

INSTRUCTIONS FOR USE

CA IX ELISA

Item No. 06490025

The logo for WILEX, featuring the word "WILEX" in a bold, black, sans-serif font. The letter "X" is stylized with a red triangle pointing upwards from its top-right corner.

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OncogeneScience

Manufactured by:
WILEX Inc.
Cambridge, MA 02140 USA

Intended Use

The CA IX ELISA is an enzyme-linked immunosorbent assay for the quantitative detection of the circulating form of the CA IX (Carbonic Anhydrase IX) protein in human serum and plasma.

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Method Principle	Solid Phase Sandwich ELISA
Analytical Range	0 pg/mL to 1500 pg/mL
Specimen Types	Human serum and plasma (EDTA, citrate, heparin)
Sample Test Volume	100.0 microliters of diluted sample per well
Sensitivity	2.5 pg/mL (<i>See Analytical Sensitivity, p. 24, for details</i>).
Purchase of this kit licenses its use under the following U.S. patents: 5,387,676; 5,981,711; and 6,004,535	

Table of Contents

Intended Use.....	1
Background	3
Principle of the Assay	6
Summary of Procedure.....	7
Materials Provided	8
Materials Required but Not Provided	10
Precautions and Recommendations	11
Sample Preparation	12
Detailed Protocol.....	13
Assay Procedure.....	15
2 Evaluation of Results	20
Sample Values	22
Assay Characteristics	24
Troubleshooting.....	27
Reagent Stability and Storage	28
References	29
Technical Support	Back Cover

Background

The transmembrane protein carbonic anhydrase IX (CAIX), also known as MN, is a member of the large family of carbonic anhydrase enzymes which share the ability to catalyze the reversible hydration of carbon dioxide to carbonic acid, leading to a decrease in pH [1]. Up-regulation of CA9 gene expression occurs in response to hypoxia via direct transcriptional activation by hypoxia inducible factor-1 alpha (HIF-1), and is believed to be involved in sensing and maintaining the acidic environment of hypoxic cells, particularly within the hypoxic regions of tumors [2,3].

Normal expression of CA IX protein is restricted primarily to gastric, intestinal, and liver mucosa [4,5]. Using IHC and RT-PCR, significant levels of CA IX protein have been detected in a variety of tumors, including those of the kidney, cervix, lung, bladder, colon, breast, liver, gall bladder, and pancreas [6]. In many of these malignancies, the presence of CAIX-positive cells is associated with earlier-stage disease. In bladder, gallbladder, and liver cancer, more CA IX staining was seen in noninvasive and dysplastic lesions than in later-stage, invasive tumors [7,8]. In breast cancer, 50% of DCIS (ductal carcinoma in situ) tumors versus 29% of invasive tumors had CA IX staining [9]. In colorectal cancer, CA IX was seen in 76% of polyps but in only 64% of carcinomas. Within the carcinomas, staining intensity was higher in the Dukes A/B than in C/D tumors. Staining levels were also higher in well-differentiated tumors [10]. In cervical neoplasms, greater than 90% stained positive for CA IX [11]. Women who had atypical cervical Pap smears and whose biopsy results showed neoplastic disease also had atypical cells in the Pap smear that stained positive for CAIX, while benign lesions showed no staining for CA IX [12]. In non-small-cell lung cancer, 80% of

tumors stained positive for CAIX, whereas all normal and dysplastic lesions were negative [13].

Many studies have analyzed CA IX levels in renal-cell carcinoma (RCC). Greater than 75% of RCC samples stain positive for CA IX by IHC or RT-PCR [14,15,16,17]. Ninety percent of all RCC are of the clear-cell type, and of these, greater than 90% are positive for CA IX [15,16,17]. Most studies were performed using tissue from the primary tumors; however, in a comparison of CA IX levels between matched primary and metastatic lesions, 11/15 patients had higher levels of CA IX in the primary lesion than in the metastatic tumor [16]. One study reported no CA IX staining in non-renal clear-cell tumors, suggesting CA IX may be a good biomarker for renal clear-cell adenocarcinoma [14].

