CART (55-102)
(Rat, Mouse, Bovine)

WESTERN BLOT KIT PROTOCOL

(Catalog# WBK-003-62)

PHOENIX PHARMACEUTICALS, INC.

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STORAGE

1. The primary antibody, blocking buffer, and antibody diluent should be stored at -20° C.
2. Secondary antibody should be stored at 2-8°C. **DO NOT FREEZE!**
3. Detection reagents A and B are light sensitive. They are stable for 6 months at 2-8°C in the dark. Please keep bottles closed to prevent microbial or dust contamination.

CAUTION:

Investigational device. Limited by law to investigational use.

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

KIT CONTENTS

1. CART (55-102) (Rat, Mouse, Bovine) Purified Rabbit Primary IgG Antibody (*1 vial, 200 µg*)
2. CART (55-102) Standard Peptide (1 vial, *20 µg*)
3. Donkey Anti-Rabbit IgG Whole Antibody HRP Conjugate (1 vial, *50 µl*)
4. 10x TBST, pH 7.6 (*2 bottles, 100 ml*)
5. 10x TBS, pH 7.6 (*1 bottle, 50 ml*)
6. 10x Blocking Buffer and Antibody Diluent (*1 bottle, 50 ml*)
7. Detection Reagent A (*1 bottle, 30 ml*)
8. Detection Reagent B (*1 bottle, 30 ml*)
9. Protocol Booklet

(Note: This kit contains enough reagents to perform 5 blots.)

REFERENCES

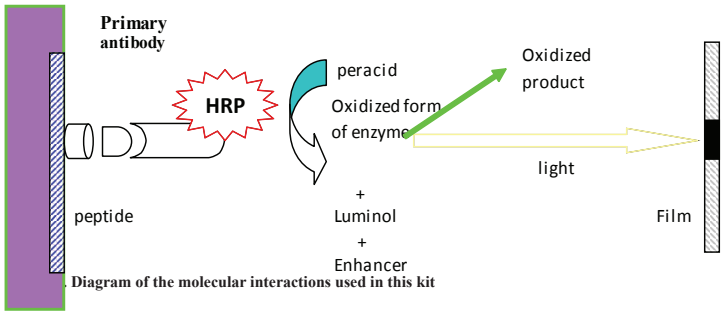
1. Yanik T, Dominguez G, Kuhar MJ, Del Giudice EM, Loh YP. The Leu34Phe ProCART mutation leads to cocaine- and amphetamine-regulated transcript (CART) deficiency: a possible cause for obesity in humans. Endocrinology. 2006 Jan;147(1):39-43.
2. Jones DC and Kuhar MJ. Cocaine-amphetamine-regulated transcript expression in the rat nucleus accumbens is regulated by adenylyl cyclase and the cyclic adenosine 5'-monophosphate/protein kinase a second messenger system. J Pharmacol Exp Ther. 2006 Apr;317(1):454-61.
3. Vicentic A, Dominguez G, Hunter RG, Philpot K, Wilson M, and Kuhar MJ CART peptide levels in blood exhibit a diurnal rhythm: regulation by glucocorticoids. Endocrinology (2004) 145: 4119–4124.
4. Kuhar MJ. Yoho LL, CART peptide analysis by Western blotting. Synapse (1999) Sep 1;33(3):163-71.

INTRODUCTION

This Western Blot kit by Phoenix Pharmaceuticals, Inc. is designed for rapid and sensitive immunoenzymatic detection of specific peptides immobilized on nitrocelluloise or polyvinylidene fluoride (PVDF) membranes. Transfering can be done from SDS-PAGE / Tricine-PAGE gels after electrophoresis (Western Blot), or binding directly from solution (Dot Blot). The purified primary antibody (IgG) used for this assay was raised against a synthetic peptide. A highly purified standard peptide is also included with this kit to be used as a positive control or to pre-block the primary antibody. All reagents necessary to provide results on nitrocellulose or PVDF membranes are included. This kit enables detection of antigen at levels of 500 ng or lower.

GENERAL DESIGN OF THIS KIT

This Western Blot kit by Phoenix Pharmaceuticals, Inc. is based on the principle of enzyme-linked immunodetection of peptide by antigen-specific antibody (supplied with this kit). Anti-IgG secondary antibodies conjugated to horseradish peroxidase (HRP) then react with a chemiluminescent substrate in the presence of a chemical enhancer. This produces a light signal that can be captured by short exposure to a blue-light sensitive autoradiography film or on a Molecular Imager system. This kit brings a new level of convenience and reliability to a non-isotopic procedure while providing fast, highly sensitive detection for membrane-bound peptides and proteins.



