KAMIYA BIOMEDICAL COMPANY

Human sCD44var (v6) ELISA

For the quantitative determination of human sCD44var (v6) in cell culture supernatants, serum, plasma, urine and other biological samples

Cat. No. KT-033

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

PRODUCT INFORMATION

Human sCD44var (v6) ELISA
Cat. No. KT-033

PRODUCT
The **K-*ASSAY*®** Human sCD44var (v6) ELISA is an enzyme-linked immunosorbent assay for the quantitative determination of human sCD44var (v6) in cell culture supernatants, serum, plasma, urine and other biological samples. 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).

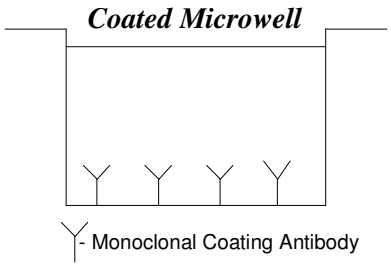
A splice variant of CD44 (exons v4-v7) confers metastatic behavior in a rat carcinoma model; aberrant expression of splice variants has been detected on a variety of human tumor cell lines as well as primary and metastatic human tumors, including lymphomas, carcinomas (colon, thyroid, mamma, bladder),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

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

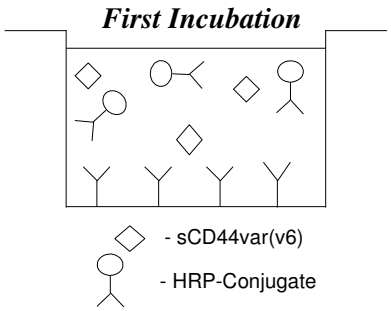
Determination of sCD44var (v6) will provide more detailed insight into different pathological modifications during cancer and other diseases.

PRINCIPLES OF THE TEST

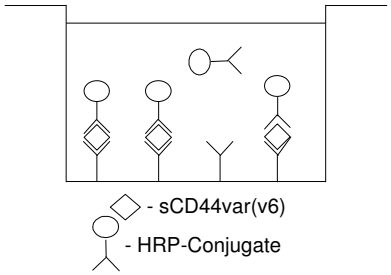
An anti-sCD44var (v6) coating antibody is adsorbed onto microwells.



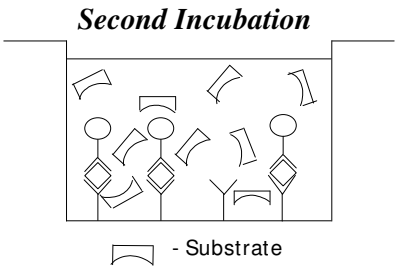
sCD44var (v6) present in the sample or calibrator binds to antibodies adsorbed to the microwells and the HRP-conjugated anti-human sCD44var (v6) antibody is added and binds to human sCD44var (v6) captured by the first antibody.

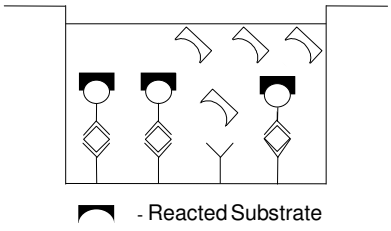


Following incubation unbound HRP-conjugated anti-human sCD44var (v6) is removed during a wash step, and substrate solution reactive with HRP is added to the wells.



A colored product is formed in proportion to the amount of human sCD44var (v6) present in the sample or calibrator. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A calibration curve is prepared from 6 human sCD44var (v6) calibrator dilutions and human sCD44var (v6) concentration determined.





