Rat/Mouse C-Peptide 2

96-Well Plate

Cat. # EZRMCP2-21K

RAT/MOUSE C-PEPTIDE 2 ELISA KIT
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RAT/MOUSE C-PEPTIDE 2 ELISA KIT

I. INTENDED USE

This kit is used for the non-radioactive quantification of rat/mouse C-peptide 2 in serum and plasma. One kit is sufficient to measure 38 unknown samples in duplicate.

This kit is for research purpose only.

II. PRINCIPLES OF ASSAY

This assay is a Sandwich ELISA based on: 1) capture of C-peptide 2 molecules in the sample by anti-C-peptide 2 IgG and immobilization of the resulting complex to the wells of a microtiter plate coated by a pre-titered amount of anchor antibodies, 2) and the simultaneous binding of a second biotinylated antibody to C-peptide 2, 3) wash away of unbound materials, followed by conjugation of horseradish peroxidase to the immobilized biotinylated antibodies, 4) wash away of free enzyme, and 5) quantification of immobilized antibody-enzyme conjugates by monitoring horseradish peroxidase activities in the presence of the substrate 3,3',5,5'-tetra-methylbenzidine. The enzyme activity is measured spectrophotometrically by the increased absorbency at 450 nm, corrected from the absorbency at 590nm, after acidification of formed products. Since the increase in absorbency is directly proportional to the amount of captured rat/mouse C-peptide 2 in the unknown sample, the concentration of C-peptide 2 can be derived by interpolation from a reference curve generated in the same assay with reference standards of known concentrations of rat C-peptide 2.

III. REAGENTS SUPPLIED

Each kit is sufficient to run one 96-well plate and contains the following reagents:

1. Microtiter Plate

Coated with pre-titered anchor antibodies.

Quantity: 1 Strip Plate

Preparation: Ready to use.

Note: Unused strips should be resealed in the foil pouch with the dessicant provided and stored at 2-8 ℃.

2. Adhesive Plate Sealer

Quantity: 2 sheets

Preparation: Ready to use.

3. 10X HRP Wash Buffer Concentrate

10X concentrate of 50 mM Tris Buffered Saline containing Tween-20.

Quantity: 2 bottles containing 50mL each

Preparation: Dilute 1:10 with distilled or de-ionized water.

4. Rat C-peptide 2 Standard

Rat C-peptide 2 reference standard, 1,600 pM.

Quantity: 1 bottle. 2 mL/bottle

Dilute with Assay Buffer according to § VIII. A.

5. Quality Controls 1 and 2

One vial each containing rat C-peptide 2 at two different levels.

Quantity: 0.5 mL/vial.

Preparation: Ready to use.

6. Matrix Solution

Processed serum matrix containing 0.08% Sodium Azide

Quantity: 0.5 mL/vial

Preparation: Ready to use. (See Section VII step 8 for alternate instructions when analyzing ob/ob serum or plasma samples)

7. Assay Buffer

0.05 M phosphosaline, pH 7.4, containing 0.025 M EDTA, 0.08% sodium azide, and 1% BSA.

Quantity: 20 mL/vial

Preparation: Ready to use.

8. Rat/Mouse C-peptide 2 Capture Antibody

Pre-titered capture antibody solution in buffer

Quantity: 3 mL/vial

Preparation: Mix 1:1 with Rat/Mouse C-peptide 2 Detection Antibody before use according to § VIII. B.

9. Rat/Mouse C-peptide 2 Detection Antibody

Pre-titered detection antibody solution in buffer

Quantity: 3 mL/vial

Preparation: Mix 1:1 with Rat/Mouse C-peptide 2 Capture Antibody before use according to § VIII. B.

10. Enzyme Solution

Pre-titered streptavidin-horseradish peroxidase conjugate in buffer.

Quantity: 12 mL/vial

Preparation: Ready to use

11. Substrate

3, 3',5,5'-tetramethylbenzidine in buffer.

Quantity: 12 mL/vial

Preparation: Ready to use. Minimize the exposure to light.

12. Stop Solution

0.3 M HCl

Quantity: 12 mL/vial

Preparation: Ready to use.