Several groups have reported an association between CA IX levels in tumors and patient outcome. Intensity of CA IX expression by IHC in rectal tumors was found to be a predictor of disease-free survival, with strong staining of tumor cells indicating worse prognosis, and weak staining predicting longer disease-free survival [18]. In bladder cancer, higher levels of CA IX staining were seen in noninvasive versus invasive tumors, in low-grade versus high-grade tumors, and in metastases versus the corresponding primary tumor. There was a trend toward increased survival for patients with strong CAIX-expressing tumors due to the significantly higher levels of CA IX staining within the noninvasive, lower-grade tumors [7,19]. For patients with invasive disease, higher CA IX expression was a strong predictor of disease progression and worse recurrence-free and overall survival [19]. Lower levels of CA IX expression were observed in metastatic clear-cell renal tumors as compared to less advanced tumors, and within the sub-group with metastatic disease, higher CA IX levels were associated with increased survival [15,16].

Invasive breast cancer patients whose tumors had higher levels of CA IX had a higher relapse rate, worse overall survival, and the CA IX level was an independent predictor for overall survival (hazard ratio = 2.61) [20]. In premenopausal patients with invasive breast cancer who were treated with radiation, positive CA IX staining in tumors was significantly associated with worse relapse-free and overall survival [21]. CA IX levels and response to radiotherapy were analyzed in high-risk post-mastectomy patients who were either postmenopausal, had one to three positive nodes, or were estrogen-receptor positive. Within each sub-group, CA IX was an independent prognostic factor for overall survival [22]. Basal-like invasive breast cancer patients treated with chemotherapy had a significantly worse prognosis if their tumors were positive for CA IX [23]. In cervical cancer, CA IX level is a significant prognostic factor for disease-specific and metastasis-free survival, with higher levels indicating a worse prognosis [24]. In lung cancer, patients whose tumors expressed EGFr alone had a better prognosis than patients whose tumors expressed EGFr with CA IX and/or MMP-9 [25]. In breast cancer, using RT-PCR, CA IX levels had a significant positive association with HER-2/neu overexpression [26].

Investigation into using soluble CA IX as a biomarker has shown increased circulating CA IX levels in serum from metastatic breast and lung cancer patients and plasma from colon, ovarian, and metastatic renal-cell cancer when compared to levels in healthy individuals [27,28]. Elevated CA IX levels in pretreatment serum samples from metastatic breast cancer patients given hormone replacement therapy predicted shorter survival [29]. In metastatic breast cancer patients treated with Herceptin, elevated pretreatment serum CA IX levels are an independent predictor of reduced

disease-free and overall survival [30]. In patients with localized clear-cell renal cancer, plasma levels of CA IX decreased after removal of the tumor, whereas in patients with metastatic clear-cell, non-clear-cell, or benign kidney disease, CA IX levels did not decrease after surgery [31]. Elevated CA IX serum levels in pre-surgery serum samples from ovarian cancer patients were significantly associated with poor progression-free survival [32].

The CA IX ELISA is designed to provide a convenient, accurate, and reproducible method to determine CA IX levels in human serum or plasma. Comparison of results between studies using a common method such as the CA IX ELISA will contribute to consistency in observations and will aid in more clearly defining the role of CA IX in the development, treatment, and progression of cancer. In addition, the CA IX ELISA may be used in conjunction with immunoassays that detect other circulating oncoproteins such as HER-2/neu.

Principle of the Assay

The CA IX ELISA is a sandwich-type immunoassay that uses a mouse monoclonal Capture Antibody (V10) and a biotinylated mouse monoclonal antibody (M75) as detector [33]. The Capture Antibody has been immobilized on the interior surface of the microtiter plate wells. To perform the test, an appropriate volume of specimen is incubated in the wells to allow binding of the antigen by the Capture Antibody. The immobilized antigen is then exposed to the biotinylated Detector Antibody. A streptavidin-HRP Conjugate is then added. Addition of Substrate to the wells allows the catalysis of a chromogen into a colored product, the intensity of which is proportional to the amount of CA IX protein, also known as MN, that is bound to the plate.

