

RayBio[®] Human/Mouse/Rat CART Enzyme Immunoassay Kit

Catalog #: EIA-CART, EIAM-CART, EIAR-CART

User Manual

Last revised December 1, 2015

Caution:
Extraordinarily useful information enclosed



ISO 13485 Certified

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Please read the entire manual carefully before starting your experiment

I. Introduction

Cocaine-and amphetamine-regulated transcript (CART) codes for a neuropeptide system with a number of biological roles. The high conservation of CART across species suggests that it has an important role in mammalian physiology. CART is widely expressed in the central nervous system and the periphery, but is particularly concentrated in the hypothalamus.

CART peptides appear to have an important function in the regulation of energy homeostasis. CART interacts with a number of central appetite circuits. Hypothalamic CART expression is regulated by a number of peripheral factors, including the adipose hormone leptin. Several research groups have shown that intracerebroventricular administration of CART (55-102) reduces appetite and stimulates energy expenditure. Animal studies show that CART knockout mice demonstrate small but significant increases in weekly food consumption, body weight and fat mass compared with their wild-type littermates when on a high-fat diet. These reports suggest that CART peptide acts as an endogenous inhibitor of food intake. On the other hand, hypothalamic CART may also play an orexigenic role under specific circumstances, as injection of CART (55-102) into specific hypothalamic nuclei increases food intake.

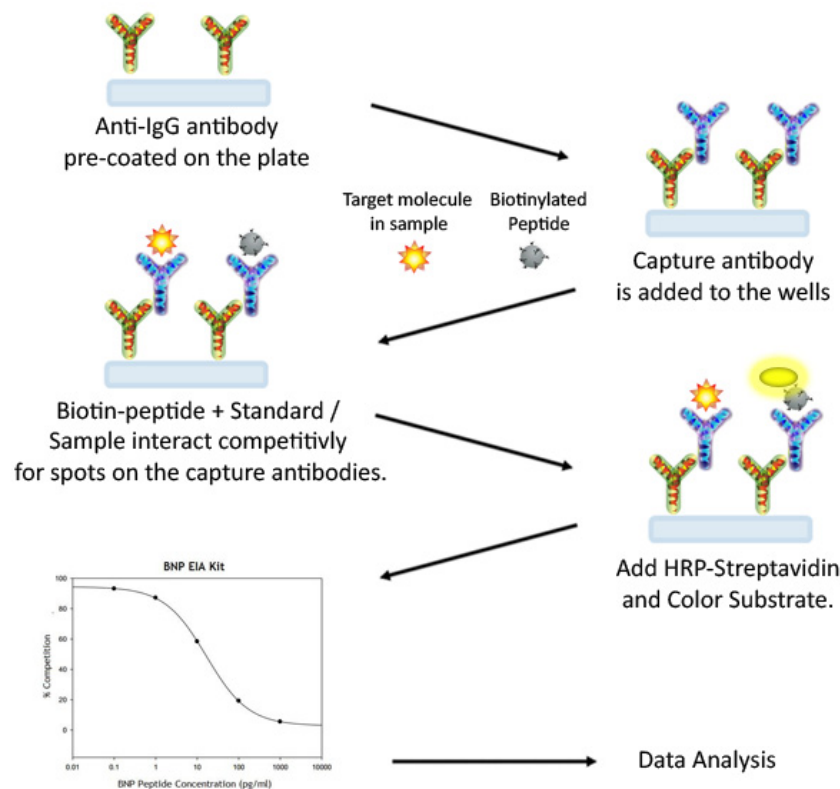
The CART system demonstrates multiple roles in energy homeostasis, depending on the internal and external milieu. The development of specific CART antagonists would be very useful in dissecting these multiple roles.

II. General Description

The RayBio® CART Enzyme Immunoassay (EIA) Kit is an in vitro quantitative assay for detecting CART peptide based on the competitive enzyme immunoassay principle.