[Caution: Corrosive Solution]

IV. STORAGE AND STABILITY

All components of the kit should be stored at 4°C. Prepare and use standard/QC solutions within a day after reconstitution and aliquot in smaller quantity and store at -20°C for later use, if necessary. Avoid further freeze/thaw cycles. Refer to expiration dates on all reagents prior to use. Do not mix reagents from different kits unless they have the same lot numbers.

V. REAGENT PRECAUTIONS

1. Sodium Azide

Sodium azide has been added to certain reagents as a preservative at a concentration of 0.08%. Although it is at a minimum concentration, sodium azide may react with lead and copper pluming to form explosive metal azides. On disposal, flush with large volume of water to prevent azide build up.

2. Hydrochloric Acid

Hydrochloric acid is corrosive, can cause eye and skin burns. Harmful if swallowed. Causes respiratory and digestive tract burns. Avoid contact with skin and eye. Do not swallow or ingest.

VI. MATERIALS REQUIRED BUT NOT PROVIDED

1. Pipettes and pipette tips: 10 µL ~ 20 µL or 20 µL ~ 100 µL
2. Multi-channel Pipettes and pipette tips: 5 ~ 50 µL and 50 ~ 300 µL
3. Buffer and Reagent Reservoirs
4. Vortex Mixer
5. De-ionized Water
6. Microtiter Plate Reader capable of reading absorbency at 450 nm and 590nm
7. Orbital Microtiter Plate Shaker
8. Absorbent Paper or Cloth

VII. SAMPLE COLLECTION AND STORAGE

1. To prepare serum, whole blood is directly drawn into a centrifuge tube that contains no anti-coagulant. Let blood clot at room temperature for 30 min.

2. Promptly centrifuge the clotted blood at 2,000 to 3,000 x g for 15 minutes at 4 ± 2°C.

3. Transfer serum samples in separate tubes. Date and identify each sample.

4. Use freshly prepared serum or store samples at –20 ± 5°C for later use. Avoid multiple (> 5) freeze/thaw cycles.

5. To prepare plasma sample, whole blood should be collected into a centrifuge tube containing enough K_3 EDTA to achieve a final concentration of 1.735 mg/mL and immediate centrifuged at 2,000 to 3,000 x g for 15 minutes at 4 ± 2°C. Transfer plasma samples in separate tubes and observe same precautions in the preparation of serum samples.

6. If heparin is to be used as anti-coagulant, the effect on the assay outcome at the dose of heparin used should be pre-determined.

7. Avoid using samples with gross hemolysis or lipemia.

8. A 5-fold dilution with assay buffer is recommended for serum/plasma samples from **ob**/**ob** mice with established phonotype because of high concentrations of C-Peptide 2. In such assays, the matrix solution should also be diluted 5-fold with assay buffer. The assay results should then be multiplied by 5.

VIII. REAGENT PREPARATION

A. Standard Preparation

Label six vials with the additional concentrations of standards to be prepared: 25 pM, 50 pM, 100 pM, 200 pM, 400 pM and 800 pM. Add 0.5 mL Assay Buffer to each vial. Make serial 2-fold dilutions of reference standard as follows: transfer 0.5 mL reference standard (1,600 pM) to the vial labeled 800 pM and mix well, then transfer 0.5 mL from 800 pM to the vial labeled 400 pM and mix well, etc, until the last vial is mixed.

Note: **Change tip for every dilution and ensure thorough mixing before and after transfer**. Wet tip with appropriate standard solution and carefully wipe the outside dry before each transfer. Unused portions of standard should be stored in small aliquots at ≤ -20°C. Avoid multiple freeze/thaw cycles.

B. Preparation of Capture and Detection Antibody Mixture

Prior to use, combine the entire contents of Rat/Mouse C-peptide 2 Capture Antibody (3mL) and Rat/Mouse C-peptide 2 Detection Antibody (3mL), or at a 1:1 ratio if less than 6 mL is needed for the assay, and invert to mix thoroughly.

IX. RAT/MOUSE C-PEPTIDE 2 ELISA ASSAY PROCEDURE

Pre-warm all reagents to room temperature immediately before setting up the assay.