Standards are provided in the kit that allow accurate, quantitative determinations of CA IX in suitable samples. Using a microtiter plate reader, one can measure simultaneously the absorbance of the colored product in the Standards and sample wells. Correlating the absorbance values of samples with the Standards allows the investigator to determine the levels of CA IX in a sample. Samples may be assigned a quantitative value of CA IX in picograms per mL (pg/mL) of serum and plasma.

For instructions, see the Detailed Protocol and Evaluation of Results sections of this booklet.

Summary of Procedure

Steps	Incubations
1. Add samples and Standards to wells	2 hours, RT* on shaker at 800 rpm
2. Wash	
3. Add Detector Antibody to wells	30 minutes, RT*
4. Wash	
5. Add Working Conjugate to wells	30 minutes, RT*
6. Wash	
7. Add Substrate to wells	25 minutes, RT*
8. Add Stop Solution to wells	
9. Read plate at 450 nm	

*Room temperature (20–27°C)

Materials Provided

The following components are supplied:

Microtiter plate—One (1) precoated microtiter plate supplied ready to use, with 96 wells (12 strips of eight) in a zip-lock bag with a desiccant pack. Wells are coated with an anti-human CA IX monoclonal antibody.

CA IX Standards—Six (6) separate vials containing 1 mL purified CA IX, supplied ready to use (do not dilute).

Standards are **labeled** with values that are two-fold greater than the actual vialled dosage. Assigning these label values to a standard curve obviates the need to correct the reported dose for the required 1:2 diluted sample (50% specimen in Sample Diluent). See Evaluation of Results section. Standards contain 0.09% sodium azide.

8

Standard#	Concentration (pg/mL)	Volume/Vial
L6	1500	1 mL
L5	1000	1 mL
L4	500	1 mL
L3	200	1 mL
L2	70	1 mL
L1	0	1 mL

Sample Diluent—One (1) 100-mL bottle containing BSA, mouse IgG, buffer salts, and 0.09% sodium azide.

Detector Antibody—One (1) 12-mL bottle containing biotinylated anti-CA IX antibody. Supplied ready to use.

Conjugate Concentrate—One (1) 0.4-mL vial containing 50X horseradish peroxidase-labeled streptavidin. Must be diluted to 1X in Conjugate Diluent to make Working Conjugate. See **Table 1**, p. 18.

Conjugate Diluent—One (1) 12-mL bottle of buffered solution (pH 7.0) containing BSA and 0.1% 2-Chloracetamide.

Substrate Solution—One (1) 12-mL bottle containing TMB Substrate. Supplied ready to use.

Stop Solution—One (1) 12-mL bottle containing 2.5 N Sulfuric Acid. Supplied ready to use.

Platewash Concentrate—One (1) 100-mL bottle. Dilute one (1) part concentrate in 19 parts high-quality deionized water prior to use.

Materials Required but Not Provided

- Barnstead Titer Plate Shaker (Model 4625), or equivalent—600–1000 rpm
- Pipettors: 20–200 μL and 200–1000 μL precision pipettors with disposable tips
- Precision manual or automated repeating pipettor for 5- and 10-mL pipets
- Disposable 5- and 10-mL pipets
- Wash bottle, multichannel dispenser, or automated 96-well microtiter plate washer
- Microcentrifuge and tubes for sample preparation
- 500-, 1000-, or 2000-mL graduated cylinder
- High-quality deionized water
- Tubes for sample preparation
- Vortex mixer
- Reagent reservoirs
- Plastic wrap or adhesive plate sealers
- Water bath
- Microtiter plate reader capable of measuring absorbance at a wavelength of 450 nm
- Liquid household bleach for inactivating clinical specimens and decontamination of plate washer
- Disposable paper towels, preferably lint-free

CA IX ELISA Controls—Controls have been developed to provide customers with control material for quality monitoring of day-to-day assay performance. Refer to CA IX ELISA Controls, Item No. 06490033, when ordering. Controls are supplied as liquid, ready to be diluted 1:2 for assay. Volumes are 1.0 mL each. Store undiluted CA IX ELISA Controls at 2–8°C.

