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ab264617
Human CKM SimpleStep ELISA® Kit

For the quantitative measurement of CKM in human serum, plasma, urine, and cell and tissue extract.

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1. Overview

CKM *in vitro* SimpleStep ELISA® (Enzyme-Linked Immunosorbent Assay) kit is designed for the quantitative measurement of CKM protein in human serum, plasma, urine, and cell and tissue extract.

The SimpleStep ELISA® employs an affinity tag labeled capture antibody and a reporter conjugated detector antibody which immunocapture the sample analyte in solution. This entire complex (capture antibody/analyte/detector antibody) is in turn immobilized via immunoaffinity of an anti-tag antibody coating the well. To perform the assay, samples or standards are added to the wells, followed by the antibody mix. After incubation, the wells are washed to remove unbound material. TMB Development Solution is added and during incubation is catalyzed by HRP, generating blue coloration. This reaction is then stopped by addition of Stop Solution completing any color change from blue to yellow. Signal is generated proportionally to the amount of bound analyte and the intensity is measured at 450 nm. Optionally, instead of the endpoint reading, development of TMB can be recorded kinetically at 600 nm.

Creatine kinase (CK), also known as creatine phosphokinase (CPK) or phospho-creatine kinase, is an enzyme that catalyzes the reversible transfer of a high-energy phosphate group between phosphocreatine and ATP. There are five known CK isozymes, three located in the cytosol (CKMM, CKMB, CKBB) and two in the mitochondria (MiSCK and MiuCK). CK isoenzymes allow for the generation of a large pool of phosphocreatine, considered to be a temporal and spatial ATP buffering system in tissues with large, fluctuating energy demands, such as skeletal muscle, heart, brain and spermatozoa. High levels of serum CKM can be found in inherited myophathies such as Duchenne and Becker muscular dystrophy and other acquired pathologies such as rhabdomyolysis, malignant hyperthermia, inflammatory myophaties, severe trauma and acute renal failure. This kit is developed to specifically recognize only CKM.

2. Protocol Summary

Prepare all reagents, samples, and standards as instructed

⇩

Add 50 µL standard or sample to appropriate wells

⇩

Add 50 µL Antibody Cocktail to all wells

⇩

Incubate at room temperature for 1 hour

⇩

Aspirate and wash each well three times with 350 µL 1X Wash Buffer PT

⇩

Add 100 µL TMB Development Solution to each well and incubate for 10 minutes.

⇩

Add 100 µL Stop Solution and read OD at 450 nm

3. Precautions

Please read these instructions carefully prior to beginning the assay.

- All kit components have been formulated and quality control tested to function successfully as a kit.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens and should be handle with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

4. Storage and Stability

Store kit at +4°C immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Refer to list of materials supplied for storage conditions of individual components.

5. Limitations

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

6. Materials Supplied

Item	Quantity	Storage Condition
Human CKM Capture Antibody 10X	600 µL	+4°C
Human CKM Detector Antibody 10X	600 µL	+4°C
Human CKM Lyophilized Recombinant Protein	2 Vials	+4°C
Antibody Diluent 5BI	6 mL	+4°C
Cell Extraction Buffer PTR 5X	10 mL	+4°C
Sample Diluent NS	50 mL	+4°C
Wash Buffer PT 10X	20 mL	+4°C
TMB Development Solution	12 mL	+4°C
Stop Solution	12 mL	+4°C
SimpleStep Pre-Coated 96-Well Microplate	96 Wells	+4°C
Plate Seal	1	+4°C

7. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform this assay:

- Microplate reader capable of measuring absorbance at 450 or 600 nm.
- Method for determining protein concentration (BCA assay recommended).
- Deionized water.
- Multi- and single-channel pipettes.
- Tubes for standard dilution.
- Plate shaker for all incubation steps.
- Optional: Phenylmethylsulfonyl Fluoride (PMSF) (or other protease inhibitors).