In this assay, a biotinylated CART peptide is spiked into the samples and standards. The samples and standards are then added to the plate, where the biotinylated CART peptide competes with endogenous (unlabeled) CART for binding to the anti-CART antibody. After a wash step, any bound biotinylated CART then interacts with horseradish peroxidase (HRP)-streptavidin, which catalyzes a color development reaction. The intensity of the colorimetric signal is directly proportional to the amount of captured biotinylated CART peptide and inversely proportional to the amount of endogenous CART in the standard or samples. A standard curve of known concentration of CART peptide can be established and the concentration of CART peptide in the samples can be calculated accordingly.

III. How It Works



IV. Storage

The entire kit may be stored at -20°C to -80°C for up to 6 months from the date of shipment. For extended storage, it is recommended to store at -80°C. **Avoid repeated freeze-thaw cycles.** For prepared reagent storage, see table below.

V. Reagents

Component	Size / Description	Storage / Stability After Preparation
CART Microplate (Item A)	96 wells (12 strips x 8 wells) coated with secondary antibody.	1 month at 4°C*
Wash Buffer Concentrate (20X) (Item B)	25 ml of 20X concentrated solution.	1 month at 4°C
Standard CART Peptide (Item C)	2 vials of CART Peptide. 1 vial is enough to run each standard in duplicate.	The first standard: 2-3 days at 4°C Additional dilutions: Do not store
Anti-CART Polyclonal Antibody (Item N)	2 vials of anti-CART.	1 month at 4°C
Assay Diluent A (Item D)	30 ml, contains 0.09% sodium azide as preservative. Diluent for standards and serum or plasma.	N/A
Assay Diluent B (Item E)	15 ml of 5X concentrated buffer. Diluent for standards, cell culture media or other sample types, and HRP-Streptavidin.	1 month at 4°C
Biotinylated CART Peptide (Item F)	2 vials of Biotinylated CART Peptide, 1 vial is enough to assay the whole plate.	2-3 days at 4°C
HRP-Streptavidin Concentrate (Item G)	600 µl 80X concentrated HRP-conjugated streptavidin.	Do not store and reuse
Positive Control (Item M)	1 vial of Positive Control.	2-3 days at 4°C
TMB One-Step Substrate Reagent (Item H)	12 ml of 3,3',5,5'-tetramethylbenzidine (TMB) in buffer solution.	N/A
Stop Solution (Item I)	8 ml of 0.2 M sulfuric acid.	N/A

*Return unused wells to the pouch containing desiccant pack, reseal along entire edge.

VI. Additional Materials Required

1. Microplate reader capable of measuring absorbance at 450 nm
2. Precision pipettes to deliver 2 µl to 1 ml volumes
3. Adjustable 1-25 ml pipettes for reagent preparation
4. 100 ml and 1 liter graduated cylinders
5. Absorbent paper
6. Distilled or deionized water
7. SigmaPlot software (or other software which can perform four-parameter logistic regression models)
8. Tubes to prepare standard or sample dilutions
9. Orbital shaker
10. Aluminum foil
11. Plastic wrap

VII. Reagent Preparation

Keep kit reagents on ice during reagent preparation steps.

Note: **Assay Diluent A** should be used for dilution of samples, Item F and Item C when testing **plasma or serum samples**. **1X Assay Diluent B** should be used for dilution of samples, Item F and Item C when testing **cell culture media or other sample types**.

