ELISA PRODUCT INFORMATION & MANUAL

Human CD44 ELISA Kit (Colorimetric)

NBP1-87599

Enzyme-linked Immunosorbent Assay for quantitative detection. For research use only.
Not for diagnostic or therapeutic procedures.

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TABLE OF CONTENTS

1 Intended Use

The human CD44var (v6) ELISA is an enzyme-linked immunosorbent assay for the quantitative detection of human CD44var (v6). **The human CD44var (v6) 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.

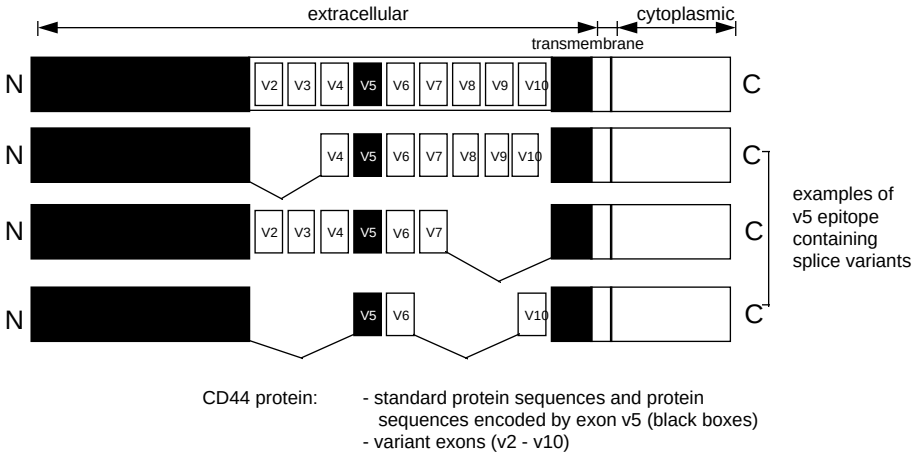
Three isoform categories of the CD44 molecule have been identified:

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.

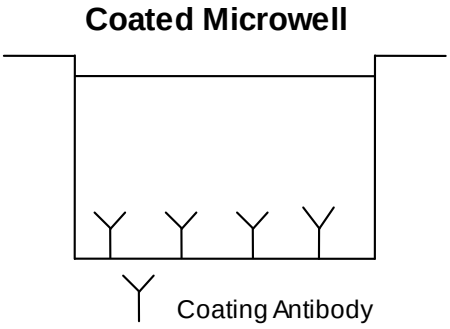
- brain tumors: CD44 is strongly expressed in high-grade gliomas and weakly expressed in meningiomas, medulloblastomas and normal brain.
- colorectal carcinomas: in human colorectal neoplasia CD44 variant proteins are found on all invasive carcinomas and during carcinoma metastasis. Variants are already 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.

3 Principles of the Test

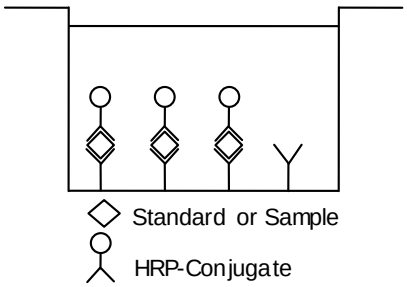
An anti-human CD44var (v6) coating antibody is adsorbed onto microwells.

Figure 1



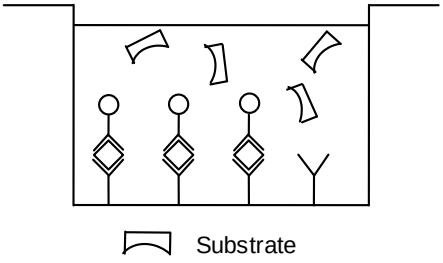
Human CD44var (v6) present in the sample or standard binds to antibodies adsorbed to the microwells and the HRP-conjugated anti-human CD44var (v6) antibody is added and binds to human CD44var (v6) captured by the first antibody.

Figure 2



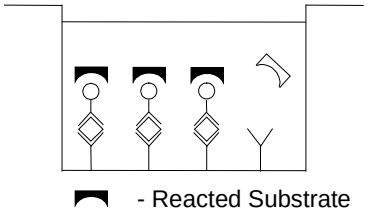
Following incubation unbound HRP-conjugated anti-human CD44var (v6) is removed during a wash step, and substrate solution reactive with HRP is added to the wells.