COMPONENTS

- 1 aluminum pouch with a Microwell Plate coated with monoclonal antibody to human sCD44var (v6)
- 2 vials (10 µL) HRP-Conjugate anti-human sCD44var (v6) monoclonal antibody
- 2 vials human sCD44var (v6) Calibrator lyophilized, 20 ng/mL upon reconstitution
- 1 bottle (50 mL) Wash Buffer Concentrate 20X, 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
- 1 vial (15 mL) Substrate Solution
- 1 vial (15 mL) Stop Solution (1M Phosphoric acid)
- 2 adhesive Plate Seals

Materials or Equipment Required but Not Provided

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

STORAGE

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

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

SPECIMEN COLLECTION

Cell culture supernatants, 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 red 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.

Samples should be aliquoted and 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, the frozen sample should be brought to room temperature slowly and mixed gently.

Pay attention to a possible "Hook Effect" due to high sample concentrations.

PROTOCOLS

Preparation of Reagents

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

Wash Buffer

Pour entire contents (50 mL) of the **Wash Buffer Concentrate** (20X) into a clean 1,000 mL graduated cylinder. Bring to final volume of 1,000 mL with glass-distilled or de-ionized water. Mix gently to avoid foaming.

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

Wash Buffer may also be prepared as needed according to the following table:

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

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

Store at 4°C. Please note that the Assay Buffer (1X) is stable for 30 days.

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

Number of Strips	Assay Buffer Conc. (20X) (mL)	Distilled Water (mL)
1-6	2.5	47.5
1-12	5.0	95.0

HRP-Conjugate
Dilute the HRP-Conjugate just prior to use by adding 760 µL of (1X) 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 (1X) in a clean plastic tube or reagent reservoir.

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

The second dilution (1:100) of the HRP-Conjugate may be prepared 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

Calibrator
Reconstitute calibrator by addition of distilled water. Reconstitution volume is stated on the label of the calibrator vial. Swirl or mix gently to insure complete and homogeneous solubilization (concentration of reconstituted calibrator = 20 ng/mL). Allow the calibrator to reconstitute for 10-30 minutes. Mix well prior to making dilutions. After usage remaining calibrator cannot be stored and has to be discarded.

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 Calibrators. Each sample, Calibrator, blank and optional control sample should be assayed in duplicate. Remove extra Microwell Strips from holder and store in the foil bag with the desiccant provided at 4°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. Add 100 µL of Sample Diluent in duplicate to the Calibrator wells. Prepare Calibrator dilutions by pipetting 100 µL of sCD44var (v6) Calibrator (20 ng/mL), in duplicate, into well A1 and A2 (see Figure 1 and 2). Mix the contents of the

wells A1 and A2 by repeated aspiration and ejection and transfer 100 µL to well B1 and B2, respectively. Take care not to scratch the inner surface of the microwells. Continue this procedure 4 times, creating two 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:

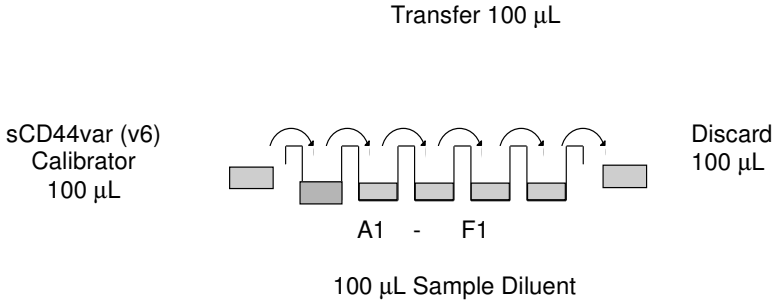


Figure 2. Table depicting an example of the arrangement of blanks, Calibrators and samples in the microwell strips:

	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

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.

i. Add 50 µL of HRP-Conjugate to all wells.

j. Cover with a Plate Seal and incubate at RT (18-25°C) for 3 hours on a rotator set at 400 rpm.

k. Remove Plate Seal 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 RT (18-25°C) for about 10 minutes. Avoid direct exposure to intense light.

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

It is recommended to add the Stop Solution when the highest calibrator has developed a dark blue color. Alternatively the color development can be monitored by the ELISA reader at 620 nm. The substrate reaction should be stopped as soon as Calibrator 1 has reached an OD of 0.9-0.95.