8. Technical Hints

- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Complete removal of all solutions and buffers during wash steps is necessary to minimize background.
- As a guide, typical ranges of sample concentration for commonly used sample types are shown below in Sample Preparation (section 11).
- All samples should be mixed thoroughly and gently.
- Avoid multiple freeze/thaw of samples.
- Incubate ELISA plates on a plate shaker during all incubation steps.
- When generating positive control samples, it is advisable to change pipette tips after each step.

- To avoid high background always add samples or standards to the well before the addition of the antibody cocktail.
- This kit is sold based on number of tests. A ‘test’ simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.

9. Reagent Preparation

- Equilibrate all reagents to room temperature (18-25°C) prior to use. The kit contains enough reagents for 96 wells. The sample volumes below are sufficient for 48 wells (6 x 8-well strips); adjust volumes as needed for the number of strips in your experiment.
- Prepare only as much reagent as is needed on the day of the experiment. Capture and Detector Antibodies have only been tested for stability in the provided 10X formulations.

9.1 1X Cell Extraction Buffer PTR (For cell and tissue extracts only):

Prepare 1X Cell Extraction Buffer PTR by diluting Cell Extraction Buffer PTR 5X to 1X with deionized water. To make 10 mL 1X Cell Extraction Buffer PTR combine 8 mL deionized water and 2 mL Cell Extraction Buffer PTR 5X. Mix thoroughly and gently. If required protease inhibitors can be added.

9.2 1X Wash Buffer PT:

Prepare 1X Wash Buffer PT by diluting Wash Buffer PT 10X with deionized water. To make 50 mL 1X Wash Buffer PT combine 5 mL Wash Buffer PT 10X with 45 mL deionized water. Mix thoroughly and gently.

9.3 Antibody Cocktail:

Prepare Antibody Cocktail by diluting the capture and detector antibodies in Antibody Diluent 5BI. To make 3 mL of the Antibody Cocktail combine 300 µL 10X Capture Antibody and 300 µL 10X Detector Antibody with 2.4 mL Antibody Diluent 5BI. Mix thoroughly and gently.

10. Standard Preparation

- Always prepare a fresh set of standards for every use.
- Discard working standard dilutions after use as they do not store well.
- The following section describes the preparation of a standard curve for duplicate measurements (recommended).

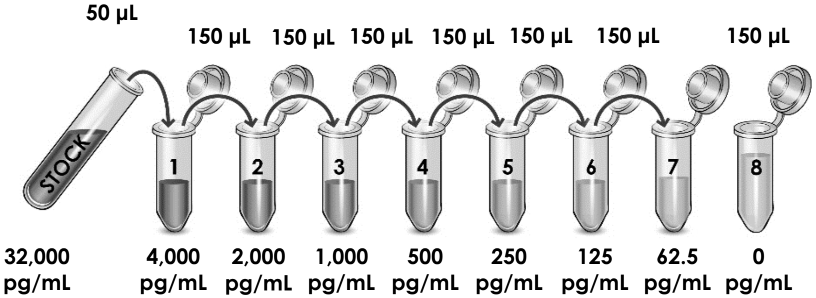
10.1 **IMPORTANT:** If the protein standard vial has a volume identified on the label, reconstitute the CKM protein standard by adding that volume of Diluent indicated on the label. Alternatively, if the vial has a mass identified, reconstitute the CKM standard by adding 500 µL Diluent. Hold at room temperature for 10 minutes and mix gently. This is the 32,000 pg/mL **Stock Standard** Solution.

For **serum and plasma samples measurements**, reconstitute the CKM protein standard by adding Sample Diluent NS. For **cell and tissue samples measurements**, reconstitute the CKM protein standard by adding 1X Cell Extraction Buffer.

10.1.1 Label eight tubes, Standards 1– 8.

10.1.2 Add 350 µL of appropriate diluent (see step 10.1) into tube number 1 and 150 µL of appropriate diluent into numbers 2-8.