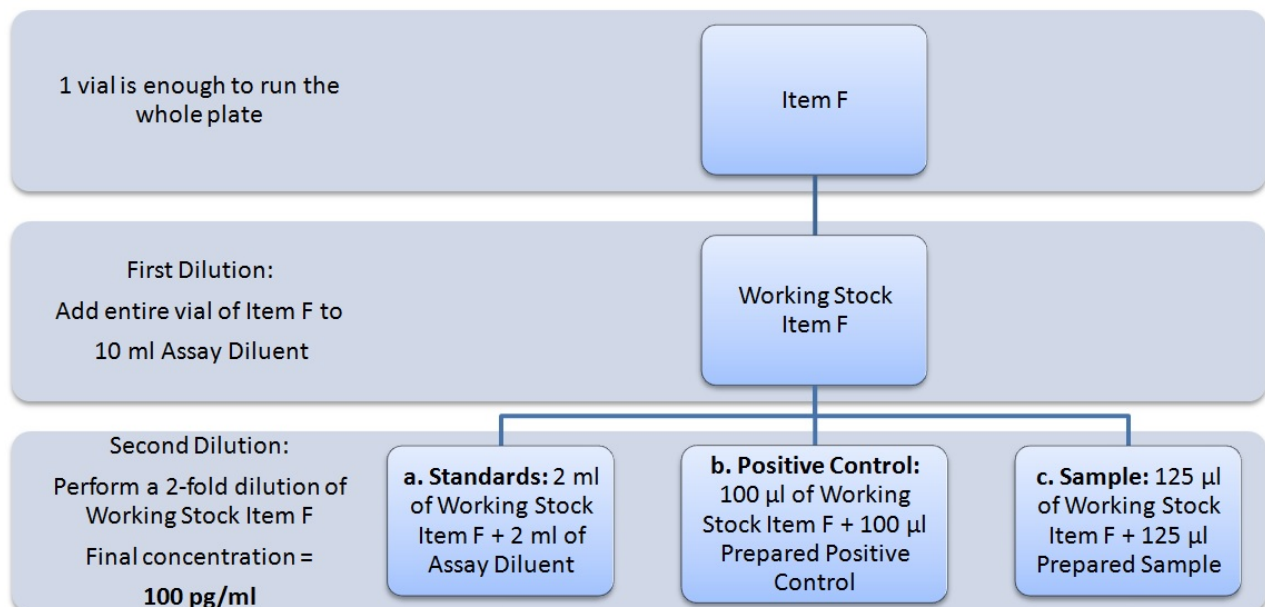
A. Preparation of Plate and Anti-CART Antibody

1. Equilibrate plate to room temperature before opening the sealed pouch.
2. Label removable 8-well strips as appropriate for your experiment.
3. 5X Assay Diluent B (Item E) should be diluted 5-fold with deionized or distilled water.
4. Briefly centrifuge the anti-CART antibody vial (Item N) Then add 50 µl of 1X Assay Diluent B to the vial to prepare the antibody concentrate. Pipette up and down to mix gently.
5. The antibody concentrate should then be diluted 100-fold with 1X Assay Diluent B. This is your anti-CART antibody working solution, which will be used in step 2 of Assay Procedure (Section VIII).

Note: The following steps may be done during the antibody incubation procedure (step 2 of Assay Procedure)

B. Preparation of Biotinylated CART (Item F)

5. Briefly centrifuge the vial of Biotinylated CART (Item F) before use.
6. See the image below for proper preparation of Item F. Transfer the entire contents of the Item F vial into a tube containing 10 ml of the appropriate Assay Diluent. This is your Working Stock of Item F. Pipette up and down to mix gently. *The final concentration of biotinylated CART will be **200 pg/ml**.*
 - a. Second Dilution of Item F for Standards: Add 2 ml of Working Stock Item F to 2 ml of the appropriate Assay Diluent. The final concentration of biotinylated CART will be **100 pg/ml**.
 - b. Second Dilution of Item F for Positive Control: Add 100 μ l of Working Stock Item F to 100 μ l of the prepared Positive Control (Item M). (See section D for Positive Control preparation) The final concentration of biotinylated CART will be **100 pg/ml**.
 - c. Second Dilution of Item F for samples: Add 125 μ l of Working Stock Item F to 125 μ l of prepared sample (see section E for sample preparation). This is a 2-fold dilution of your sample. The final concentration of biotinylated CART will be **100 pg/ml**.

