The mean human sCD44var (v6) concentration and the coefficient of variation for each sample.

Positive Sample	Experiment	Human sCD44var (v6) Concentration (ng/ml)	Coefficient of Variation (%)
1	1	296	10
	2	224	2
	3	241	6
2	1	210	4
	2	182	12
	3	186	7
3	1	185	2
	2	181	12
	3	193	8
4	1	151	9
	2	118	13
	3	128	6
5	1	161	8
	2	124	9
	3	138	9
6	1	121	13
	2	100	12
	3	113	7
7	1	116	14
	2	91	12
	3	116	6
8	1	118	11
	2	111	7
	3	122	5

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 sCD44var (v6). 2 standard curves were run on each plate. Data below show the mean human sCD44var (v6) 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 10%.

Table 4
The mean human sCD44var (v6) concentration and the coefficient of variation of each sample

Sample	Mean Human sCD44var (v6) Concentration (ng/ml)	Coefficient of Variation (%)
1	254	14.7
2	193	8.1
3	187	3.3
4	132	13.0
5	141	13.4
6	111	9.7
7	108	13.2
8	117	4.8

13.3 Spike Recovery

The spike recovery was evaluated by spiking 4 levels of human sCD44var (v6) into serum. Recoveries were determined in 3 independent experiments with 6 replicates each. The unspiked serum was used as blank in these experiments.
Average recovery ranged from 75% to 113% with an overall mean recovery of 90%.

13.4 Dilution Parallelism

Serum samples with different levels of human sCD44var (v6) were analysed at serial 2 fold dilutions with 4 replicates each. The recovery ranged between 82% and 120% with an overall recovery of 104% (see Table 5).

Table 5

Sample	Dilution	Mean human sCD44var (v6) Concentration (n/ml)		% Recovery of Exp. Val.
		Expected Value	Observed Value	
1	1:100	--	718	--
	1:200	359	428	119
	1:400	214	236	110
	1:800	118	111	95
2	1:100	--	358	--
	1:200	179	199	112
	1:400	99	91	91
	1:800	46	45	99
3	1:100	--	444	--
	1:200	222	259	117
	1:400	129	143	111
	1:800	72	59	82
4	1:100	--	263	--
	1:200	132	144	110
	1:400	72	57	79
	1:800	28	34	120

13.5 Sample Stability

13.5.1 Freeze-Thaw Stability

Aliquots of serum samples (unspiked or spiked) were stored at -20°C and thawed 5 times, and the human sCD44var (v6) levels determined. There was no significant loss of human sCD44var (v6) immunoreactivity detected by freezing and thawing.

13.5.2 Storage Stability

Aliquots of serum samples (spiked or unspiked) were stored at -20°C, 2-8°C, room temperature (RT) and at 37°C, and the human sCD44var (v6) level determined after 24 h.
There was no significant loss of human sCD44var (v6) immunoreactivity detected during storage under above conditions.

13.6 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 human CD44var(v6) determinations, although human 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.

13.7 Specificity

The assay detects both natural and recombinant human sCD44var (v6). To define the specificity of this ELISA several proteins were tested for cross reactivity. There was no cross reactivity observed, notably not with human sCD44-polypeptides lacking the protein sequence encoded by exon 6.

13.8 Expected Values

A panel of 40 serum samples from randomly selected apparently healthy donors (males and females) was tested for human sCD44var (v6).
The detected human sCD44var (v6) levels ranged between 119 and 355 ng/ml with a mean level of 230 ng/ml and a standard deviation of 68 ng/ml.
The levels measured may vary with the sample collection used.

14 Ordering Information

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15 Reagent Preparation Summary

15.1 Wash Buffer (1x)

Add **Wash Buffer Concentrate** 20 x (25 ml) to 475 ml distilled water

16 Test Protocol Summary

- Predilute samples 1:20 Sample Diluent
- Place standard strips in position A1/A2 to H1/H2.
- Add distilled water, in duplicate, to all standard and blank wells as indicated on the label of the standard strips.
- Add 130 µl distilled water to sample wells.
- Add 20 µl of 1:20 prediluted sample to designated wells.
- Cover microwell strips and incubate 3 hours at room temperature (18° to 25°C) if available on a microplate shaker at 400 rpm.
- Empty and wash microwell strips 3 times with 400 µl Wash Buffer.
- Add 100 µl of TMB Substrate Solution to all wells including blank wells.
- Incubate the microwell strips for about 10 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: Samples have been diluted 1:100, thus the concentration read from the standard curve must be multiplied by the dilution factor (x 100)