Precautions and Recommendations

- Store components at 2–8°C, except for Platewash Concentrate, which can be stored at room temperature. Do not expose reagents to excessive light.
- Do not use kit components beyond the indicated kit expiration date.
- Open kit components are stable up to three months when stored as specified.
- Use only the microtiter wells provided with the kit.
- Do not mix reagents from different kits.
- The buffers and reagents used in this kit contain sodium azide as a preservative. Care should be taken to avoid direct contact with these reagents.
- Wear disposable gloves and eye protection when handling Stop Solution (2.5 N Sulfuric Acid).
- Do not mouth pipet or ingest any of the reagents.
- Do not smoke, eat, or drink when performing the assay or in areas where samples or reagents are handled.

- Human samples may be contaminated with infectious agents. Do not ingest, expose to open wounds, or breathe aerosols. Wear protective gloves and dispose of biological samples properly.

Sample Preparation

Suitable samples for analysis by the CA IX ELISA include human plasma treated with heparin, citrate, or EDTA, and human serum. Due to possible interfering factors, special care must be taken in the preparation and assay of human serum and plasma. Remove any flocculent material from samples by microcentrifugation prior to dilution. Prior to assay, predilute serum and plasma 1:2 in Sample Diluent as described.

SERUM OR PLASMA

The initial concentration of the serum or plasma sample to be examined should not exceed a concentration of 50% (a 1:2 dilution of sample in Sample Diluent). For example, 0.150 mL of sample may be diluted into 0.150 mL of Sample Diluent and 100 μ L added to the microtiter plate wells.

Detailed Protocol

RECOMMENDED PROCEDURES

1. Addition of reagents must be in the order specified.
2. All six Standards and the diluted Controls and test specimens should be run in duplicate. Change tips between each sample, Control, and Standard. Avoid carryover of one Standard into another.
3. Equilibrate all reagents to room temperature (20–27°C) prior to use.
4. CAUTION: When inverting the microtiter plate to decant or blot, press the side tabs of the frame inward to prevent the strips from falling out. If performing a partial plate assay, fill the open portion of the frame with uncoated or used strips when 96-port mechanical washers are used.
5. Preparation of Platewash
 - a. If the Platewash Concentrate is cold, allow it to reach room temperature (20–27°C) before use (about 45 minutes). Make sure all crystals are dissolved. If necessary, warm at 37°C and swirl to mix.
 - b. Dilute one (1) volume of Platewash Concentrate with 19 volumes of distilled or deionized water. Mix well. This solution is Platewash. The total volume required will depend on the washing method/instrument used. Approximately 1 L of this solution is required to prime an automated washer and run one microtiter plate; about 700 mL is required for each microtiter plate when manual washing is performed.

- c. Platewash must be freshly prepared each day. Do not store Platewash.
6. Microtiter plate washing may be automated, semi-automated, or manual, but must be carried out with care to ensure optimal performance of the assay. Plate washing equipment must be properly adjusted, cleaned, and maintained. Whichever method is used, the solution used to wash plates is Platewash.
 - a. Automatic Microtiter Plate Washer—Set the fill volume to 300 μL /well. Prime the instrument with Platewash. For washing the plates, use two 3-cycle washes. After the initial 3-cycle wash, rotate the plate 180° and repeat.
 - b. Manual Microtiter Plate Washer—Wash six times, using 300 μL per well per wash. Fill the entire plate, then aspirate in the same order.
 - c. Hand-Held Syringe—Wash six times, using 300 μL per well per wash. Blot the plate upside-down between washes.

After the final wash, invert the microtiter plate and tap it on an absorbent surface to remove excess liquid. Wells should not be completely dry. Residual liquid protects the bound reagents from desiccation. This is particularly important for maintaining enzyme activity.

7. The transfer of diluted samples and Controls from tubes to the microtiter wells can be greatly simplified by using semi-automated pipets. The hand-held expandable and programmable 8-place pipet, Impact EXP® (Matrix Technologies Corp., Hudson, NH), is ideal for this purpose.

Assay Procedure

The CA IX ELISA is provided with removable strips of wells so the assay can be carried out on multiple occasions. A standard curve must be included in each separate assay. Standards and diluted Controls and samples should be assayed in duplicate. Disposable pipet tips and clean reagent troughs must be used for all transfers to avoid cross-contamination of reagents and samples.