Figure 3



A coloured product is formed in proportion to the amount of human CD44var (v6) present in the sample or standard. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from 6 human CD44var (v6) standard dilutions and human CD44var (v6) concentration determined.

Figure 4



4 Reagents Provided

4.1 Reagents for human CD44var (v6) ELISA NBP1-87599 (96 tests)

1 aluminium pouch with a **Microwell Plate coated** with monoclonal antibody to human CD44var (v6)

2 vials (10 µl) **HRP-Conjugate** anti-human CD44var (v6) monoclonal antibody

2 vials human CD44var (v6) **Standard** lyophilized, 20 ng/ml upon reconstitution

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

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

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

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

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

2 **Adhesive Films**

5 Storage Instructions – ELISA Kit

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

6 Specimen Collection and Storage Instructions

Cell culture supernatant, serum, plasma (EDTA, citrate, heparin) 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.

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

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

Samples should be aliquoted and must be stored frozen at -20°C to avoid loss of bioactive human CD44var (v6). If samples are to be run within 24 hours, they may be stored at 2° to 8°C (for sample stability refer to 13.5).
Avoid repeated freeze-thaw cycles. Prior to assay, the frozen sample should be brought to room temperature slowly and mixed gently.

7 Materials Required But Not Provided

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

8 Precautions for Use

- All 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 statement(s) for specific advice.
- Reagents are intended for research use only and are not for use in diagnostic or therapeutic procedures.
- Do not mix or substitute reagents with those from other lots or other sources.
- Do not use kit reagents beyond expiration date on label.
- Do not expose kit reagents to strong light during storage or incubation.
- Do not pipette by mouth.
- Do not eat or smoke in areas where kit reagents or samples are handled.
- Avoid contact of skin or mucous membranes with kit reagents or specimens.
- Rubber or disposable latex gloves should be worn while handling kit reagents or specimens.
- Avoid contact of substrate solution with oxidizing agents and metal.
- Avoid splashing or generation of aerosols.
- In order to avoid microbial contamination or cross-contamination of reagents or specimens which may invalidate the test use disposable pipette tips and/or pipettes.
- Use clean, dedicated reagent trays for dispensing the conjugate and substrate reagent.

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

9 Preparation of Reagents

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

9.1 Wash Buffer (1x)

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

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

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

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

9.2 Assay Buffer (1x)

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

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

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

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

9.3 HRP-Conjugate

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

Dilute the **HRP-Conjugate** just prior to use by adding 760µl Assay Buffer (1x) to the tube containing the HRP-Conjugate concentrate. Mix the contents of the tube well.
Make a further 1:100 dilution with Assay Buffer (1x) in a clean plastic tube or reagent reservoir.

The second dilution (1:100) of the HRP-Conjugate may be prepared as needed according to the following table:

Number of Strips	Prediluted HRP-Conjugate (ml)	Assay Buffer (1x)(ml)
1 - 6	0.03	2.97
1 - 12	0.06	5.94

9.4 Human CD44var (v6) Standard

Reconstitute **human CD44var (v6) standard** by addition of distilled water.
Reconstitution volume is stated on the label of the standard vial. Swirl or mix gently to insure complete and homogeneous solubilization (concentration of reconstituted standard = 20 ng/ml).
Allow the standard to reconstitute for 10-30 minutes. Mix well prior to making dilutions.

After usage remaining standard cannot be stored and has to be discarded.

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

9.4.1 External Standard Dilution

Label 6 tubes, one for each standard point.

