Peptide-Carrier™ Kit

E6600S

50 Reactions Store at –70°C (see note below)

Description: The Peptide-Carrier Kit allows peptides of interest to be detected on Western blots or peptide arrays through ligation to a carrier protein. The resulting peptide-carrier ligation product is compatible with a variety of applications including Western blotting, peptide arrays and kinase assays. Simply mixing a peptide (N-terminal cysteine required) with the supplied Carrier Protein 39 (CP39, ~39 kDa) results in a peptide-carrier ligation product via intein-mediated protein ligation (IPL; Appendix 1) (1-3).

Since most antigenic peptides are routinely synthesized with a cysteine, a single peptide containing an N-terminal cysteine can now be used to generate antibodies as well as a control for Western blots (4). The antigenic peptide may contain modified residues, such as a phosphorylated tyrosine. The ligated product has one antigenic peptide per CP39 resulting in a single, sharp band on Western blots (Figure 1).

In addition, peptide substrates ligated to Carrier Proteins 39 or 27 can be used for enzymatic assays and subsequent Western blot analysis (Figure 3) (5). This kit also makes it possible to produce peptide substrates for enhanced sensitivity in array analysis (Figures 4 & 5) (6).

Applications:

- Generation of positive controls for Western blot analysis.
- Production of substrates for enhanced sensitivity in peptide array or dot blot assay
- Production of substrates for protein modification, such as kinase assays
- Ligation of a peptide containing natural or unnatural amino acids.

Can be stored for 1 week or less at –20°C
Should be stored at –70°C upon arrival
Shipped on wet ice. Avoid freeze/thaw cycles

Kit Components:

Carrier Protein 39 (Western) 50 µl (10 µg)
~39 kDa protein to which a peptide or protein with an N-terminal cysteine can be ligated. Aliquot into 5 µl fractions. **Store at –70°C**.
Thaw at room temperature and use immediately.
Other components can be stored at –20°C.

10X Carrier Protein (CP) Reaction Buffer 0.5 ml

2X Control Peptide, PB1 50 µl (1 mM)
Peptide derived from mouse Bad protein synthesized with an N-terminal cysteine (CTRSHSSYPNEYEEDEEMEEEL; MW, 2862 Da).

3X SDS Sample Buffer (Reducing) 0.5 ml
70 mM Tris-HCl (pH 6.8), 33 mM NaCl, 1 mM Na_2EDTA, 2% (w/v) SDS, 40 mM dithiothreitol, 0.01% (w/v) Bromophenol Blue and 10% glycerol.

Protocols

This kit provides carrier protein (0.2 mg/ml) for 50 ligation reactions (at least 250 lanes) for Western blot analysis (Protocol I). The extent of ligation is typically 75–90% when a final peptide concentration of 0.5 mM is used. **It is not necessary to run Coomassie Blue stained SDS-PAGE unless you wish to check the extent of ligation.**

Note: Please refer to Table 1 for a list of reagents that may affect the extent of ligation.

Protocol I: Western Blotting

1. Dissolve your peptide in water to a concentration of 1 mM.
2. Thaw the carrier protein at room temperature.
3. Mix the following (in order):

Peptide Solution (1 mM)	12.5 µl
10X CP Reaction Buffer	2.5 µl
dH_20	9.0 µl
Carrier Protein 39 (0.2 mg/ml)	1. 0 µl
Total volume	25.0 µl

4. Incubate at room temperature for 15–60 minutes or at 4°C overnight.
5. Add 12.5 µl of 3X SDS Sample Buffer.
6. Heat at 95°C for 5 minutes.
7. Load 5–10 µl/lane (~ 25–50 ng) for Western blotting.

Note: The sample can be stored at –20°C after the addition of 3X SDS Sample Buffer.

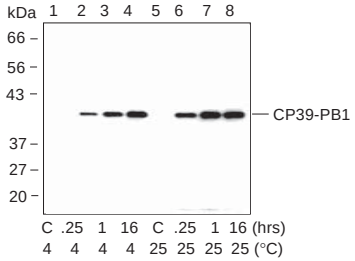


Figure 1: *Western blot analysis of antiserum raised against control peptide, PB1. PB1 was ligated to Carrier Protein 39 (Western) (NEB #E6602) as in Protocol I. The reaction times and temperatures are indicated. The samples were electrophoresed on a 12% SDS polyacrylamide gel and transferred to nitrocellulose. Anti-PB1 Peptide Antibody (NEB #E6608S) was used as the primary antibody. Lanes 1 and 5 are controls (C) which contain only Carrier Protein 39 (Western).*

Protocol II: Coomassie Blue SDS-PAGE

We recommend the use of a Coomassie stained SDS-PAGE to check your ligated product (by a shift in mobility) only if you suspect that the ligation is not working.