1. Dilute the 10X concentrated HRP wash buffer 10 fold by mixing the entire contents of both buffer bottles with 900 mL de-ionized or glass distilled water.

2. Remove the required number of strips from the Microtiter Assay Plate. Unused strips should be resealed in the foil pouch and stored at 2-8°C. Assemble the strips in an empty plate holder and fill each well with 300 µl diluted Wash Buffer. Decant wash buffer and remove the residual amount by inverting the plate and tapping it smartly onto absorbent towels several times. Wash assay plate using this procedure 2 additional times. **Do not let wells dry before proceeding to the next step.** If an automated machine is used for the assay, follow the manufacturer's instructions for all washing steps described in this protocol.

3. Add 20 µL Matrix Solution to Blank, Standards and Quality Control wells (refer to § X. for suggested well orientations).

4. Add 30 µL assay buffer to each of the Blank and sample wells.

5. Add 10 µL assay buffer to each of the Standard and Quality Control wells.

6. Add in duplicate 20 µL Rat C-peptide 2 Standards in the order of ascending concentrations to the appropriate wells.

7. Add in duplicate 20 µL QC1 and 20 µL QC2 to the appropriate wells.

8. Add sequentially 20 µL of the unknown samples in duplicate to the remaining wells**.**

9. Transfer the Antibody Solution Mixture (1:1 mixture of capture and detection antibody) to a buffer or reagent reservoir and add 50 µL to each well with a multi-channel pipette.

10. Cover the plate with plate sealer and incubate at room temperature for 2 hours on an orbital microtiter plate shaker set to rotate at moderate speed, about 400 to 500 rpm.

11. Remove plate sealer and decant solutions from the plate. Tap as before to remove residual solutions in well.

12. Wash wells 3 times with diluted Wash Buffer, 300 µL per well per wash. Decant and tap after each wash to remove residual buffer.

13. Add 100 µL Enzyme Solution to each well. Cover plate with sealer and incubate with moderate shaking at room temperature for 30 min on the micro-titer plate shaker.

14. Remove sealer, decant solutions from the plate and tap plate to remove the residual fluid.

15. Wash wells 6 times with diluted Wash Buffer, 300 µL per well per wash. Decant and tap after each wash to remove residual buffer.

16. Add 100 µL of Substrate solution to each well, cover plate with sealer and shake in the plate shaker for **approximately** 5 to 20 minutes. (**Note:** Please be aware that the color may develop more quickly or more slowly than the recommended incubation time depending on the localized room temperature. Please visually monitor the color development to optimize the incubation time.) Blue color should be formed in the standard wells with intensity proportional to increasing concentrations of C-peptide 2. Remove sealer and add 100 µl stop solution **[CAUTION: CORROSIVE SOLUTION]** and shake plate by hand to ensure complete mixing of solution in all wells. The blue color should turn into yellow after acidification. Read absorbance at 450 nm and 590nm in a plate reader within 5 minutes and ensure that there is no air bubbles in any well.

Assay Procedure for Rat/Mouse C-peptide 2 ELISA Kit (Cat. # EZRMCP2-21K)

	Step 1	Step 2	Step 3	Step 4-5	Step 6-8	Step 9	Step 10-12	Step 13	Step 14-15	Step 16			
Well #	Dilute both bottles of 10X HRP Wash Buffer with 900 mL de-ionized water.	Wash plate 3X with 300 µL diluted HRP wash buffer. Remove residual buffer by tapping smartly on absorbent towels	Matrix Solution	Assay Buffer	Standards/QCs/ Samples	Capture/ Detection Antibody Mixture	Seal, Agitate, Incubate 2 hours at Room Temperature. Wash 3X with 300 µL Wash Buffer.	Enzyme Solution	Seal, Agitate, Incubate 30 minutes at Room Temperature. Wash 6X with 300 µL Wash Buffer.	Substrate	Seal, Agitate, Incubate 12~15 minutes at Room Temperature.	Stop Solution	Read Absorbance at 450 nm and 590 nm.
A1, B1			20 µl	30 µl	--	50 µl		100 µl		100 µl		100 µl	
C1, D1			20 µl	10 µl	20 µl of 25 pM Standard								
E1, F1			20 µl	10 µl	20 µl of 50 pM Standard								
G1, H1			20 µl	10 µl	20 µl of 100 pM Standard								
A2, B2			20 µl	10 µl	20 µl of 200 pM Standard								
C2, D2			20 µl	10 µl	20 µl of 400 pM Standard								
E2, F2			20 µl	10 µl	20 µl of 800 pM Standard								
G2, H2			20 µl	10 µl	20 µl of 1,600 pM Standard								
A3, B3			20 µl	10 µl	20 µl of QC 1								
C3, D3			20 µl	10 µl	20 µl of QC 2								
E3, F3			--	30 µl	20 µl of Sample 1								
G3, H3 Etc.			--	30 µl	20 µl of sample 2								