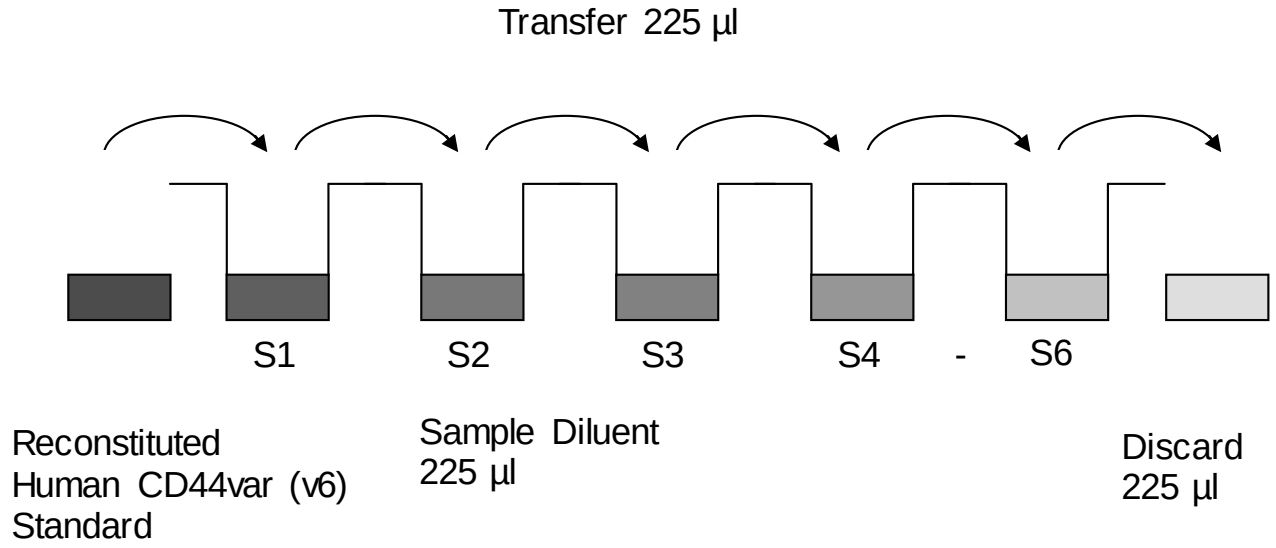
S1, S2, S3, S4, S5, S6

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

Pipette 225 µl of reconstituted standard (concentration of standard = 20 ng/ml) into the first tube, labelled S1, and mix (concentration of standard 1 = 10 ng/ml).
Pipette 225 µl of this dilution into the second tube, labelled S2, and mix thoroughly before the next transfer.
Repeat serial dilutions 4 more times thus creating the points of the standard curve (see Figure 5).

Sample Diluent serves as blank.

Figure 5


Transfer 225 µl
S1
S2
S3
S4
-
S6
Reconstituted Human CD44var (v6) Standard
Sample Diluent 225 µl
Discard 225 µl

10 Test Protocol

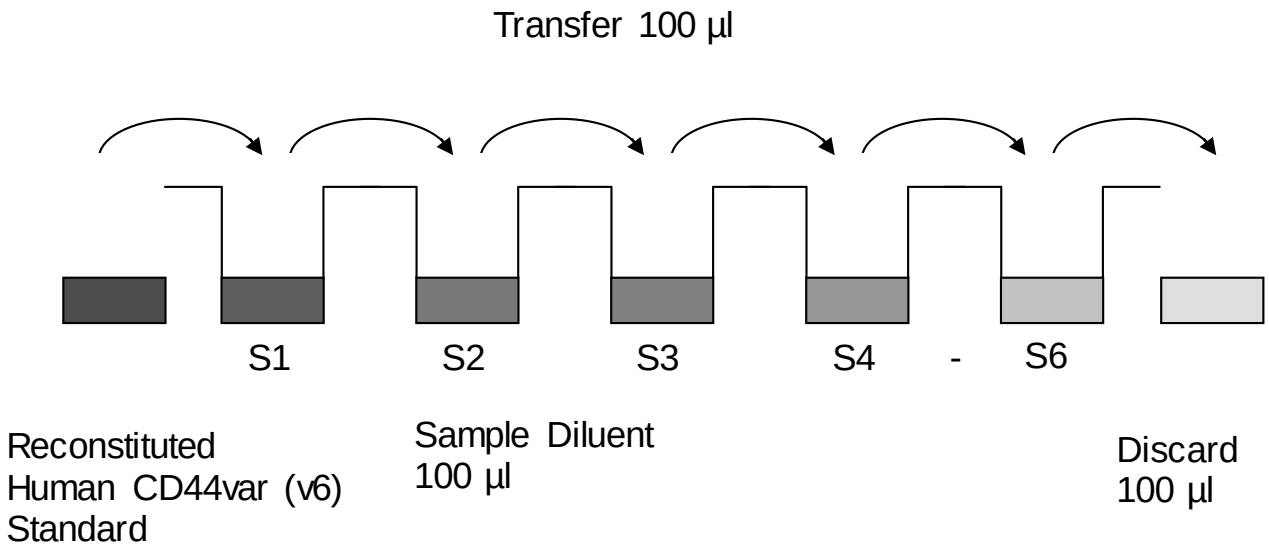
a. Predilute your samples before starting with the test procedure. Dilute serum, plasma and urine samples 1:20 with Sample Diluent according to the following scheme:
10 µl sample + 190 µl Sample Diluent