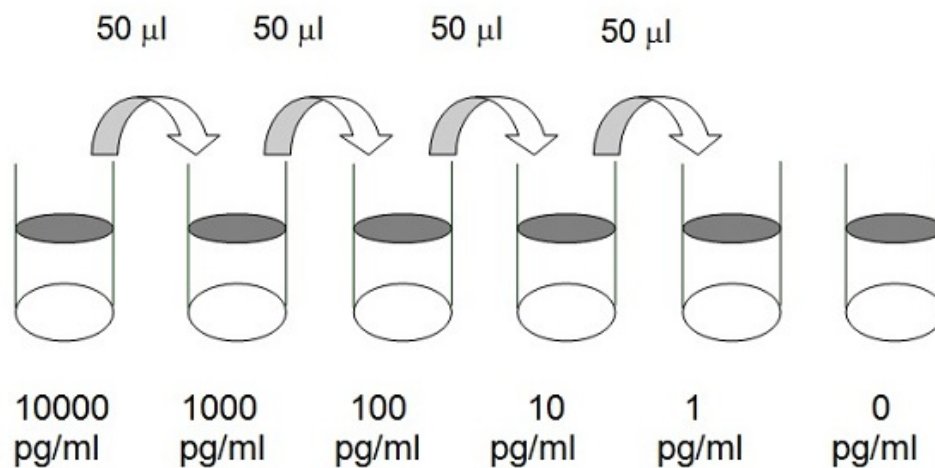


C. Preparation of Standards

7. Label 6 microtubes with the following concentrations: 10,000 pg/ml, 1,000 pg/ml, 100 pg/ml, 10 pg/ml, 1 pg/ml and 0 pg/ml. Pipette 450 μ l of biotinylated CART Item F working solution (prepared in step 6a) into each tube, except the 1,000 pg/ml (leave this one empty).

It is very important to make sure the concentration of biotinylated CART is 100 pg/ml in all standards.

8. Briefly centrifuge the vial of CART Standard (Item C). Pipette 8 μ l of Item C and 792 μ l of 100 pg/ml biotinylated CART working solution (prepared in step 6a) into the tube labeled 10,000 pg/ml. Mix thoroughly. This solution serves as the first standard (10,000 pg/ml CART standard, 100 pg/ml biotinylated CART).
9. To make the 1,000 pg/ml standard, pipette 50 μ l of the 10,000 pg/ml CART standard into the tube labeled 1,000 pg/ml. Mix thoroughly.
10. Repeat this step with each successive concentration, preparing a dilution series as shown in the illustration below. Each time, use 450 μ l of biotinylated CART and 50 μ l of the prior concentration until the 1 pg/ml is reached. Mix each tube thoroughly before the next transfer.



D. Positive Control Preparation

11. Briefly centrifuge the Positive Control vial (Item M).
12. Refer to step 6b. This is a 2-fold dilution of the Positive Control. The final concentration of biotinylated CART should still be 100 pg/ml.

The Positive Control is a cell culture media sample that serves as a system control to verify that the kit components are working. The resulting OD will not be used in any calculations; if no positive competition is observed please contact RayBiotech Technical Support. The Positive Control may be diluted further if desired, but be sure the final concentration of biotinylated CART is 100 pg/ml.

E. Sample Preparation

13. If you wish to perform a 2-fold dilution of your sample, proceed to step 6c. If you wish to perform a higher dilution of your sample, dilute your sample with the appropriate Assay Diluent before performing step 6c.
EXAMPLE (to make a 4-fold dilution of sample):
 - a. Dilute sample 2-fold (62.5 µl of sample + 62.5 µl of the appropriate Assay Diluent.).
 - b. Perform step 6c (125 µl of working solution Item F + 125 µl of sample prepared above).

The total volume is 250 µl, enough for duplicate wells on the microplate.

It is very important to make sure the final concentration of the biotinylated CART is **100 pg/ml**.

Note: Optimal sample dilution factors should be determined empirically, however you may reference below for recommended dilution factors for serum: Human=4X
Mouse=4X Rat=2X.

If you have any questions regarding the recommended dilutions you may contact technical support at 888-494-8555 or techsupport@raybiotech.com.

F. Preparation of Wash Buffer and HRP

14. If Item B (20X Wash Concentrate) contains visible crystals, warm to room temperature and mix gently until dissolved.
15. Dilute 20 ml of Wash Buffer Concentrate into deionized or distilled water to yield 400 ml of 1X Wash Buffer.
16. Briefly centrifuge the HRP-Streptavidin vial (Item G) before use.
17. Dilute the HRP-Streptavidin concentrate 80-fold with 1X Assay Diluent B.