10.1.3 Use the Stock Standard to prepare the following dilution series. Standard #8 contains no protein and is the Blank control:



11. Sample Preparation

Typical Sample Dynamic Range	
Sample Type	**Range**
Serum	1:128 - 1:16
Plasma - Citrate	1:128 - 1:8
Plasma - EDTA	1:64 - 1:8
Plasma - Heparin	1:128 - 1:8
Urine*	< 25%
Skeletal Muscle Tissue extract	31.25 ng/mL - 500 ng/mL

*Based on spiked sample

11.1 Plasma:

Collect plasma using citrate, EDTA or heparin. Centrifuge samples at 2,000 x g for 10 minutes. Dilute samples at least 1:8 into Sample Diluent NS and assay. Store un-diluted plasma samples at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.

11.2 Serum:

Samples should be collected into a serum separator tube. After clot formation, centrifuge samples at 2,000 x g for 10 minutes and collect serum. Dilute samples at least 1:16 into Sample Diluent NS and assay. Store un-diluted serum at -20°C or below. Avoid repeated freeze-thaw cycles.

11.3 Urine:

Centrifuge urine at 2,000 x g for 10 minutes to remove debris. Dilute samples at least 1:4 into Sample Diluent NS and assay. Store un-diluted urine samples at -20°C or below. Avoid repeated freeze-thaw cycles.

11.4 Preparation of extracts from cell pellets:

11.4.1 Collect non-adherent cells by centrifugation or scrape to collect adherent cells from the culture flask. Typical centrifugation conditions for cells are 500 x g for 5 minutes at 4°C.

11.4.2 Rinse cells twice with PBS.

11.4.3 Solubilize pellet at $2x10^7$ cell/mL in chilled 1X Cell Extraction Buffer PTR.

11.4.4 Incubate on ice for 20 minutes.

11.4.5 Centrifuge at 18,000 x g for 20 minutes at 4°C.

11.4.6 Transfer the supernatants into clean tubes and discard the pellets.

11.4.7 Assay samples immediately or aliquot and store at -80°C. The sample protein concentration in the extract may be quantified using a protein assay.

11.4.8 Dilute samples to desired concentration in 1X Cell Extraction Buffer PTR.

11.5 Preparation of extracts from tissue homogenates:

11.5.1 Tissue lysates are typically prepared by homogenization of tissue that is first minced and thoroughly rinsed in PBS to remove blood (dounce homogenizer recommended).

11.5.2 Homogenize 100 to 200 mg of wet tissue in 500 µL – 1 mL of chilled 1X Cell Extraction Buffer PTR. For lower amounts of tissue adjust volumes accordingly.

11.5.3 Incubate on ice for 20 minutes.

11.5.4 Centrifuge at 18,000 x g for 20 minutes at 4°C.

11.5.5 Transfer the supernatants into clean tubes and discard the pellets.

11.5.6 Assay samples immediately or aliquot and store at -80°C. The sample protein concentration in the extract may be quantified using a protein assay.

11.5.7 Dilute samples to desired concentration in 1X Cell Extraction Buffer PTR.

12. Plate Preparation

- The 96 well plate strips included with this kit are supplied ready to use. It is not necessary to rinse the plate prior to adding reagents.
- Unused plate strips should be immediately returned to the foil pouch containing the desiccant pack, resealed and stored at 4°C.
- For each assay performed, a minimum of two wells must be used as the zero control.
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).
- Differences in well absorbance or “edge effects” have not been observed with this assay.

13. Assay Procedure

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- We recommend that you assay all standards, controls and samples in duplicate.

13.1 Prepare all reagents, working standards, and samples as directed in the previous sections.

13.2 Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, reseal and return to 4°C storage.