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 4°C in the dark.

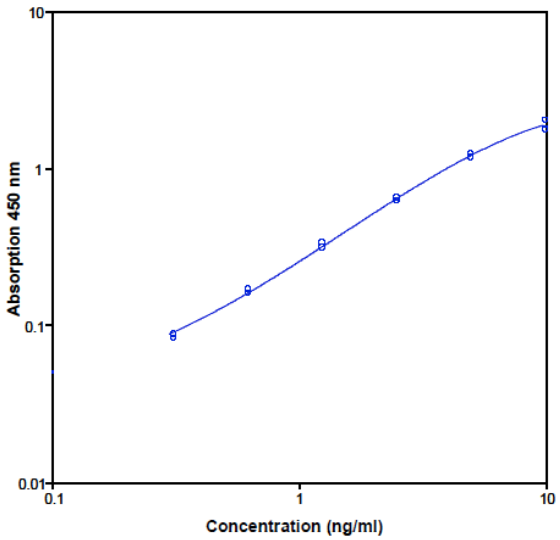
o. Read absorbance of each microwell on a spectrophotometer using 450 nm as the primary wavelength (optionally 620 nm as the reference wavelength; 610 nm to 650 nm is acceptable). Blank the plate reader according to the manufacturer's instructions by using the blank wells. Determine the absorbance of both the samples and the 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 (a 5-parameter curve fit is recommended).
- 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 according to the instructions given in this manual, 1:100, the concentration read from the calibration curve must be multiplied by the dilution factor (x 100).
- Calculation of samples with a concentration exceeding calibrator 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 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 below. This curve cannot be used to derive test results. Each laboratory must prepare a calibration curve for each group of microwell strips assayed.

Representative calibration curve for human sCD44var (v6) ELISA. Human sCD44var (v6) was diluted in serial 2-fold steps in Sample Diluent.



Typical Data Using the sCD44var (v6) ELISA

Measuring wavelength: 450 nm Reference wavelength: 620 nm

Calibrator	sCD44var (v6) Conc. (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	0.63	0.168	0.164	2.8
	0.63	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		

The OD values of the calibration curve may vary according to the conditions of assay performance (e.g. operator, pipetting technique, washing technique or temperature effects). Furthermore, shelf life of the kit may effect enzymatic activity and thus color intensity. Values measured are still valid.

LIMITATIONS

- Since exact conditions may vary from assay to assay, a calibration curve must be established for every run.
- Bacterial or fungal contamination of either screen samples or reagents or cross-contamination between reagents may cause erroneous results.
- Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergent before use.
- Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Completely empty wells before dispensing fresh Wash Buffer, fill with Wash Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.
- The use of radioimmunotherapy has significantly increased the number of patients with human anti-mouse IgG antibody (HAMA). HAMA may interfere with assays utilizing mouse monoclonal antibodies leading to both false positive and false negative results. Serum samples containing antibodies to mouse immunoglobulins can still be analyzed in such assays when mouse immunoglobulins (serum, ascitic fluid, or monoclonal antibodies of irrelevant specificity) are added to the sample.

PERFORMANCE CHARACTERISTICS

Sensitivity

The limit of detection for sCD44var (v6), defined as the analyte concentration resulting in an absorption significantly higher than that of the dilution medium (mean plus two standard deviations), was determined to be 0.05 ng/mL (mean of 10 independent assays).

Reproducibility

a. 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 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%.

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

b. 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 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

Spike Recovery

The spike recovery was evaluated by spiking 4 levels of human sCD44var (v6) into serum. Recoveries were determined in 2 independent experiments with 6 replicates each.
The amount of endogenous human sCD44var (v6) in unspiked serum was subtracted from the spike values.
The recovery ranged from 89% to 115% with an overall mean recovery of 106%.