X. MICROTITER PLATE ARRANGEMENT

Rat/Mouse C-peptide 2 ELISA

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	200 pM	QC1	Etc.								
B	Blank	200 pM	QC1	Etc.								
C	25 pM	400 pM	QC2									
D	25 pM	400 pM	QC2									
E	50 pM	800 pM	Sample 1									
F	50 pM	800 pM	Sample 1									
G	100 pM	1,600 pM	Sample 2									
H	100 pM	1,600 pM	Sample 2									

XI. CALCULATIONS

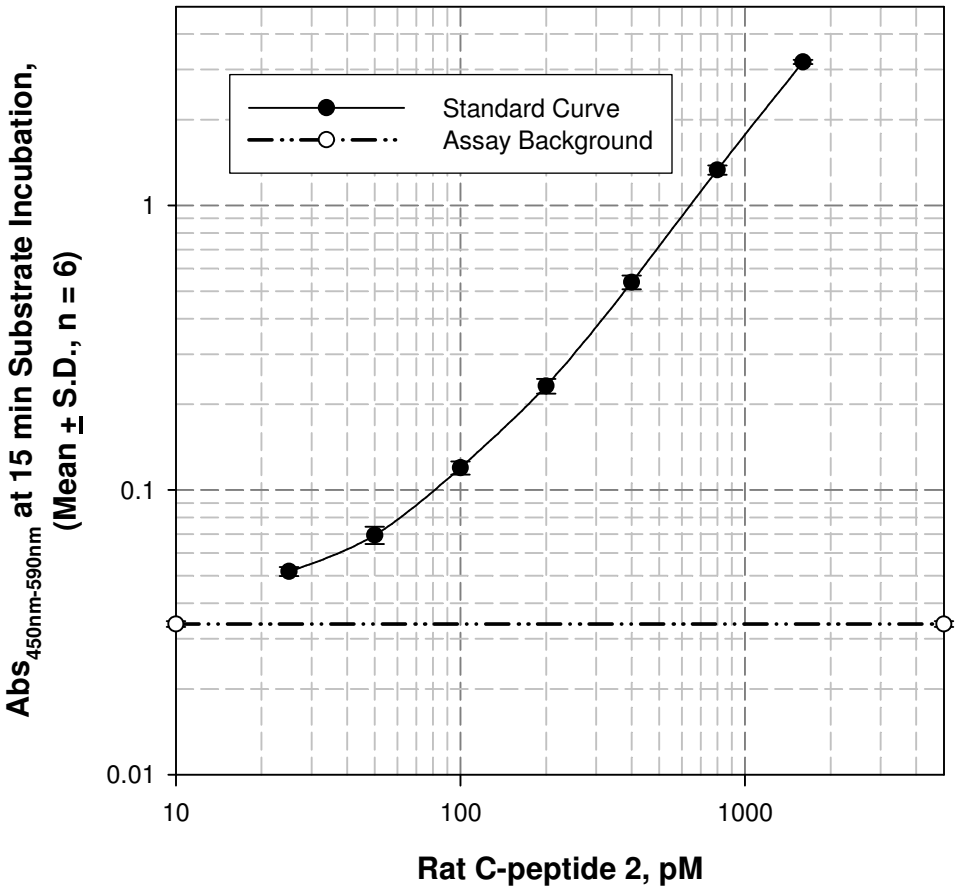
Graph a reference curve by plotting the absorbance unit of 450nm, less unit at 590nm, on the Y-axis against the concentrations of C-peptide 2 standard on the X-axis. The dose-response curve of this assay fits best to a sigmoidal 4- or 5-parameter logistic equation. The results of unknown samples can be calculated with any computer program having a 4- or 5-parameter logistic function.

