ab187396 – Rat CKM SimpleStep ELISA® Kit

Instructions for Use

For the quantitative measurement of CKM in rat serum, plasma-citrate, and tissue extracts.

This product is for research use only and is not intended for diagnostic use.

Version 3 Last Updated 13 January 2022

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1. BACKGROUND

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

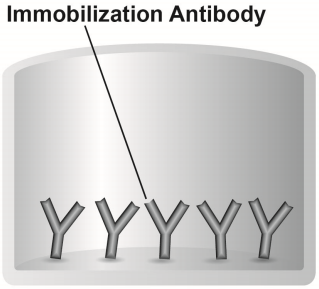
The SimpleStep ELISA® employs an affinity tag labeled capture antibody and a reporter conjugated detector antibody which immunocapture the sample analyte in solution. This entire complex (capture antibody/analyte/detector antibody) is in turn immobilized via immunoaffinity of an anti-tag antibody coating 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 (Mi_SCK and Mi_uCK). Structurally, cytosolic CK is a 86kDa dimeric enzyme of two identical or non-identical chains, CKM and/or CKB whereas mitochondrial CK is an octameric enzyme associated with mitochondrial membranes. The compartmentation of CK isoenzymes allows for direct association with ATP-providing or consuming processes both linked via metabolite channeling through the creatine phosphate shuttle. Thus 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.

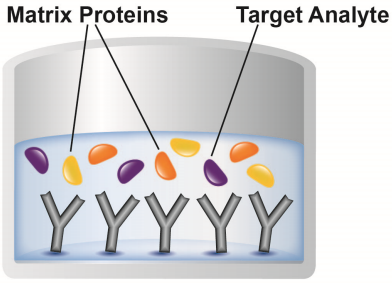
INTRODUCTION

Expression of cytosolic isoenzyme patterns differ between tissues, with skeletal muscle expressing high levels of CK-MM (98%) and low levels of CK-MB (1%), the myocardium (heart muscle) expressing CK-MM at 70% and CK-MB at 25–30% and the brain and neural tissue expressing mainly CK-BB. CKM is also found in serum from normal animals and levels vary based on muscle mass, age, physical activity and underlying pathologies.

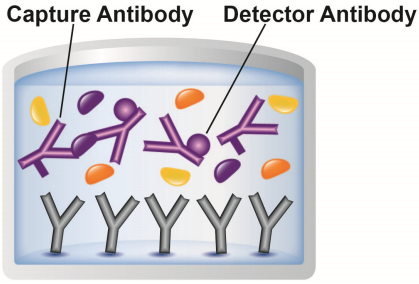
2. ASSAY SUMMARY



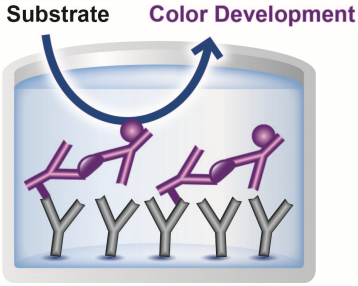
Remove appropriate number of antibody coated well strips. Equilibrate all reagents to room temperature. Prepare all reagents, samples, and standards as instructed.



Add standard or sample to appropriate wells.



Add Antibody Cocktail to all wells. Incubate at room temperature.



Aspirate and wash each well. Add TMB Development Solution to each well and incubate. Add Stop Solution at a defined endpoint.

Alternatively, record color development kinetically after TMB substrate addition.

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. Modifications to the kit components or procedures may result in loss of performance.

4. STORAGE AND STABILITY

Store kit according to instructions in the Material Supplied table immediately upon receipt.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in sections 9 & 10.

5. MATERIALS SUPPLIED

Item	Amount	Storage Condition (Before Preparation)
10X CKM Capture Antibody	600 µL	+2-8ºC
10X CKM Detector Antibody	600 µL	+2-8ºC
CKM Rat Lyophilized Purified Protein	2 Vials	-80ºC
Antibody Diluent 5BI	6 mL	+2-8ºC
10X Wash Buffer PT	20 mL	+2-8ºC
5X Cell Extraction Buffer PTR	10 mL	+2-8ºC
50X Cell Extraction Enhancer Solution	1 mL	+2-8ºC
TMB Development Solution	12 mL	+2-8ºC
Stop Solution	12 mL	+2-8ºC
Sample Diluent NS	50 mL	+2-8ºC
SimpleStep Pre-Coated 96 Well Microplate (12 x 8 well strips)	96 Wells	+2-8ºC
Plate Seal	1	+2-8ºC