13.3 Add 50 µL of all sample or standard to appropriate wells.

13.4 Add 50 µL of the Antibody Cocktail to each well.

13.5 Seal the plate and incubate for 1 hour at room temperature on a plate shaker set to 400 rpm.

13.6 Wash each well with 3 x 350 µL 1X Wash Buffer PT. Wash by aspirating or decanting from wells then dispensing 350 µL 1X Wash Buffer PT into each well. Wash Buffer PT should remain in wells for at least 10 seconds. Complete removal of liquid at each step is essential for good performance. After the last wash invert the plate and tap gently against clean paper towels to remove excess liquid.

13.7 Add 100 µL of TMB Development Solution to each well and incubate for 10 minutes in the dark on a plate shaker set to 400 rpm.
Given variability in laboratory environmental conditions, optimal incubation time may vary between 5 and 20 minutes. *Note: The addition of Stop Solution will change the color from blue to yellow and enhance the signal intensity about 3X. To avoid signal saturation, proceed to the next step before the high concentration of the standard reaches a blue color of O.D.600 equal to 1.0.*

13.8 Add 100 µL of Stop Solution to each well. Shake plate on a plate shaker for 1 minute to mix. Record the OD at 450 nm. This is an endpoint reading.

13.9 Alternative to 13.7 – 13.8: Instead of the endpoint reading at 450 nm, record the development of TMB Substrate kinetically. Immediately after addition of TMB Development Solution begin recording the blue color development with elapsed time in the microplate reader prepared with the following settings:

Mode	**Kinetic**
Wavelength:	600 nm
Time:	up to 20 min
Interval:	20 sec - 1 min
Shaking:	Shake between readings

Δ **Note**: that an endpoint reading can also be recorded at the completion of the kinetic read by adding 100 µL Stop Solution to each well and recording the OD at 450 nm.

13.10 Analyze the data as described below.

14. Calculations

14.1 Calculate the average absorbance value for the blank control (zero) standards. Subtract the average blank control standard absorbance value from all other absorbance values.

14.2 **Create a standard curve** by plotting the average blank control subtracted absorbance value for each standard concentration (y-axis) against the target protein concentration (x-axis) of the standard. Use graphing software to draw the best smooth curve through these points to construct the standard curve.

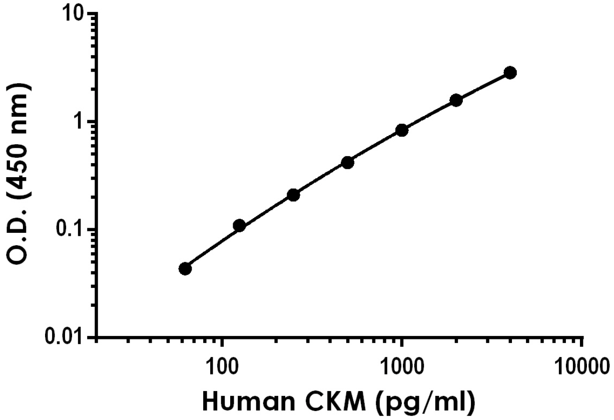
Δ **Note:** Most microplate reader software or graphing software will plot these values and fit a curve to the data. A four-parameter curve fit (4PL) is often the best choice; however, other algorithms (e.g. linear, semi-log, log/log, 4-parameter logistic) can also be tested to determine if it provides a better curve fit to the standard values.

14.3 Determine the concentration of the target protein in the sample by interpolating the blank control subtracted **absorbance values against the standard curve**. Multiply the resulting value by the appropriate sample dilution factor, if used, to obtain the concentration of target protein in the sample.

14.4 Samples generating absorbance values greater than that of the highest standard should be further diluted and reanalyzed. Similarly, samples which measure at an absorbance values less than that of the lowest standard should be retested in a less dilute form.