*Note: do **not** use Assay Diluent A for HRP-Streptavidin preparation in step 17*

VIII. Assay Procedure

1. Keep kit reagents on ice during reagent preparation steps. It is recommended that all standards and samples be run at least in duplicate.
2. Add 100 μ l of Anti-CART Antibody (Item N) (See Reagent Preparation step 3) to each well. Incubate for 1.5 hours at room temperature with gentle shaking (1-2 cycle/sec). You may also incubate overnight at 4°C.
3. Discard the solution and wash wells 4 times with 1X Wash Solution Buffer (200-300 μ l each). Washing may be done with a multichannel pipette or an automated plate washer. Complete removal of liquid at each step is essential to good assay performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
4. Add 100 μ l of each standard (see Reagent Preparation Section C), Positive Control (see Reagent Preparation Section D) and sample (see Reagent Preparation Section E) in appropriate wells. Be sure to include a blank well (Assay Diluent only). Cover wells and incubate for 2.5 hours at room temperature with gentle shaking (1-2 cycles/sec) overnight or at 4°C.
5. Discard the solution and wash 4 times as directed in Step 3.
6. Add 100 μ l of prepared HRP-Streptavidin solution (see Reagent Preparation step 7) to each well. Incubate for 45 minutes at room temperature with gentle

shaking. It is recommended that incubation time should not be shorter or longer than 45 minutes.

7. Discard the solution and wash 4 times as directed in Step 3.
8. Add 100 µl of TMB One-Step Substrate Reagent (Item H) to each well. Incubate for 30 minutes at room temperature in the dark with gentle shaking (1-2 cycles/sec).
9. Add 50 µl of Stop Solution (Item I) to each well. Read at 450 nm immediately.

IX. Assay Procedure Summary

1. Prepare all reagents, samples and standards as instructed.
2. Add 100 µl anti-CART to each well. Incubate 1.5 hours at room temperature or overnight at 4°C.
3. Add 100 µl standard or sample to each well. Incubate 2.5 hours at room temperature or overnight at 4°C.
4. Add 100 µl prepared Streptavidin solution. Incubate 45 minutes at room temperature.
5. Add 100 µl TMB One-Step Substrate Reagent to each well. Incubate 30 minutes at room temperature.
6. Add 50 µl Stop Solution to each well. Read at 450 nm immediately.

X. Calculation of Results

Calculate the mean absorbance for each set of duplicate stands, controls, and samples and subtract the blank optical density. Plot the standard curve using SigmaPlot software (or other software which can perform four-parameter logistic regression models), with standard concentration on the x-axis and percentage of absorbance (see calculation below) on the y-axis. Draw the best-fit curve through the standard points.

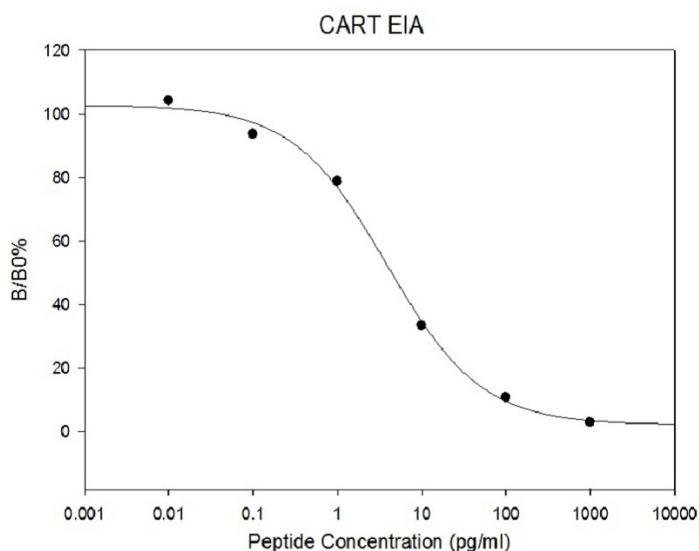
Percentage absorbance = $(B - \text{blank OD}) / (B_0 - \text{blank OD})$ where

B = OD of sample or standard and

B_0 = OD of zero standard (total binding)

A. Typical Data

These standard curves are for demonstration only. A standard curve must be run with each assay.