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

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

d. **Standard dilution on the microwell plate** (Alternatively the standard dilution can be prepared in tubes - see 9.4.1):
Add 100 µl of Sample Diluent in duplicate to **standard wells** A1/2-F1/2. Pipette 100 µl of prepared **standard** (see Preparation of Standard 9.4, concentration = 20.00 ng/ml) in duplicate into well A1 and A2 (see Table 1). Mix the contents of wells A1 and A2 by repeated aspiration and ejection (concentration of standard 1, S1 = 10.00 ng/ml), and transfer 100 µl to wells B1 and B2, respectively (see Figure 6). Take care not to scratch the inner surface of the microwells. Continue this procedure 4 times, creating two rows of human CD44var (v6) standard dilutions ranging from 10.00 to 0.32 ng/ml. Discard 100 µl of the contents from the last microwells (F1, F2) used.

Figure 6


Transfer 100 µl
S1
S2
S3
S4
-
S6
Reconstituted Human CD44var (v6) Standard
Sample Diluent 100 µl
Discard 100 µl

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

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

	1	**2**	**3**	**4**
A	Standard 1 (10.00 ng/ml)	Standard 1 (10.00 ng/ml)	Sample 2	Sample 2
B	Standard 2 (5.00 ng/ml)	Standard 2 (5.00 ng/ml)	Sample 3	Sample 3
C	Standard 3 (2.50 ng/ml)	Standard 3 (2.50 ng/ml)	Sample 4	Sample 4
D	Standard 4 (1.25 ng/ml)	Standard 4 (1.25 ng/ml)	Sample 5	Sample 5
E	Standard 5 (0.63 ng/ml)	Standard 5 (0.63 ng/ml)	Sample 6	Sample 6
F	Standard 6 (0.32 ng/ml)	Standard 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

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

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

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

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

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

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

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

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

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

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

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

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

o. Read absorbance of each microwell on a spectro-photometer using 450 nm as the primary wave length (optionally 620 nm as the reference wave length; 610 nm to 650 nm is acceptable). Blank the plate reader according to the manufacturer's instructions by using the blank wells. Determine the absorbance of both the samples and the standards.

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

11 Calculation of Results

- Calculate the average absorbance values for each set of duplicate standards and samples. Duplicates should be within 20 per cent of the mean value.
- Create a standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the human CD44var (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 CD44var (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 CD44var (v6) concentration.
- **If instructions in this protocol have been followed, samples have been diluted 1:100 (1:20 external predilution, 1:5 dilution on the plate: 20 µl sample + 80 µl Sample Diluent) and 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 CD44var (v6) levels (Hook Effect). Such samples require further external predilution according to expected human CD44var (v6) values with Sample Diluent in order to precisely quantitate the actual human CD44var (v6) level.**
- It is suggested that each testing facility establishes a control sample of known human CD44var (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 7. This curve cannot be used to derive test results. Each laboratory must prepare a standard curve for each group of microwell strips assayed.

Figure 7

Representative standard curve for human CD44var (v6) ELISA. Human CD44var (v6) was diluted in serial 2-fold steps in Sample Diluent. Do not use this standard curve to derive test results. A standard curve must be run for each group of microwell strips assayed.