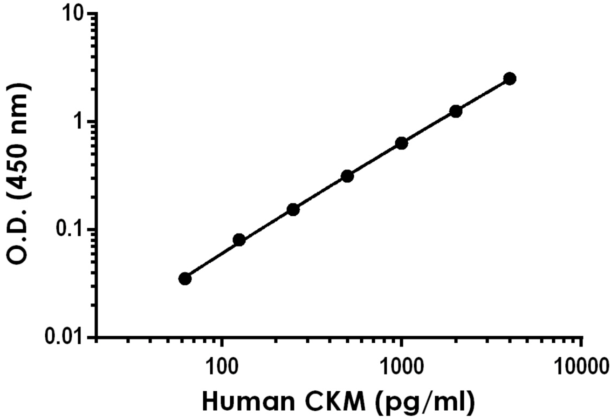
15. Typical Data

Typical standard curve – data provided **for demonstration purposes only.** A new standard curve must be generated for each assay performed.



Standard Curve Measurements			
Concentration (pg/mL)	O.D 450 nm		Mean O.D
	1	2	
0	0.067	0.061	0.064
62.5	0.112	0.104	0.108
125	0.178	0.170	0.174
250	0.274	0.276	0.275
500	0.487	0.482	0.484
1000	0.885	0.918	0.901
2000	1.630	1.677	1.654
4000	2.921	2.920	2.921

Figure 1. Example of human CKM standard curve in Sample Diluent NS. The CKM standard curve was prepared as described in Section 10. Raw data values are shown in the table. Background-subtracted data values (mean +/- SD) are graphed.



Standard Curve Measurements			
Concentration (pg/mL)	O.D 450 nm		Mean O.D
	1	2	
0	0.071	0.061	0.066
62.5	0.104	0.099	0.101
125	0.147	0.147	0.147
250	0.221	0.220	0.221
500	0.385	0.378	0.382
1000	0.679	0.727	0.703
2000	1.315	1.325	1.320
4000	2.570	2.594	2.582

Figure 2. Example of human CKM standard curve in 1X Cell Extraction Buffer. The CKM standard curve was prepared as described in Section 10. Raw data values are shown in the table. Background-subtracted data values (mean +/- SD) are graphed.

16. Typical Sample Values

SENSITIVITY –

The MDD was determined by calculating the mean of zero standard replicates and adding 2 standard deviations then extrapolating the corresponding concentration.

Sample Diluent Buffer	n=	Minimal Detectable Dose
Sample Diluent NS	24	18.9 pg/mL
1X Cell Extraction Buffer	20	28.9 pg/mL

RECOVERY –

Three concentrations of CKM recombinant protein were spiked in duplicate to the indicated biological matrix to evaluate signal recovery in the working range of the assay.

Sample Type	Average % Recovery	Range (%)
3.125% Serum	112	100 - 120
3.125% Plasma - Citrate	108	102 - 114
3.125% Plasma - EDTA	104	103 - 106
3.125% Plasma - Heparin	103	97 - 113
25% Urine	84	82 - 86
125ng/mL Skeletal Muscle Tissue extract	105	103 - 106

Linearity of Dilution

Linearity of dilution is determined based on interpolated values from the standard curve. Linearity of dilution defines a sample concentration interval in which interpolated target concentrations are directly proportional to sample dilution.

Native CKM was measured in the following biological samples in a 2-fold dilution series. Sample dilutions are made in Sample Diluent NS.

Dilution Factor	Interpolated value	6.25% Human Serum	12.5% Human Plasma (Citrate)	12.5% Human Plasma (EDTA)	12.5% Human Plasma (Heparin)
Undiluted	pg/mL	2232.1	1765.0	949.9	3187.8
	% Expected value	100	100	100	100
2	pg/mL	1097.3	924.2	480.9	1480.4
	% Expected value	98	105	101	93
4	pg/mL	560.5	440.4	244.3	723.9
	% Expected value	100	100	103	91
8	pg/mL	287.2	241.2	102.1	387.3
	% Expected value	103	109	86	97
16	pg/mL	NL	93.4	NL	158.5
	% Expected value		85		80

NL – Non-Linear

Recombinant CKM was spiked into the following biological samples and diluted in a 2-fold dilution series in Sample Diluent NS.