IMPORTANT: Warm kit reagents to room temperature before addition to assay plate wells.

1. Cut the foil pouch between the notches at the zip-lock end, break the zip-lock seal, and remove the plate from the foil pouch. Select the number of 8-well strips needed. Remove unused strips from the frame and return to the pouch. Seal the zip lock and store at 2–8°C. (Save the frame for future assays.)
2. Dilute specimens and Controls 1:2 using the Sample Diluent. The Standards are provided ready to use; do not dilute them.
3. Pipet 100 μ L of each of the six **undiluted** CA IX Standards (L1 to L6) and each of the diluted Controls and samples into appropriate wells. Use clean pipet tips for each of the samples, Controls, and Standards. Add Standard L1 to one additional well to be used for determination of Substrate blank.

4. Cover wells with clean plastic wrap or plate sealer. **Incubate plate for two hours at RT (20–27°C) on a titer plate shaker at ~ 800 rpm.** CAUTION: When the shaker speed is too high, there is a potential of cross-contaminating plate wells and creation of aerosols when removing the plate seal.
5. Prepare a working solution (1X) of Platewash. Add one (1) part Platewash Concentrate to 19 parts of deionized water. Mix well.
6. Carefully remove the plastic wrap or plate sealer. Take appropriate precautions to prevent contamination from aerosols if splashing onto the sealer has occurred. Wash wells for six cycles using 300 μ L per well of Platewash. (Wash for three cycles, rotate the plate 180°, and wash for three more cycles.) After the final wash, invert the plate and tap it on an absorbent surface to remove excess liquid. CAUTION: Do not allow plates to dry out. Proceed immediately to Step 7.
7. Pipet 100 μ L of Detector Antibody into all wells **except the Substrate blank well.** Cover the wells with a fresh piece of plastic wrap or plate sealer. **Incubate plate at room temperature (20–27°C) for 30 minutes.**
8. Prepare Working Conjugate by diluting an appropriate volume of Conjugate Concentrate into Conjugate Diluent (1:50 dilution). Refer to **Table 1**, p. 18, for volumes appropriate for the number of strips used.

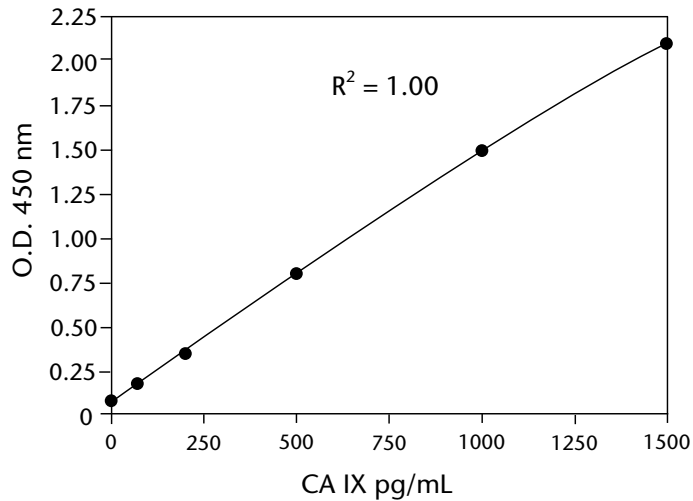
9. Wash wells as in Step 6. CAUTION: Do not allow plates to dry out. Proceed immediately to Step 10.
10. Pipet 100 μL of Working Conjugate into all wells **except the Substrate blank well**. Cover the wells with a fresh piece of plastic wrap or plate sealer. **Incubate plate at room temperature (20–27°C) for 30 minutes.**
11. Dispense Substrate into a clean reagent trough and do not dispense more than needed. Pipet 100 μL into each well. Discard unused Substrate.
12. Wash wells as in Step 6. CAUTION: Do not allow plates to dry out. Proceed immediately to Step 13.
13. Pipet 100 μL of Substrate into all wells and cover the plate with plastic wrap or plate sealer. **Incubate plate at room temperature (20–27°C) for 25 minutes.**
14. Pipet 100 μL of Stop Solution into all wells.
15. Measure absorbance in each well using a spectrophotometric plate reader at a wavelength of 450 nm. Wells should be read within 30 minutes of adding the Stop Solution.