6. MATERIALS REQUIRED, NOT SUPPLIED

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

- Microplate reader capable of measuring absorbance at 450 or 600 nm
- Method for determining protein concentration (BCA assay recommended)
- Deionized water
- PBS (1.4 mM KH2PO4, 8 mM Na2HPO4, 140 mM NaCl, 2.7 mM KCl, pH 7.4)
- Multi- and single-channel pipettes
- Tubes for standard dilution
- Plate shaker for all incubation steps
- Phenylmethylsulfonyl Fluoride (PMSF) (or other protease inhibitors)

7. 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

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
- The provided 50X Cell Extraction Enhancer Solution may precipitate when stored at + 4ºC. To dissolve, warm briefly at + 37ºC and mix gently. The 50X Cell Extraction Enhancer Solution can be stored at room temperature to avoid precipitation
- **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 5X Cell Extraction Buffer PTR and 50X Cell Extraction Enhancer Solution to 1X with deionized water. To make 10 mL 1X Cell Extraction Buffer PTR combine 7.8 mL deionized water, 2 mL 5X Cell Extraction Buffer PTR and 200 µL 50X Cell Extraction Enhancer Solution Mix thoroughly and gently. If required protease inhibitors can be added.

Alternative – Enhancer may be added to 1X Cell Extraction Buffer PTR after extraction of cells or tissue. Refer to note in Section 19.

9.2 1X Wash Buffer PT

Prepare 1X Wash Buffer PT by diluting 10X Wash Buffer PT with deionized water. To make 50 mL 1X Wash Buffer PT combine 5 mL 10X Wash Buffer PT 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

Prepare serially diluted standards immediately prior to use. Always prepare a fresh set of positive controls for every use.

The following table 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 standard by adding that volume of Sample Diluent NS indicated on the label. Alternatively, if the vial has a mass identified, reconstitute the CKM standard by adding 500 µL Sample Diluent NS. Hold at room temperature for 3 minutes and mix gently. This is the 1,000 ng/mL Stock Standard Solution.

10.2 Label eight tubes with numbers 1 – 8.

10.3 Add 150 µL Sample Diluent NS into tube numbers 2-8.

10.4 Prepare 100 ng/mL **Standard #1** by adding 50 µL of the 1,000 ng/mL Stock Standard Solution to 450 µL of Sample Diluent NS to tube #1. Mix thoroughly and gently.

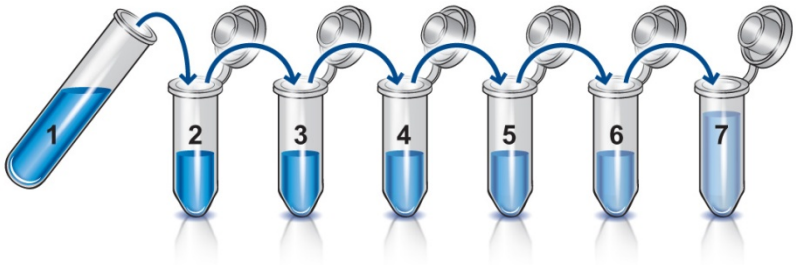
10.5 Prepare **Standard #2** by transferring 300 µL from Standard #1 to tube #2. Mix thoroughly and gently.

10.6 Prepare **Standard #3** by transferring 300 µL from Standard #2 to tube #3. Mix thoroughly and gently.

10.7 Using the table below as a guide, repeat for tubes #4 through #7.

10.8 **Standard #8** contains no protein and is the Blank control.

Standard #	Sample to Dilute	Volume to Dilute (µL)	Volume of Diluent (µL)	Starting Conc. (ng/mL)	Final Conc. (ng/mL)
1	Stock	50	450	1,000	100
2	Standard #1	300	150	100	66.7
3	Standard #2	300	150	66.7	44.4
4	Standard #3	300	150	44.4	29.6
5	Standard #4	300	150	29.6	19.8
6	Standard #5	300	150	19.8	13.2
7	Standard #6	300	150	13.2	8.8
8 (Blank)	none	--	--	--	0



11. SAMPLE PREPARATION

TYPICAL SAMPLE DYNAMIC RANGE	
Sample Type	**Range**
Rat Serum	1:40 – 1:135X
Rat Plasma	1:40 – 1:135X
Rat Heart Homogenate	8.9 – 30 µg/mL
Rat Skeletal Muscle Homogenate	0.3 – 3 µg/mL

11.1 **Plasma**

Collect plasma using citrate. Centrifuge samples at 2,000 x g for 10 minutes. Dilute samples into Sample Diluent NS and assay. Store un-diluted plasma samples at -20ºC 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 into Sample Diluent NS and assay. Store un-diluted serum at -20ºC or below. Avoid repeated freeze-thaw cycles.