Dilution Factor	**Interpolated value**	**25% Human Urine**
Undiluted	pg/mL	977.6
	% Expected value	100
2	pg/mL	537.9
	% Expected value	110
4	pg/mL	280.6
	% Expected value	115
8	pg/mL	144.6
	% Expected value	118
16	pg/mL	70.5
	% Expected value	115

Native CKM was measured in the following biological samples in a 2-fold dilution series. Sample dilutions are made in 1X Cell Buffer Extraction.

Dilution Factor	**Interpolated value**	**500 ng/mL Human Skeletal Muscle Tissue Extract**
Undiluted	pg/mL	2116.9
	% Expected value	100
2	pg/mL	1072.5
	% Expected value	101
4	pg/mL	544.7
	% Expected value	103
8	pg/mL	278.2
	% Expected value	105
16	pg/mL	132.3
	% Expected value	100

PRECISION –

Mean coefficient of variations of interpolated values of CKM from a single concentration of human serum within the working range of the assay.

	Intra-Assay	Inter-Assay
n =	8	3.6
CV (%)	3	2.3

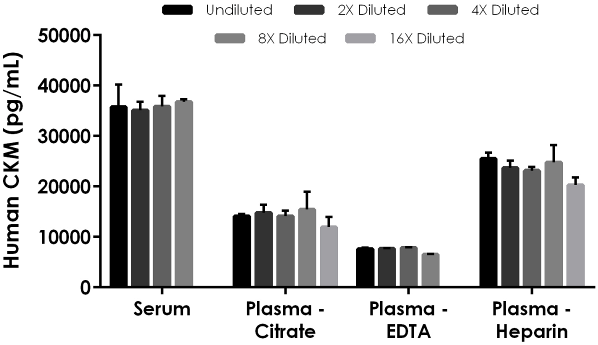


Figure 3. Interpolated concentrations of native CKM in human serum and plasma samples. The concentrations of CKM were measured in duplicates, interpolated from the CKM standard curves and corrected for sample dilution. Undiluted samples are as follows: serum 6.25%, plasma (citrate) 12.5%, plasma (EDTA), and plasma (heparin) 12.5%. The interpolated dilution factor corrected values are plotted (mean +/- SD, n=2). The mean CKM concentration was determined to be 35,863 pg/mL in serum, 14,078 pg/mL in plasma (citrate), 7,412 pg/mL in plasma (EDTA) and 23.485 pg/mL in plasma (heparin).

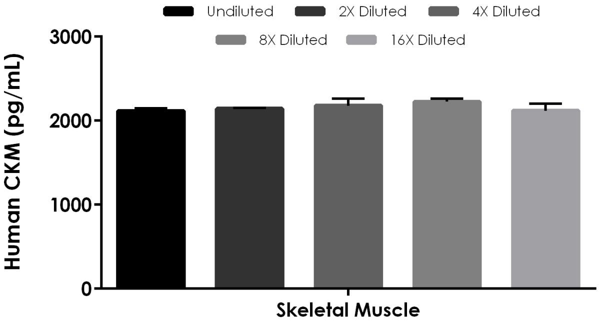


Figure 4. Interpolated concentrations of native CKM in human skeletal muscle tissue sample based on a 500 ng/mL extract load. The concentrations of CKM were measured in duplicate and interpolated from the CKM standard curve and corrected for sample dilution. The interpolated dilution factor corrected values are plotted (mean +/- SD, n=2). The mean CKM concentration was determined to be 2,157 pg/mL in skeletal muscle tissue sample.