. Diagram of the molecular interactions used in this kit

Note: Read this protocol in its entirety before starting the assay. Each kit contains reagents sufficient for 5 Western Blots.

REAGENT PREPARATION

1. **10x TBST Buffer:** Dilute each bottle with 900ml distilled, deionized water. Stir to homogeneity.
2. **10x TBS Buffer:** Dilute with 450ml distilled, deionized water. Stir to homogeneity.
3. **Primary antibody IgG:** Add 200 µl of Antibody Diluent to rehydrate the primary antibody IgG.
4. **Detection reagent:** Mix equal volumes of Detection Reagent A and Detection Reagent B to make the enhanced chemiluminescent developing solution. This mixed detection reagent is stable for 6-8hours at room temperature.
5. **10x blocking buffer and antibody diluent:** Dilute with 450 ml distilled, deionized water. Stir to homogeneity.

ASSAY PROTOCOL

Blocking of the membrane (blot):

To saturate nonspecific protein binding sites, incubate the membrane for in blocking buffer / antibody diluent (TBST containing 1% blot-qualified BSA). For nitrocellulose membranes, incubate for 30 minutes. For PVDF membranes, incubate for 60 minutes.

Primary Antibody Binding:

1. To bind the primary antibody, replace the blocking solution (which can be re-used several times) with blocking buffer / antibody diluent containing an appropriate dilution of primary antibody. The recommended primary antibody dilution is 1:500 ~ 1:2000. Incubate the blot for 30 ~ 60 minutes with gentle agitation at room temperature (or overnight at 2 ~ 8 °C).
2. Wash the membrane three times with TBST for 5 ~ 10 minutes each to remove any unbound primary antibody.

Secondary Antibody Binding:

1. Incubate blot with blocking buffer / antibody diluent containing the appropriate dilution of anti-rabbit IgG-HRP conjugate for 30 minutes with gentle agitation. The recommended secondary antibody dilution is 1:3000 ~ 1:6000.
2. Wash the blot with TBST three times for 10 minutes each to remove any unbound secondary antibody.
3. Briefly rinse twice with TBS to remove Tween 20 from the blot surface.

ECL development:

1. Mix equal volumes of detection reagent A and detection reagent B to cover the membranes (based on 0.125 ml / cm^2 membrane).
2. Drain the excess buffer from the washed blot (membrane) and place it on a piece of plastic (Saran) wrap or on a glass plate with the protein or peptide side facing up. Add mixed detection solution to the peptide or protein side of the membrane. Make sure the entire surface of the membrane is covered with the mixed detection solution. Incubate for exactly 5 minute without agitation. Drain off excess detection solution and wrap membranes in plastic (Saran) wrap+. Using gloved fingers, smooth out all air pockets on the membrane surface by pressing gently.
3. Place the blot with the peptide or protein face up, in the film cassette. Work as quickly as possible to minimize the delay between incubation of the blot in the mixed detection solution and exposure it to the film. In a dark room, place a sheet of blue light sensitive film on top of the blot, close the cassette and expose for 1 minute according to signal strength. The second exposure may vary from 30 seconds to 7 minutes depending on target signal and background. Perform this step in a dark room using a red safe light..

EXAMPLE SAMPLE PREPARATION

1. Cultured cells:

   The target cells or CART gene transfected cells were extracted in lysis buffer (Pierce, Rockford, IL) plus protease inhibitors (complete mini protease inhibitor cocktail; Roche Molecular Biochemicals, Indianapolis, IN). The soluble extracts, obtained after centrifugation of the total cell lysates at 15,000 x g for 10 min, were assayed for protein content. Forty micrograms of protein from each extract were analyzed by Western blot. The blots were probed with anti-CART (55–102) IgG (1:5000 dilution; Phoenix Pharmaceuticals Inc.) overnight at 4°C.

2. Rat brain section:

   The rats weighing ~300g were used in all experiments. Animals were group-housed prior to surgery and individually housed thereafter. Animals were allowed access to food and water ad libitum and maintained on a 12 hour light/dark cycle. According to previous studies, CART peptides are partly located in the ventral tegmental area and nucleus accumbens, regions of the brain included in the mesolimbic dopamine pathway. Therefore, the region of hypothalamus, nucleus accumbens, frontal lobe region has been used as described in previous publications . Briefly, rats were decapitated, and the designated region was dissected out and frozen. Total protein was extracted in 50 µl of lysis buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 5 nM acidic acid, 1 µg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Fifteen to 20 µg of total protein was loaded in 1× sample buffer [62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% glycerol, and 0.01% (w/v) bromphenol blue] onto 16% Novex precast SDS-Tris-glycine gel (Invitrogen, Carlsbad, CA). Proteins were transferred at 30V at 4°C overnight.

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diagnostic procedures

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