B. Sensitivity

The minimum detectable concentrations of CART is 13.5 pg/ml.

C. Detection Range

1-10,000 ng/ml

D. Reproducibility

Intra-Assay: CV<10%

Inter-Assay: CV<15%

E. Assay Diagram

Recommended Plate Layout:

Blank	Blank	SA1	SA1	SA9	SA9	SA17	SA17	SA25	SA25	SA33	SA33
Total Binding	Total Binding	SA2	SA2	SA10	SA10	SA18	SA18	SA26	SA26	SA34	SA34
Standard1	Standard1	SA3	SA3	SA11	SA11	SA19	SA19	SA27	SA27	SA35	SA35
Standard2	Standard2	SA4	SA4	SA12	SA12	SA20	SA20	SA28	SA28	SA36	SA36
Standard3	Standard3	SA5	SA5	SA13	SA13	SA21	SA21	SA29	SA29	SA37	SA37
Standard4	Standard4	SA6	SA6	SA14	SA14	SA22	SA22	SA30	SA30	SA38	SA38
Standard5	Standard5	SA7	SA7	SA15	SA15	SA23	SA23	SA31	SA31	SA39	SA39
Pos Control	Pos Control	SA8	SA8	SA16	SA16	SA24	SA24	SA32	SA32	SA40	SA40

Key:

Blank = Buffer Only

Total Binding = Biotin-CART only

Standard 1 = 10,000 pg/ml

Standard 2 = 1000 pg/ml

Standard 3 = 100 pg/ml

Standard 4 = 10 pg/ml

Standard 5 = 1 pg/ml

Pos Control = Biotin with Item M

XI. Specificity

Cross Reactivity: This EIA kit shows no cross-reactivity with any of the cytokines tested: Ghrelin, Nesfatin, Angiotensin II, NPY and APC.

XIV. Publications Citing This Product

1. Hehir MP, Laursen H, Higgins MF, Brennan DJ, O'Connor DP, McAullife FM. Maternal and fetal cocaine- and amphetamine-regulated transcript in diabetic and non-diabetic pregnancy. *Gynecol Endocrinol*. 2012;28(9):682–685.

Species: Human

Sample Type: Serum

2. Wysokinski A., et al. Blood serum levels of CART peptide in patients with schizophrenia. *Psychiatry Research*. 2014 August, epub ahead of print. DOI: 10.1016/j.psychres.2014.08.017 on clozapinemonotherapy

Species: Human

Sample Type: Serum

XIII. Troubleshooting Guide

Problem	Cause	Solution
Poor standard curve	• Inaccurate pipetting • Improper standard dilution	• Check pipettes • Briefly centrifuge Item C and dissolve the powder thoroughly by gently mixing
Low signal	• Improper preparation of standard and/or biotinylated antibody • Too brief incubation times • Inadequate reagent volumes or improper dilution	• Briefly spin down vials before opening. Dissolve the powder thoroughly. • Ensure sufficient incubation time; assay procedure step 2 may be done overnight • Check pipettes and ensure correct preparation
Large CV	• Inaccurate pipetting • Air bubbles in wells	• Check pipettes • Remove bubbles in wells
High background	• Plate is insufficiently washed • Contaminated wash buffer	• Review the manual for proper wash. If using a plate washer, ensure that all ports are unobstructed. • Make fresh wash buffer
Low sensitivity	• Improper storage of the ELISA kit • Stop solution	• Follow storage recommendations in sections IV and V. Keep substrate solution protected from light. • Add stop solution to each well before reading plate

RayBio[®] ELISA Kits

Over 2,000 ELISA kits available, visit www.RayBiotech.com/ELISA-Kits.html for details.

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