Note: When sample volumes assayed differ from 20 µl, an appropriate mathematical adjustment must be made to accommodate for the dilution factor (e.g., if 10 µl of sample is used, then calculated data must be multiplied by 2). When sample volume assayed is less than 20 µl, compensate the volume deficit with matrix solution.

XII. INTERPRETATION

1. The assay will be considered accepted when all Quality Control values fall within the calculated QC range. If any QCs fall outside of the control range, review results with a supervisor.
2. If the difference between duplicate results of a sample is >15% CV, repeat the sample.
3. The theoretical minimal detecting concentration of this assay is 6 pM C-peptide 2 (20 µL sample size).
4. The appropriate range of this assay is 25 pM to 1,600 pM C-peptide 2 (20 µL sample size). Any result greater than 1,600 pM in a 20 µL sample should be diluted using matrix solution and the assay repeated until the results fall within range.

XIII. GRAPH OF TYPICAL REFERENCE CURVE



For Demonstration Only - Do not use for calculations

XIV. ASSAY CHARACTERISTICS

A. Analytical Sensitivity

The lowest level of C-peptide 2 that can be detected by this assay is 6 pM when using a 20 µL sample size.

B. Specificity

Rat C-peptide 2	100%
Mouse C-peptide 2	100%
Rat C-peptide 1	10%
Mouse C-peptide 1	0%
Canine C-peptide	0%
Porcine C-peptide	0%
Human C-peptide	0%

C. Precision

Intra and Inter-Assay Variations

Sample	C-peptide 2 (pM) Mean, n = 5	Intra-assay CV (%)	Inter-assay CV (%)
Rat serum 1	156	< 10%	< 10%
Rat serum 2	324	< 10%	< 10%
Rat serum 3	552	< 10%	< 10%
Mouse serum 1	60	< 10%	< 10%
Mouse serum 2	283	< 10%	< 10%
Mouse serum 3	464	< 10%	< 10%

Serum samples from rats and mice are used for measurement of C-peptide 2 by ELISA. Intra-assay variations were calculated from results of five duplicate determinations in one assay. Inter-assay variations were calculated from results of five separate assays with duplicate samples in each assay.

XIV. ASSAY CHARACTERISTICS (continued)

D. Spike Recovery Rate of Rat/Mouse C-peptide 2 in Assay Samples

Sample Source	I.D. #	Basal C-peptide 2, pM	C-peptide 2 Spike Recovery Rate at		
			+ 100 pM	+ 400 pM	+ 800 pM
Rat Serum	**49427**	173	78.0 %	83.5 %	84.6 %
	49428	118	97.0 %	88.0 %	89.4 %
	49429	120	86.0 %	84.5 %	87.5 %
	49430	113	97.0 %	84.8 %	88.3 %
	49431	177	95.0 %	95.8 %	94.6 %
	49432	99	71.0 %	82.8 %	84.9 %
	Mean ± S.D. (n = 6)		**91.8 ± 11.0 %**	**86.6 ± 4.9 %**	**88.2 ± 3.7 %**
Rat Plasma	**49439**	103	98.0 %	96.5 %	91.0 %
	49440	216	97.0 %	95.0 %	94.9 %
	49441	153	86.0 %	91.0 %	92.0 %
	49442	191	106.0 %	90.3 %	92.6 %
	49443	173	90.0 %	95.3 %	93.8 %
	49444	165	91.0 %	91.8 %	92.1 %
	Mean ± S.D. (n = 6)		**94.7 ± 7.1 %**	**93.3 ± 2.6 %**	**92.7 ± 1.4 %**
Mouse Serum	**24074**	107	88.0 %	89.3 %	88.9 %
	24077	154	82.0 %	88.3 %	87.6 %
	24080	176	90.0 %	83.0 %	81.5 %
	24081	131	86.0 %	88.3 %	87.6 %
	Mean ± S.D. (n = 4)		**86.5 ± 3.4 %**	**87.2 ± 2.9 %**	**86.4 ± 3.3 %**
Mouse Plasma	**38365**	119	86.0 %	94.3 %	97.0 %
	38366	120	96.0 %	98.0 %	99.0 %
	38371	175	87.0 %	94.5 %	93.5 %
	38374	274	105.0 %	97.8 %	96.3 %
	Mean ± S.D. (n = 4)		**93.5 ± 8.9 %**	**96.2 ± 2.0 %**	**96.5 ± 2.3 %**