TABLE 1. CA IX ELISA—PREPARATION OF WORKING CONJUGATE

# Strips Used	Conj. Concentrate	Conj. Diluent
1	20 μ L	0.98 mL
2	40 μ L	1.96 mL
3	60 μ L	2.94 mL
4	80 μ L	3.92 mL
5	100 μ L	4.90 mL
6	120 μ L	5.88 mL
7	140 μ L	6.86 mL
8	160 μ L	7.84 mL
9	180 μ L	8.82 mL
10	200 μ L	9.80 mL
11	220 μ L	10.78 mL
12	240 μ L	11.76 mL

Figure 1

Sample Standard Curve



Evaluation of Results

1. Average the absorbance values for each Standard, Control, and all sample dilutions to obtain the mean absorbance.
2. Do not assign “blank” wells using software. This will subtract the average blank readings from all other wells. The Substrate blank should be labeled as a sample so no subtraction occurs. It is useful for quality control and troubleshooting purposes to be able to inspect the absorbance values reported for the Substrate blank without adjustments applied to the raw data.
3. Design a standard curve (**Figure 1**, p. 19) by plotting the mean absorbance for each Standard on the y-axis versus the concentration of the Standards on the x-axis. There are a variety of microtiter plate reader software packages available for analysis of microtiter plate data (SoftMax Pro™, Molecular Devices Corporation, Sunnyvale, CA; KC4™, Bio-Tek Instruments, Inc., Winooski, VT) that simplify this process. The standard curve concentrations are assigned the value indicated on the vial. See next page. Use a quadratic curve fit algorithm (second order polynomial).

Standard#	Assigned Value (pg/mL)
L6	1500
L5	1000
L4	500
L3	200
L2	70
L1	0

- Results for samples and Controls are expressed in pg/mL by reading directly from the standard curve concentrations as designated on the vials (see above). **For convenience, no mathematical dilution correction is needed for 1:2 diluted samples since the actual concentration in the Standard preparations is at 50% of the labeled dosage** (i.e., they have been prediluted at 1:2).
- For samples that give absorbance (OD) values exceeding the range of the standard curve, a subsequent assay at greater dilutions will be necessary. Any such sample result will require correcting the value obtained from the assay for any dilution beyond 1:2.

Example:

Sample Dilution	Dilution Correction Factor (multiply reported result by)
1:4	2
1:8	4
1:16	8

Sample Values

The levels listed in the following tables should be used as a guideline only. The determination of normal ranges should be carried out by each laboratory using appropriate samples.

CA IX IN NORMAL HUMAN SERUM AND EDTA PLASMA SAMPLES (pg/mL)

22

	Serum (N=34)	Plasma (N=10)
Range	25–1795	58–432
Mean	269	178
Mean+2SD	648	397
95% fall below	678	280

CA IX IN MATCHED NORMAL HUMAN SERUM AND PLASMA SAMPLES (N=10)

Sample	Serum (pg/mL)	Plasma (pg/mL)
1	136	142
2	166	172
3	175	183
4	37	62
5	323	280
6	106	161
7	54	58
8	121	124
9	150	164
10	398	432

CA IX IN MATCHED ANTI-COAGULANT PLASMA SAMPLES (N=5)

Sample ID	Heparin (pg/mL)	Citrate (pg/mL)	EDTA (pg/mL)
11	284	218	257
12	267	241	263
13	195	158	171
14	339	304	279
15	194	167	166

Assay Characteristics

ANALYTICAL SENSITIVITY

A minimal detectable concentration of analyte was determined by repeated measurement of a zero dose sample (Standard Level 1) and calculation of mean + 2 standard deviations. The CA IX ELISA will detect 5.0 pg/mL of CA IX when read off a curve using the values indicated on Standards labels and in this booklet. Because the Standards are labeled with values that are two-fold greater than the actual vialled dosage (see Materials Provided—Standards), the actual analytical sensitivity of the CA IX ELISA is 2.5 pg/mL.