1. Dissolve your peptide in water to a concentration of 1 mM.
2. Thaw the carrier protein at room temperature.
3. Mix the following (in order):

Peptide Solution (1 mM)	12.5 µl
10X CP Reaction Buffer	2.5 µl
Carrier Protein 39 (0.2 mg/ml)	10.0 µl
Total volume	25.0 µl

4. Incubate at room temperature for 15–60 minutes or at 4°C overnight.
5. Add 12.5 µl of 3X SDS Sample Buffer.
6. Heat at 95°C for 5 minutes.
7. Load 15 µl/lane (~ 0.8 µg carrier protein) for Coomassie Blue staining. Dilute 10–20 fold for Western blot analysis.

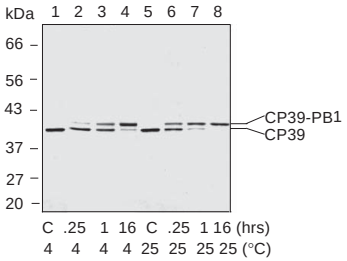


Figure 2: Analysis of peptide ligation by Coomassie blue stained SDS-PAGE*. The control peptide, PB1, was ligated to CP39, as in Protocol II. The ligation samples were electrophoresed on a 12% SDS polyacrylamide gel and examined by Coomassie blue staining. Lanes 1 and 5 are controls (C) which contain only CP39.*

Table I: *Effect of Reagents on the Ligation Reaction.*

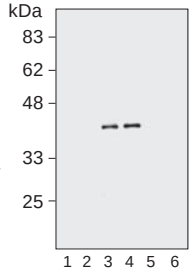
Reagent	Concentration	Extent of ligation (Control = 100%)
NaCl	0.05–1 M	> 75%
Triton X-100	0.1–1%	> 75%
Tween-20	0.05–0.5%	> 75%
NP-40	0.5–2%	> 75%
DMSO	1–25%	> 75%
Glycerol	0–50%	> 75%
Dithiothreitol	0–5 mM	> 75%
Urea	0–6 M	> 25–75%
SDS	0.05–1%	> 25–75%

Note: The data were produced by analysis of a Coomassie blue stained SDS-PAGE. CP39 was ligated to PB1, in the presence of the reagents overnight at 4°C. 10% ligation was sufficient to generate a positive signal on Western blots.

Additional Applications:

Assay Substrates for Enzymatic Modification: In the example below, the phosphorylation activity of Abl Protein Tyrosine Kinase was examined.

Figure 3: *Kinase Assay. A peptide containing a candidate phosphorylation site was synthesized with an additional N-terminal cysteine (CGSNEAIYAAPFAKKK; 7) and ligated to CP39. The ligated product was phosphorylated with Abl Protein Tyrosine Kinase (NEB #P6050) and then subjected to Western blot analysis using anti phospho tyrosine antibody (Cell Signaling Technology). A positive signal was detected only in lanes 3 and 4 where the ligated product was treated with Abl Kinase.*



Lane 1: CP39.
Lane 2: CP39 + Abl Kinase (100 units).
Lane 3: CP39-peptide + Abl Kinase (50 units).
Lane 4: CP39-peptide + Abl Kinase (100 units).
Lane 5: CP39-peptide.
Lane 6: Abl Kinase (100 units).

Peptide Array or Dot Blot Assay: This kit allows production of peptide substrates for array analysis in research laboratories (5). CP39 is utilized as a carrier to improve the binding of peptide substrates to commonly used membranes (such as nitrocellulose, nylon and PVDF). The ligated carrier peptide is retained due to the high affinity of CP39 for these membranes, thereby significantly enhancing the sensitivity of detection in antibody binding assays, such as epitope mapping. Since the ligation stoichiometry is precisely one-to-one, synthetic peptides ligated to a carrier protein become normalized during array production.

