



HOME

NEW PRODUCTS

SEARCH

PEPTIDE ANTIBODIES

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RADIOACTIVE PEPTIDES

CATALOG PEPTIDES

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TECHNICAL SUPPORT



**PHOENIX
PHARMACEUTICALS,
INC.**
The Peptide Elite

DOT BLOT KIT ASSAY PROTOCOL

TABLE OF CONTENTS

- [Introduction](#)
- [Contents](#)
- [Storage Conditions](#)
- [Preparation of Reagents](#)
- [Principal of Western Blot Kit](#)
- [Standard Protocol](#)

▶ [For print-friendly PDF file, click here.](#)

INTRODUCTION

Phoenix's Immunodot Blot Kit is designed for the rapid and sensitive immunoenzymatic detection of specific peptides immobilized on nitrocellulose or polyvinylidene fluoride (PVDF) membranes with dot-blot apparatus . The purified primary antibody (IgG) used for this assay was raised against a synthetic peptide. A highly purified standard peptide is included with the kit to be used as a positive

control or to pre-block the primary antibody. All reagent necessary to produce fast and sensitive results on nitrocellulose or PVDF membrane are included. This kit enable the detection of antigen levels of 100ng or lower.

CONTENTS

1. Purified rabbit primary antibody (IgG), (lyophilized powder), 200 μ g
2. Donkey anti-rabbit IgG whole antibody HRP conjugate, 50 μ l
3. Standard peptide, (lyophilized powder), 12.8 μ g
4. 10 x TBST pH 7.6 (Catalog No. WB-B001), 100 ml
5. 10x TBS pH 7.6, (Catalog No. WB-B002), 50 ml
6. Blocking Buffer and Antibody Diluent, (Catalog No. WB-B003), 400ml
7. Detection Reagent A, (Catalog No. WB-B006), 30 ml
8. Detection Reagent B, (Catalog No. WB-B007), 30 ml
9. Kit Protocol

Notes: This kit supplies sufficient reagents to perform 5 blots.



STORAGE CONDITIONS:

1. The primary antibody, Blocking Buffer and Antibody Diluent should be stored below -20° C.

3. Detection Reagent A and B are light sensitive. They are stable for 6 months at 2 ~ 8 ° C in the dark. Keep bottles closed to prevent microbial or dust contamination.

PREPARATION OF REAGENTS:

1. **10 X TBST buffer:** Dilute each bottle with 900ml distilled deionized water . Stir to homogeneity.
2. **10 X TBS buffer:** Dilute with 450ml distilled deionized water . Stir to homogeneity.
3. **Primary antibody IgG :** Add 200 μ l of Antibody Diluent to rehydrate the primary antibody IgG.
4. **Detection Reagent:** Mix 10 ml of Detection Reagent A with 5 μ l of Detection Reagent B to make enhanced chemiluminescent developing solution. It should be used within 30 minutes.



PRINCIPAL OF THE PHOENIX WESTERN BLOT KIT

Phoenix's Western Blot Kit is based on the enzyme-linked immunodetection of antigen-specific antibody (supplied with kit) using anti-IgG secondary antibodies conjugated to horseradish peroxidase (HRP), which reacts 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 the Molecular Imager system. This kit brings a new level of convenience and reliability to a non-isotopic procedure while providing a fast, highly sensitive detection for membrane bound peptides / proteins.

STANDARD PROTOCOL

Load Sample to Dot Blot Apparatus.

1. Preparation of standard peptide dilution with TBS.

Blocking of Membrane (Blot)

2. Cut nitrocellulose membrane to the appropriate size to fit in the dot-blot apparatus. Wet the membrane by immersing it in deionized water. PVDF membrane pre-wet with methanol for seconds and soak in deionized water. Add 100 μ l of TBS and 100 μ l of standard peptide sample to each well. Draw the liquid through the membrane by applying suction pump, and then remove the nitrocellulose from the apparatus.
3. To saturate nonspecific protein binding sites, incubate the membrane 30 minutes in Blocking Solution and Antibody Diligent (TBST containing 1% Blot-qualified BSA) for nitrocellulose membrane; incubate 60 minutes for PVDF membrane.

Primary Antibody Binding

4. To bind primary antibody, replace the blocking solution with Blocking Buffer and Antibody Diluent containing appropriate dilution of primary antibody (the rabbit anti-CNP22 purified IgG 1:500~ 1000 in a minimal volume). Incubate the membrane for 30~ 60 minutes with gentle agitation at room temperature (or overnight at 2~ 8° C).
5. To remove unbound antibody, wash the membrane three times with TBST for 5~ 10 minutes each.

Secondary Antibody Binding

6. Incubate membrane with Blocking Buffer and Antibody Diluent containing the appropriate

dilution of anti-rabbit IgG-HRP conjugate for 30 minutes with gentle agitation. We recommend that the secondary antibody dilution be in the range of 1:5000~ 10000.

7. Wash membrane with TBST three times for 10 minutes each to remove unbound secondary antibody.
8. Briefly rinse twice with TBS to remove Tween 20 from the membrane surface.

ECL Developing

9. Mix 10 ml of Detection Reagent A and 5 μ l of Detection Reagent B to cover the membrane (based on 0.125~ 0.25 ml/ cm^2 membrane). The mixed solution should be used within 30 minutes.
10. Drain the excess buffer from the washed membrane and place it on a piece of Saran Wrap or 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 and wrap membrane in Saran Wrap. Using gloved fingers, smooth out all air pockets on the membrane surface by pressing gently.
11. Place the blot with the peptide or protein face up, in the film cassette. Work as quickly as possible to minimize the delay between incubating the blot in the mixed detection solution and exposing it to the film. In a dark room, place a sheet of blue light sensitive film on top of the membrane, close the cassette and expose for 5 seconds ~ 10 minutes according to signal strength. The second exposure can vary from 1 minute to 30 minutes depending on target signal and background. Perform this step in a dark room using red safe light.