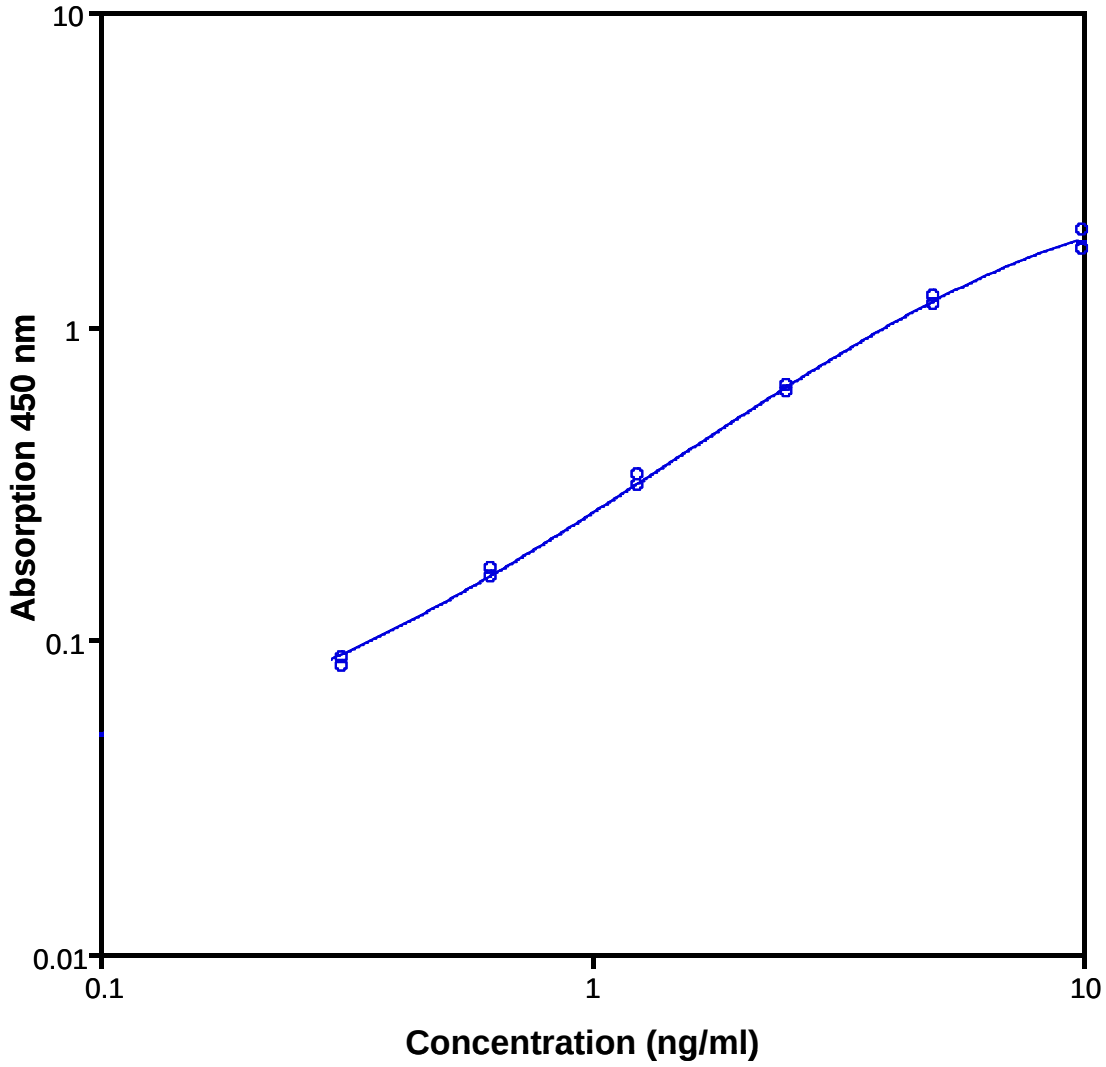


Table 2
Typical data using the human CD44var (v6) ELISA
Measuring wavelength: 450 nm
Reference wavelength: 620 nm

Standard	Human CD44var (v6) Concentration (ng/ml)	O.D. at 450 nm	Mean O.D. at 450 nm	C.V. (%)
1	10.00	1.749	1.884	7.2
		2.019		
2	5.00	1.246	1.203	3.6
		1.159		
3	2.50	0.638	0.628	1.7
		0.617		
4	1.25	0.334	0.323	3.6
		0.311		
5	0.63	0.168	0.164	2.8
		0.159		
6	0.32	0.087	0.085	3.0
		0.082		
Blank	0.00	0.010	0.013	16.5
		0.015		
		0.011		
		0.018		

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 CD44var (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 0.05 ng/ml (mean of 10 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 CD44var (v6). 2 standard curves were run on each plate. Data below show the mean human CD44var (v6) concentration and the coefficient of variation for each sample (see Table 3). The calculated overall intra-assay coefficient of variation was 3.0%.

Table 3
The mean human CD44var (v6) concentration and the coefficient of variation for each sample

Sample	Experiment	Mean Human CD44var (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

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 CD44var (v6). 2 standard curves were run on each plate. Data below show the mean human CD44var (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 4.2%.