ANALYTICAL SPECIFICITY

Cross-reactivity: CA II and CA XII are two related members of the carbonic anhydrase family. The CA IX ELISA was challenged with high levels of each analyte. Both enzymes showed less than 5% cross-reactivity with CA IX.

POTENTIAL INTERFERING SUBSTANCES

Serum samples with high and low concentrations of cholesterol, triglycerides, T. bilirubin, and RF+ were run on the assay with and without added 500 pg/mL CA IX. There did not appear to be any loss in recovery due to elevated levels of the above interferents.

PRECISION

1. Inter-Assay

Normal human serum and plasma were spiked with CA IX at three different concentrations and tested in four assays with eight replicates per test point. Between assay variability was less than 12.0%.

Sample	High	Medium	Low
n	32	32	32
Serum Mean (pg/mL)	1391	768	381
Plasma Mean (pg/mL)	1521	1046	734
Serum Inter-Assay % C.V.	10.2	11.3	9.1
Plasma Inter-Assay % C.V.	9.4	10.3	8.0

2. Intra-Assay

Normal human serum and plasma were spiked with CA IX at three different concentrations and tested in four assays with eight replicates per test point. The intra-assay variability was less than 10.0%.

Sample	High	Medium	Low
n	8	8	8
Serum Mean (pg/mL)	1269	778	382
Plasma Mean (pg/mL)	1461	1153	725
Serum Intra-Assay % C.V.	8.6	9.9	6.2
Plasma Intra-Assay % C.V.	9.6	7.3	4.4

PARALLELISM

Parallelism tests the ability of the CA IX ELISA to report accurate values for serial dilutions of samples. Serum and plasma samples were initially diluted 1:2 as per protocol. Subsequent two-fold dilutions were made in Sample Diluent, and all dilutions were tested in the CA IX ELISA. Values (pg/mL) for each dilution were calculated by correcting with the appropriate dilution factor. Results show that plasma and serum samples diluted in Sample Diluent result in accurate values of CA IX.

26

Dilution	Plasma (EDTA)		Serum	
	OD	pg/mL	OD	pg/mL
1:2	1.805	1028	1.761	998
1:4	1.055	1095	1.035	1072
1:8	0.617	1167	0.582	1086

Troubleshooting

A. ASSAY DOES NOT DEVELOP COLOR OR ODs ARE LOW

- Plate allowed to dry out after Conjugate step.
- Step(s) omitted or in wrong sequence.
- Room temperature during incubations fell below 20°C.
- Substrate was not warmed to room temperature (20–27°C) prior to addition to wells.
- Sample incubation without using the plate shaker at 800 rpm (600–1000 rpm suitable) will lead to lower ODs.

B. HIGH BACKGROUND SIGNAL

- Insufficient washing between steps.
- The Substrate blank well should read ≤ 0.06 absorbance units. Zero Standard should read ≤ 0.13 absorbance units. Higher readings indicate deterioration of Substrate or exposure of Substrate to light before or during the incubation step.
- Be certain the plate is read at the correct wavelength.

C. POOR DUPLICATES

- Insufficient washing, especially when accompanied by high background. Take special care when washing plates by hand, or have automatic washer serviced.

- Tilting the plate during sample incubation will lead to high CVs. Use a plate shaker that shakes in one plane, not a type that tilts plates during the rotation step.
- Sporadic high signals may indicate contamination of Substrate by Conjugate. Be sure to use a fresh piece of plastic wrap or adhesive plate sealer for this step. Residual droplets of Conjugate on re-used plastic wrap or adhesive plate sealer may lead to false positive signals.
- Generating bubbles in wells on addition of reagents. Use care in pipetting.
- Splashing of reagents between wells will lead to erroneous results. Avoid jarring the plate.

Reagent Stability and Storage

All of the reagents included with the CA IX ELISA have been tested for stability. Reagents should not be used beyond the stated expiration date. Kit reagents, with the exception of Platewash Concentrate, should be stored at 2–8°C. Coated assay plates should be stored in the original foil bag sealed by the zip lock and containing a desiccant pack. Platewash Concentrate can be stored at room temperature (20–27°C). Opened kits should be used within three months of opening.

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