Dilution Parallelism

Serum samples with different levels of sCD44var (v6) were assayed at serial two-fold dilutions with 4 replicates each. Recoveries ranged from 98% to 106% with an overall mean recovery of 102%.

		sCD44var(v6) Concentration (ng/mL)		
Sample	Dilution	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 5 times and the sCD44var (v6) level determined. There was no significant loss of sCD44var (v6) immunoreactivity detected by freezing and thawing.

Storage Stability

Aliquots of serum samples 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 detected during storage under above conditions.

Comparison of Serum and Plasma

Sera, as well as EDTA, citrate and heparin plasmas from 22 individuals were obtained at the same time. All these blood preparations were found suitable for sCD44var (v6) determinations, although sCD44var (v6) levels in citrate and EDTA plasma were slightly lower than serum levels. It is, therefore, highly recommended to assure the uniformity of sample preparations.

Specificity

This assay recognizes both natural and recombinant sCD44var(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.

PRECAUTIONS

- All reagents should be considered as potentially hazardous. We therefore recommend that only those persons who have been trained in laboratory techniques handle this product. It should be used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water.
- Reagents are intended for research use only and are not for use in diagnostic or therapeutic procedures.
- Do not mix or substitute reagents with those from other lots or other sources.
- Do not use kit reagents beyond expiration date on label.
- Do not expose kit reagents to strong light during storage or incubation.

- Do not pipette by mouth.
- Do not eat or smoke in areas where kit reagents or samples are handled.
- Avoid contact of skin or mucous membranes with kit reagents or specimens.
- Rubber or disposable latex gloves should be worn while handling kit reagents or specimens.
- Avoid contact of substrate solution with oxidizing agents and metal.
- Avoid splashing or generation of aerosols.
- In order to avoid microbial contamination or cross-contamination of reagents or specimens which may invalidate the test use disposable pipette tips and/or pipettes.
- Use clean, dedicated reagent trays for dispensing the conjugate and substrate reagents.
- Exposure to acids will inactivate the conjugate.
- Glass-distilled water or de-ionized water must be used for reagent preparation.
- Substrate solution must be at RT prior to use.
- Decontaminate and dispose specimens and all potentially contaminated materials as if they could contain infectious agents. The preferred method of decontamination is autoclaving for a minimum of 1 hour at 121.5°C.
- Liquid wastes not containing acid and neutralized waste may be mixed with sodium hypochlorite in volumes such that the final mixture contains 1.0 % sodium hypochlorite. Allow 30 minutes for effective decontamination. Liquid waste containing acid must be neutralized prior to the addition of sodium hypochlorite.

REAGENT PREPARATION SUMMARY

A. Wash Buffer

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

B. Assay Buffer

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

C. HRP Conjugate

Predilution: Add 760 µL Assay Buffer to tube containing HRP-Conjugate concentrate. Mix. Make further dilution according to table.

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

TEST PROTOCOL SUMMARY

- Predilute sample with Sample Diluent 1:20.
- Wash Microwell Strips twice with Wash Buffer.
- Add 100 µL Sample Diluent, in duplicate, to Calibrator wells.
- Pipette 100 µL sCD44var (v6) Calibrator into the first Calibrator wells and create Calibrator dilutions 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.
- Prepare HRP-Conjugate.
- Add 50 µL diluted HRP-Conjugate to all wells, including the blank wells.

- Cover microwell strips and incubate 3 hours at RT (18-25℃).
- Empty and wash microwell strips 3 times with Wash Buffer.
- Add 100 µL of TMB Substrate Solution to all wells, including blank wells.
- Incubate the microwell strips for approximately 10 minutes at RT (18-25℃).
- 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).

FOR RESEARCH USE ONLY

KAMIYA BIOMEDICAL COMPANY
12779 Gateway Drive, Seattle WA 98168
Tel: (206) 575-8068 Fax: (206) 575-8094
Email: LifeScience@k-assay.com
www.k-assay.com