11.3 **Cell Culture Supernatants**

Centrifuge cell culture media at 2,000 x g for 10 minutes to remove debris. Collect supernatants and assay. Store 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 4ºC.

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 adherent cells by direct lysis (alternative protocol)**

11.5.1 Remove growth media and rinse adherent cells 2 times in PBS.

11.5.2 Solubilize the cells by addition of chilled 1X Cell Extraction Buffer PTR directly to the plate (use 750 µL - 1.5 mL 1X Cell Extraction Buffer PTR per confluent 15 cm diameter plate).

11.5.3 Scrape the cells into a microfuge tube and incubate the lysate on ice for 15 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.

11.6 **Preparation of extracts from tissue homogenates**

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

11.6.2 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.

11.6.3 Incubate on ice for 20 minutes.

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

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

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

11.6.7 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.**
- **It is recommended to 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 4ºC 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 blot it against clean paper towels to remove excess liquid.

13.7 Add 100 µL of TMB Development Solution to each well and incubate for 5 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.

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

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.

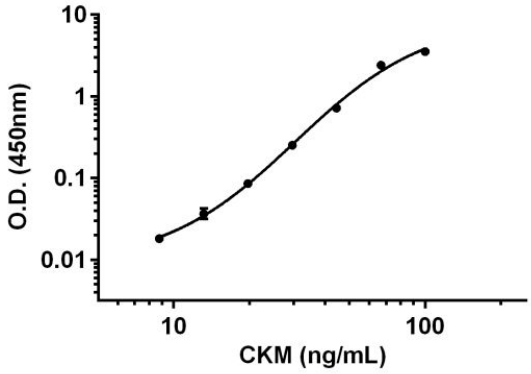
13.9 Analyze the data as described below.

14. CALCULATIONS

Subtract average zero standard from all readings. Average the duplicate readings of the positive control dilutions and plot against their concentrations. Draw the best smooth curve through these points to construct a standard curve. Most plate reader software or graphing software can plot these values and curve fit. A four parameter algorithm (4PL) usually provides the best fit, though other equations can be examined to see which provides the most accurate (e.g. linear, semi-log, log/log, 4 parameter logistic). Interpolate protein concentrations for unknown samples from the standard curve plotted. Samples producing signals greater than that of the highest standard should be further diluted and reanalyzed, then multiplying the concentration found by the appropriate dilution factor.

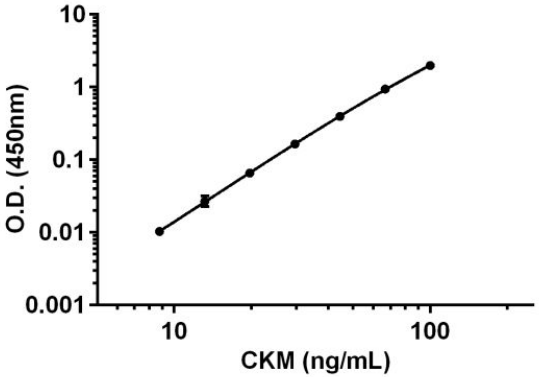
15. TYPICAL DATA

TYPICAL STANDARD CURVE – Data provided for **demonstration purposes only**. A new standard curve must be generated for each assay performed.



Standard Curve Measurements			
Conc. (ng/mL)	O.D. 450 nm		Mean O.D.
	1	2	
0	0.048	0.051	0.05
8.8	0.068	0.068	0.068
13.2	0.09	0.083	0.086
19.8	0.137	0.134	0.135
29.6	0.309	0.3	0.303
44.4	0.755	0.785	0.77
66.7	2.43	2.5	2.46
100	3.6	3.61	3.6

Figure 1. Example of 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			
Conc. (ng/mL)	O.D. 450 nm		Mean O.D.
	1	2	
0	0.048	0.05	0.049
8.8	0.063	0.062	0.063
13.2	0.082	0.076	0.079
19.8	0.121	0.115	0.118
29.6	0.227	0.208	0.218
44.4	0.485	0.414	0.45
66.7	1.07	0.918	0.994
100	2.15	1.95	2.05

Figure 2. Example of CKM standard curve in Extraction Buffer PTR. 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 calculated minimal detectable dose (MDD) was determined by calculating the mean of zero standard replicates and adding 2 standard deviations then extrapolating the corresponding concentrations:

Sample Diluent Buffer	n=	Minimal Detectable Dose
Sample Diluent NS	24	5.83 ng/mL
1X Cell Extraction Buffer PTR	24	11.1 ng/mL

RECOVERY –

Three concentrations of CKM 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 (%)
Rat Serum	94	89 - 97
1X Cell Extraction Buffer PTR	96	87 - 101

LINEARITY OF DILUTION –

Native CKM was measured in the following biological samples in a 2-fold dilution series. Sample dilutions are made in Sample Diluent NS for serum, plasma and 1X Cell Extraction Buffer PTR for tissue extracts.