Rat C-peptide 2 at indicated concentrations are spiked to rat samples and mouse C-peptide 2 to mouse samples. Analyte recovery rate is calculated as: (Level after Spike – Basal Level) / Spiked Level x 100%

XIV. ASSAY CHARACTERISTICS (continued)

E. Linearity of Sample Dilution

Sample I.D.	Volume Assayed	Serum C-peptide 2		Plasma C-peptide 2	
		pM	% of Expected	pM	% of Expected
Rat	**20 µl**	200	**100%**	232	**100%**
	15 µl	139	93%	167	96%
	10 µl	91	91%	111	96%
	5 µl	44	88%	59	102%
Rat	**20 µl**	491	**100%**	565	**100%**
	15 µl	366	99%	416	98%
	10 µl	250	102%	275	97%
	5 µl	130	106%	136	96%
Rat	**20 µl**	835	**100%**	938	**100%**
	15 µl	611	98%	702	100%
	10 µl	413	99%	466	99%
	5 µl	216	104%	231	99%
MEAN ± S.D. (n = 3)	**20 µl**	**--**	**100%**	--	**100%**
	15 µl	--	**96.6 ± 3.5%**	--	**98.0 ± 1.9%**
	10 µl	--	**97.2 ± 5.6%**	--	**97.5 ± 1.9%**
	5 µl	--	**99.1 ± 9.7%**	--	**98.8 ± 2.7%**
Mouse	**20 µl**	228	**100%**	189	**100%**
	15 µl	171	100%	154	109%
	10 µl	117	103%	102	108%
	5 µl	61	107%	59	125%
Mouse	**20 µl**	492	**100%**	534	**100%**
	15 µl	371	101%	401	100%
	10 µl	254	103%	276	103%
	5 µl	140	113%	141	106%
Mouse	**20 µl**	853	**100%**	969	**100%**
	15 µl	665	104%	711	98%
	10 µl	461	108%	474	98%
	5 µl	247	116%	241	99%
MEAN ± S.D. (n = 3)	**20 µl**	--	**100%**	--	**100%**
	15 µl	--	**101.5 ± 2.2%**	--	**102.2 ± 5.7%**
	10 µl	--	**104.7 ± 3.0%**	--	**103.0 ± 5.1%**
	5 µl	--	**112.0 ± 4.5%**	--	**110.1 ± 13.3%**