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Example I: Dot blot assay using CP39-PB1 ligation product.

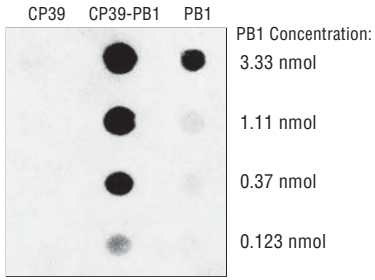


Figure 4: *Comparison of CP39-PB1 and unligated PB1 peptide by dot blot analysis on a nitrocellulose membrane.*

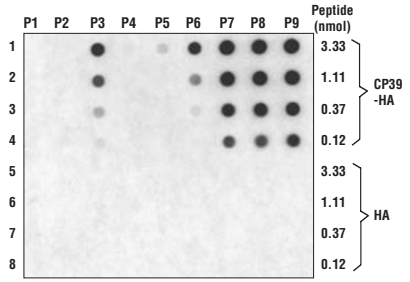
The ligation was carried out in the presence of 0.5 mM PBI peptide and 0.08 mg/ml CP39 overnight at 4°C. Ligation product (CP39-PBI) and the controls (CP39 and PB1 peptide) were diluted into phosphate buffered saline (PBS) in a microtiter plate (10 µl into 140 µl PBS) followed by three-fold serial dilutions in PBS. The samples (each in 100 µl volume) were applied to a nitrocellulose membrane (0.45 µm) using a Bio-Dot Microfiltration Apparatus (Bio-Rad, Hercules, CA). Immunoblotting was performed using polyclonal anti-PB1 antibody. The signal from the CP39-PB1 ligation product was significantly better than the unligated PB1 peptide.

Example II: Alanine scan of the hemagglutinin (HA) epitope. HA peptide library consisting of nine peptides was synthesized with a N-terminal cysteine (Figure 5A). The ligation was carried out in the presence of 0.5 mM peptide and 0.8 mg/ml CP39 overnight at 4°C. The ligation product was diluted into PBS in a microtiter plate (10 µl into 140 µl PBS) followed by serial three-fold dilutions in PBS. The ligated samples (rows 1–4) and unligated peptide (rows 5–8) were arrayed on a 0.45 µM nitrocellulose membrane (Figure 5B). The blot was then reacted with an anti-HA monoclonal antibody (Cell Signaling Technology, 1:2000 dilution). The data indicated that the residues mutated in P1, P2, P4 and P5 are essential for antibody recognition and the unligated peptide (rows 5-8) did not produce a detectable signal.

Figure 5: *Alanine scan of the hemagglutinin (HA) epitope*

HA tag	P1	CAGAG **A** P Y D V P D Y A
HA tag	P2	CAGAG Y **A** Y D V P D Y A
HA tag	P3	CAGAG Y P **A** D V P D Y A
HA tag	P4	CAGAG Y P Y **A** V P D Y A
HA tag	P5	CAGAG Y P Y D **A** P D Y A
HA tag	P6	CAGAG Y P Y D V **A** D Y A
HA tag	P7	CAGAG Y P Y D V P **A** Y A
HA tag	P8	CAGAG Y P Y D V P D **A** A
HA tag	P9	CAGAG Y P Y D V P D Y A

A) *HA peptide library was synthesized with an N-terminal cysteine. P9 contains the wild type sequence corresponding to residues Tyr98 to Ala106 of the hemagglutinin (HA) protein. Each of the other 8 peptides (P1-P8) carries a single substitution with an alanine residue.*



B) *The ligated products (rows 1–4) and the unligated peptides (rows 5–8) were arrayed on a 0.45 µm nitrocellulose membrane. Each peptide was ligated to Carrier Protein 39 (Array) (NEB #E6603) overnight at 4°C. The blot was reacted with an anti-HA monoclonal antibody.*

Frequently Asked Questions:

Do I have to run a Coomassie-stained SDS-PAGE gel before the Western blot?

No. We recommend the Coomassie-stained SDS-PAGE gel to check your ligated product only if you suspect that your ligation is not working. To check ligation run a control of carrier protein.You may not detect a shift of the ligation product on SDS-PAGE if CP39 is ligated to a short peptide. