User Guide

Rat/Mouse C-Peptide 2 ELISA

96-Well Plate

EZRMCP2-21K

For research use only. Not for use in diagnostic procedures.

Intended Use

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

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Principles of Assay

This assay is a Sandwich ELISA based on:

- 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
- The simultaneous binding of a second biotinylated antibody to C-peptide 2
- Wash away of unbound materials
- Conjugation of horseradish peroxidase to the immobilized biotinylated antibodies
- Wash away free enzyme
- 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 590 nm, 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.

Reagents Supplied

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

Note: Store all reagents at 2-8 °C

Reagents Supplied	Volume	Quantity	Cat. No.
Microtiter Plate with 2 plate sealers **Note:** Unused strips should be resealed in the foil pouch with the desiccant provided and stored at 2–8 °C	-	1 plate 2 sealers	EPDAG
Rat/Mouse C-Peptide 2 Standard	2 mL	1 vial	E8021-K
Quality Controls 1 and 2	0.5 mL	1 vial each	E6021-K
Matrix Solution	0.5 mL	1 vial	EMTX-RMI
Assay Buffer	20 mL	1 vial	AB-PHK
10X Wash Buffer	50 mL	2 bottles	EWB-HRP
Rat/Mouse C-Peptide 2 Detection Antibody	3 mL	1 bottle	E1021-D
Rat/Mouse C-Peptide 2 Capture Antibody	3 mL	1 bottle	E1021-C
Enzyme Solution	12 mL	1 bottle	EHRP-88
Substrate Solution	12 mL	1 bottle	ESS-TMB2
Stop Solution (**Caution:** Corrosive Solution)	12 mL	1 bottle	ET-TMB

Storage and Stability

Recommended storage for kit components is 2-8 °C.

All components are shipped and stored at 2-8 °C. Reconstituted standards and controls can be frozen for future use but repeated freeze/thaw cycles should be avoided. Refer to expiration dates on all reagents prior to use. Do not mix reagents from different kits unless they have the same lot numbers.

Reagent Precautions

Sodium Azide

Sodium azide or Proclin™ has been added to some reagents as a preservative. Although the concentrations are low, Sodium azide and Proclin™ may react with lead and copper plumbing to form highly explosive metal azides. Dispose of unused contents and waste in accordance with international, federal, state, and local regulations.

Hydrochloric Acid

Hydrochloric acid is corrosive and 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.

Note: see next page for full Hazardous Component labels

Symbol Definitions

Ingredient	Cat. No.	Full Label	
Rat/Mouse C-Peptide 2 Capture Antibody	E1021-C		**Warning:** Causes serious eye irritation. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
Rat/Mouse C-Peptide 2 Detection Antibody	E1021-D		**Warning:** Causes serious eye irritation. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
Rat/Mouse C-Peptide 2 Standard	E8021-K		**Warning:** Causes serious eye irritation. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
Rat/Mouse C-Peptide 2 Quality Control 1 and 2	E6021-K		**Warning:** Causes serious eye irritation. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
Stop Solution	ET-TMB		**Warning:** May be corrosive to metals.
10X HRP Wash Buffer Concentrate	EWB-HRP		**Warning:** May cause an allergic skin reaction. Wear protective gloves. IF ON SKIN: Wash with plenty of soap and water.

Materials Required (Not Provided)

- Multi-channel Pipettes and pipette tips: 5 µL-50 µL and 50-300 µL
- Pipettes and pipette tips: 10 µL-20 µL and 20 µL-200 µL
- Buffer and Reagent Reservoirs
- Vortex Mixer
- De-ionized water
- Microtiter Plate Reader capable of reading absorbency at 450 nm and at 590 nm
- Orbital Microtiter Plate Shaker
- Absorbent Paper or Cloth

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_3EDTA 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.

Reagent Preparation

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.

Preparation of Capture and Detection Antibody Mixture

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

Rat/Mouse C-Peptide 2 ELISA Assay Procedure

Warm all reagents to room temperature 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. 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 Microtiter Plate Arrangement 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.

17. 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. Wipe the bottom of the microtiter plate to remove any residue prior to reading on plate reader. Read absorbance at 450 nm and 590 nm in a plate reader within 5 minutes and ensure that there are no air bubbles in any wells.

Assay Procedure for Rat/Mouse C-Peptide 2 ELISA Kit

Well #	Step 1	Step 2	Step 3	Step 4	Step 6-8	Step 9	Step 10-12	Step 13	Step 14-15	Step 16		
			Matrix Solution	Assay Buffer	Standards/ QCs/Samples	Capture/Detection Antibody Mixture		Enzyme Solution		Substrate		Stop Solution
A1, B1	Dilute both bottles of 10X Wash Buffer with 900 mL Deionized Water.	Wash plate 3X with 300 L diluted HRP wash buffer. Remove residual buffer by tapping smartly on absorbent towels.	20 µL	30 µL	-	50 µL	Seal, Agitate, Incubate 2 hours at Room Temperature. Wash 3X with 300 µL Wash Buffer.	100 µL	Seal, Agitate, Incubate 30 mins at Room Temperature. Wash 6X with 300 µL Wash Buffer.	100 µL	Seal, Agitate, Incubate 5-20 mins at Room Temperature.	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 1600 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							
			-	30 µL	20 µL of Sample 2							Read Absorbance at 450 nm and 590 nm.

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									

Calculations

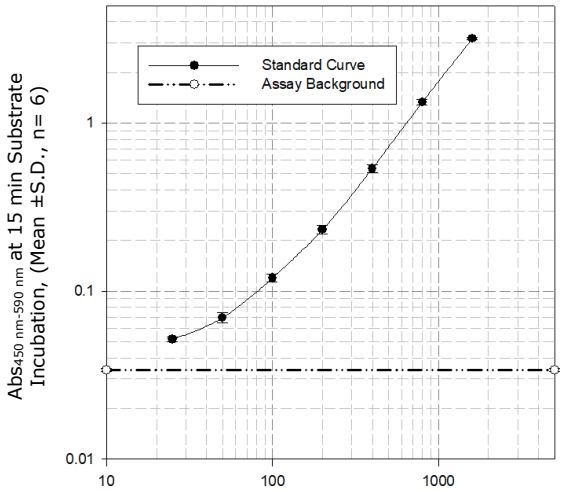
Graph a reference curve by plotting the absorbance unit of 450 nm, less unit at 590 nm, on the Y-axis against the concentrations of C-peptide 2 standard on the X-axis. The dose-response curve of this assay fits best to a sigmoidal 4- or 5-parameter logistic equation. The results of unknown samples can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

Interpretation

- 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.
- If the difference between duplicate results of a sample is >15% CV, repeat the sample.
- The theoretical minimal detecting concentration of this assay is 15 pM C-peptide 2 (20 µL sample size).
- 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.

Graph of Typical Reference Curve



For Demonstration Only – Do not use for calculations

Assay Characteristics

Analytical Sensitivity

The lowest level of C-peptide 2 that can be detected by this assay is 15 pM when using a 20 µL sample size.

Specificity

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

Precision

Intra- and Inter- Assay Variations

Sample No.	Mean C-peptide 2 Levels (pM/mL)	Intra-Assay Variations % CV	Inter-Assay Variations % 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.

Recovery

Spike and Recovery 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%

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 than 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.

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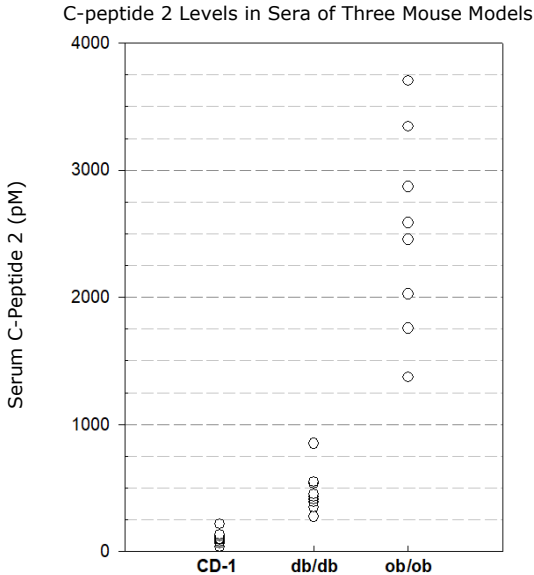
Normal Range of C-Peptide 2 Levels in Rat/Mouse Blood

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:



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

Quality Controls

The ranges for each analyte in Quality Control 1 and 2 are provided on the card insert, or available at our website SigmaAldrich.com.

Troubleshooting

- 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.
- Throughout the assay the operator should adhere strictly to the procedures with good laboratory practice.
- 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.
- Avoid cross contamination of any reagents or samples to be used in the assay.
- Make sure all reagents and samples are added to the bottom of each well.
- 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.
- Remove any air bubble formed in the well after acidification of substrate solution because bubbles interfere with spectrophotometric readings.
- High absorbance in background or blank wells could be due to
    - cross well contamination by standard solution or sample or
    - inadequate washing of wells with HRP Wash Buffer or
    - overexposure to light after substrate has been added.

Product Ordering

Products are available for online ordering at SigmaAldrich.com.

Replacement Reagents

Reagents	Cat. No.
ELISA Plate	EPDAG
10X HRP Wash Buffer Concentrate	EWB-HRP
Rat/Mouse C-Peptide 2 ELISA Standard	E8021-K
Rat/Mouse C-Peptide 2 Quality Control 1 and 2	E6021-K
Matrix Solution	EMTX-RMI
Assay Buffer	AB-PHK
Rat/Mouse C-Peptide 2 Detection Antibody	E1021-D
Rat/Mouse C-Peptide 2 Capture Antibody	E1021-C
Enzyme Solution	EHRP-88
Substrate	ESS-TMB2
Stop Solution	ET-TMB

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