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.

Dilution Factor	Interpolated value	2.5% Rat Serum	2.5% Rat Plasma	Rat HH	Rat SKM
1:1	ng/mL	94.6	99.4	49.3	89.6
	% Expected value	**100**	**100**	**100**	**100**
1:2	ng/mL	43.3	68.5	31	58.6
	% Expected value	**92**	**103**	**94**	**98**
1:4	ng/mL	22.7	46.3	20.3	38.1
	% Expected value	**96**	**105**	**92**	**96**
1:8	ng/mL	14.0	34.9	13.6	25.4
	% Expected value	**118**	**119**	**90**	**96**

*Rat HH = Rat Heart Homogenate, Rat SKM = Rat Skeletal Muscle Homogenate.

PRECISION –

Mean coefficient of variations of interpolated values from 3 concentrations of serum within the working range of the assay.

	Intra-Assay	Inter-Assay
n=	8	3
CV (%)	4	5

SAMPLE DATA –

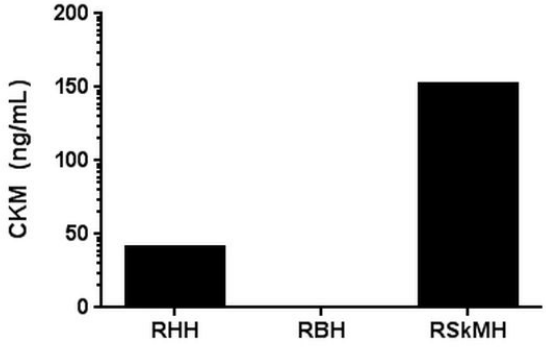


Figure3. Tissue specificity of CKM in rat tissue extracts. Rat Heart (RHH), rat brain (RBH) and rat skeletal muscle (RSkMH) homogenates were extracted following the procedure as described in Section 11. Levels of CKM in 10µg/mL extract were interpolated from a standard curve in Extraction Buffer PTR.

17. ASSAY SPECIFICITY

This kit recognizes both native and purified CKM protein.

18. SPECIES REACTIVITY

This kit recognizes rat CKM protein.

Species cross-reactivity was tested using native protein from serum loaded within the dynamic range of the assay

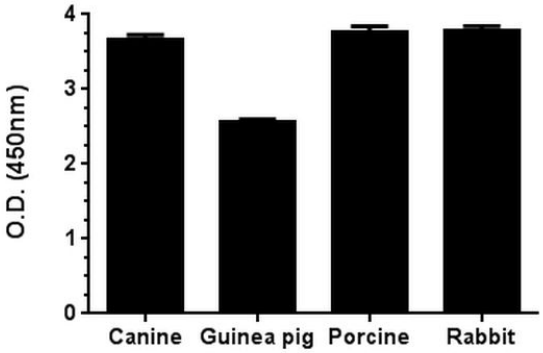


Figure 4 Serum species reactivity. Canine, guinea pig, porcine and rabbit serum was loaded in the assay at 2.5% in Sample Diluent NS. Absorbance levels after background subtraction are displayed.

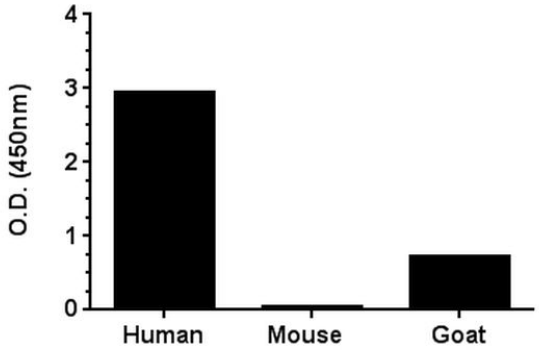


Figure 5. Serum species reactivity. Human, mouse and goat serum was loaded in the assay at 1% in Sample Diluent NS. Absorbance levels after background subtraction are displayed.

Please contact our Technical Support team for more information.

19. TROUBLESHOOTING

Problem	Cause	Solution
Difficulty pipetting lysate; viscous lysate.	Genomic DNA solubilized	Prepare 1X Cell Extraction Buffer PTR (without enhancer). Add enhancer to lysate after extraction.
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.

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)