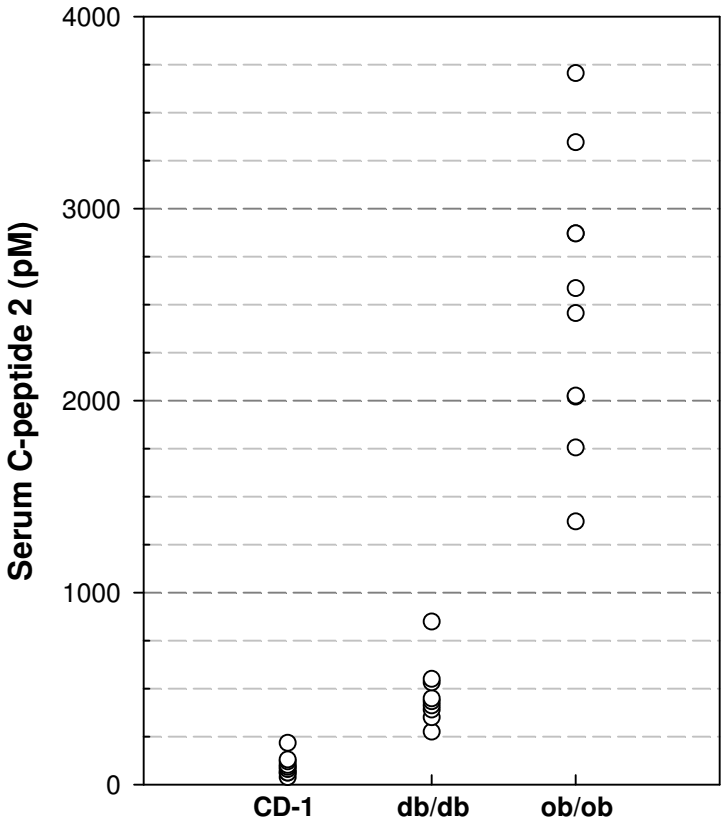
Serum and plasma samples from separate animals are assayed at 20, 15, 10 and 5 µL each for C-peptide 2 by ELISA. Samples less then 20 µL are reconstituted to 20 µL total with enough matrix solution. Rat C-peptide 2 are spiked into some rat samples and mouse C-peptide to some mouse samples before assay to achieve intermediate and high levels shown. Measured C-peptide 2 levels are corrected for various dilution factors and then divided by levels found at 20 µL sample size to obtain the % of expected values.

XV. NORMAL RANGE OF C-peptide 2 LEVELS IN RAT/MOUSE BLOOD

The range of c-peptide 2 in non-fasted rat (Sprague Dawley) blood is 70 ~600 pM.

The range of serum c-peptide 2 in mice varies greatly, depending on the disease models:

C-peptide 2 Levels in Sera of Three Mouse Models



Serum samples of 9 to 10 animals of each mouse model are used in this study.

XVI. QUALITY CONTROLS

The ranges for Quality Control 1 and 2 are provided on the card insert or can be located at the Millipore website www.millipore.com/bmia.

XII. TROUBLESHOOTING GUIDE

1. To obtain reliable and reproducible results the operator should carefully read this manual and fully understand all aspects of each assay step before attempting to run the assay.
2. Throughout the assay the operator should adhere strictly to the procedures with good laboratory practice.
3. Have all necessary reagents and equipment ready on hand before starting. Once the assay has been started all steps should be completed with precise timing and without interruption.
4. Avoid cross contamination of any reagents or samples to be used in the assay.
5. Make sure all reagents and samples are added to the bottom of each well.
6. Careful and complete mixing of solutions in the well is critical. Poor assay precision will result from incomplete mixing or cross well contamination due to inappropriate mixing.
7. Remove any air bubble formed in the well after acidification of substrate solution because bubbles interfere with spectrophotometric readings.
8. High absorbance in background or blank wells could be due to 1) cross well contamination by standard solution or sample or 2) inadequate washing of wells with HRP Wash Buffer or 3) overexposure to light after substrate has been added.

XIII. REPLACEMENT REAGENTS

Reagents	**Cat. #**
Microtiter Plate	EPDAG
10X HRP Wash Buffer Concentrate (50 mL)	EWB-HRP
Rat C-peptide 2 Standard	E8021-K
Rat/Mouse C-peptide 2 Quality Controls 1 and 2	E6021-K
Matrix Solution	EMTX-RMI
Assay Buffer	AB-PHK
Rat/Mouse C-peptide 2 Capture Antibody	E1021-C
Rat/Mouse C-peptide 2 Detection Antibody	E1021-D
Enzyme Solution	EHRP-4
Substrate	ESS-TMB2
Stop Solution	ET-TMB

XIX. ORDERING INFORMATION

A. To place an order:

For USA Customers:

Please provide the following information to our customer service department to expedite your telephone, fax or mail order:

1. Your name, telephone and/or fax number
2. Customer account number
3. Shipping and billing address
4. Purchase order number
5. Catalog number and description of product
6. Quantity and product size

TELEPHONE ORDERS:

Toll Free US: (800) MILLIPORE

FAX ORDERS: (636) 441-8050

MAIL ORDERS: Milllipore
6 Research Park Drive
St. Charles, Missouri 63304 U.S.A.

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