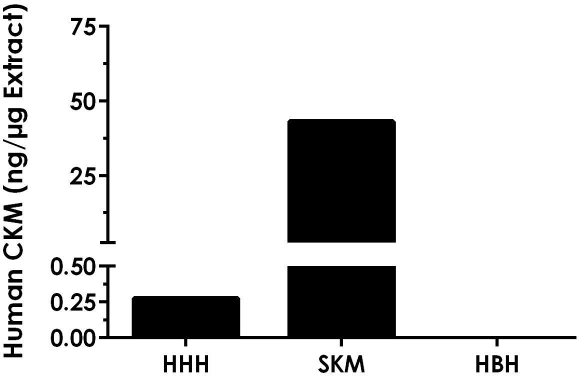


Figure 5. Tissue specificity of CKM in human tissue extracts. Human heart (HHH), human skeletal muscle (HSKM), and human brain (HBH) homogenates were extracted following the procedure as described in Section 11. Levels of CKM in 100 µg/mL extract were interpolated from a standard curve in 1X Cell Extraction Buffer.

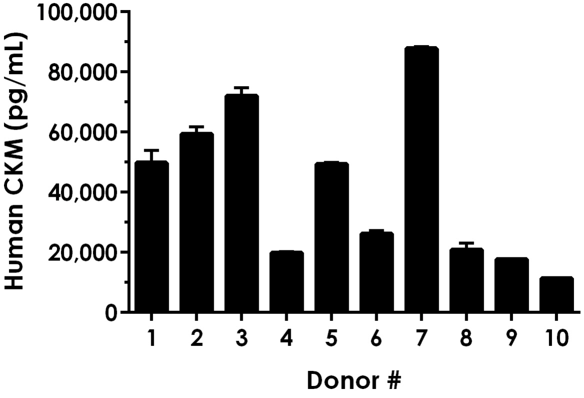


Figure 6. Serum from ten individual healthy human male donors was measured in duplicate. Interpolated dilution factor corrected values are plotted (mean +/- SD, n=2). The mean CKM concentration was determined to be 41,464 pg/mL with a range of 11,349 – 88,217 pg/mL.

17. Assay Specificity

This kit recognizes both native and recombinant human CKM protein in serum, plasma, and, cell and tissue extract samples only.

Cell culture supernatants, saliva, and milk samples have not been tested with this kit.

CROSS REACTIVITY

Recombinant human CKMB and human CKBB were prepared at 50 ng/mL respectively and assayed for cross reactivity. No cross-reactivity was observed.

INTERFERENCE

Recombinant human CKMB and human CKBB were prepared at 50 ng/mL respectively and tested for interference. No interference with was observed.

18. Species Reactivity

This kit recognizes human and monkey CKM protein.

Other species reactivity was determined by measuring 1:16 serum samples of various species, interpolating the protein concentrations from the human standard curve, and expressing the interpolated concentrations as a percentage of the protein concentration in human serum assayed at the same dilution.

Reactivity < 3% was determined for the following species:

- Mouse
- Rat
- Cow

Other species reactivity not determined.

Please contact our Technical Support team for more information.

19. Troubleshooting

Problem	Reason	Solution
Poor standard curve	Inaccurate Pipetting	Check pipettes
	Improper standard dilution	Prior to opening, briefly spin the stock standard tube and dissolve the powder thoroughly by gentle mixing
Low Signal	Incubation times too brief	Ensure sufficient incubation times; increase to 2 or 3 hour standard/sample incubation
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
	Incubation times with TMB too brief	Ensure sufficient incubation time until blue color develops prior addition of Stop solution
Large CV	Plate is insufficiently washed	Review manual for proper wash technique. If using a plate washer, check all ports for obstructions.
	Contaminated wash buffer	Prepare fresh wash buffer
Low sensitivity	Improper storage of the ELISA kit	Store your reconstituted standards at -80°C, all other assay components 4°C. Keep TMB Development Solution protected from light.
Precipitate in Diluent	Precipitation and/or coagulation of components within the Diluent.	Precipitate can be removed by gently warming the Diluent to 37°C.

20. Notes

Technical Support

For all technical or commercial enquiries please go to:

www.abcam.com/contactus
www.abcam.cn/contactus (China)
www.abcam.co.jp/contactus (Japan)