Table 4
The mean human CD44var (v6) concentration and the coefficient of variation of each sample

Sample	Mean Human CD44var (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

13.3 Spike Recovery

The spike recovery was evaluated by spiking 4 levels of human CD44var (v6) into serum. Recoveries were determined in 2 independent experiments with 6 replicates each.
The amount of endogenous human CD44var (v6) in unspiked serum was subtracted from the spike values.
The recovery ranged from 89% to 115% with an overall mean recovery of 106% (see Table 5).

Table 5

Sample matrix	Spike high (%)	Spike medium (%)	Spike low (%)
Serum	115	107	97

13.4 Dilution Parallelism

Serum samples with different levels of human CD44var (v6) were analysed at serial 2 fold dilutions with 4 replicates each.
The recovery ranged from 98% to 106% with an overall recovery of 102% (see Table 6).

Table 6 shows detailed recovery data of 4 serum samples.

Table 6

Sample	Dilution	Expected Human CD44var (v6) Concentration (ng/ml)	Observed Human CD44var (v6) Concentration (ng/ml)	Recovery of Expected Human CD44var (v6) Concentration (%)
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

13.5 Sample Stability

13.5.1 Freeze-Thaw Stability

Aliquots of serum samples were stored at -20°C and thawed 5 times, and the human CD44var (v6) levels determined. There was no significant loss of human CD44var (v6) immunoreactivity detected by freezing and thawing.

13.5.2 Storage Stability

Aliquots of serum samples were stored at -20°C, 2-8°C, room temperature (RT) and at 37°C, and the human CD44var (v6) level determined after 24 h. There was no significant loss of human CD44var (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 CD44var (v6) determinations, although CD44var (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 CD44var (v6). The interference of circulating factors of the immune systeme was evaluated by spiking these proteins at physiologically relevant concentrations into positive serum.
There was no crossreactivity detected, notably not with CD44-polypeptides lacking the protein sequence encoded by exon 6.

13.8 Expected Values

A panel of 40 sera samples from randomly selected apparently healthy donors (males and females) was tested for human CD44var (v6).
The detected human CD44var (v6) levels ranged between 119.3 and 354.9 ng/ml with a mean level of 230.4 ng/ml and a standard deviation of 67.9 ng/ml.
The levels measured may vary with the sample collection used.

14 Reagent Preparation Summary

14.1 Wash Buffer (1x)

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

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

14.2 Assay Buffer (1x)

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

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

14.3 HRP-Conjugate

Make a predilution of the **HRP-Conjugate** by adding 760 µl Assay Buffer (1x). Make a further 1:100 dilution in Assay Buffer (1x):

Number of Strips	Prediluted HRP-Conjugate (ml)	Assay Buffer (1x) (ml)
1 - 6	0.03	2.97
1 - 12	0.06	5.94

14.4 Human CD44var (v6) Standard

Reconstitute lyophilized **human CD44var (v6) standard** with distilled water. (Reconstitution volume is stated on the label of the standard vial.)

15 Test Protocol Summary

1. Predilute sample with Sample Diluent 1:20.
2. Determine the number of microwell strips required.
3. Wash microwell strips twice with Wash Buffer.
4. Standard dilution on the microwell plate: Add 100 µl Sample Diluent, in duplicate, to standard wells A1/2 - F1/2. Pipette 100 µl prepared standard into the first wells and create standard dilutions by transferring 100 µl from well to well. Discard 100 µl from the last wells.
   Alternatively external standard dilution in tubes (see 9.4.1): Pipette 100 µl of these standard dilutions in the microwell strips.
5. Add 100 µl Sample Diluent in duplicate to the blank wells.
6. Add 80 µl Sample Diluent to sample wells.
7. Add 20 µl sample in duplicate, to designated sample wells.
8. Prepare HRP-Conjugate.
9. Add 50 µl HRP-Conjugate to all wells.
10. Cover microwell strips and incubate 3 hours at room temperature (18° to 25°C).
11. Empty and wash microwell strips 3 times with Wash Buffer.
12. Add 100 µl of TMB Substrate Solution to all wells.
13. Incubate the microwell strips for about 10 minutes at room temperature (18° to 25°C).
14. Add 100 µl Stop Solution to all wells.
15. Blank microwell reader and measure colour intensity at 450 nm.

Note: If instructions in this protocol have been followed, samples have been diluted 1:100 (1:20 external predilution, 1:5 dilution on the plate: 20 µl sample + 80 µl Sample Diluent) and the concentration read from the standard curve must be multiplied by the dilution factor (x 100).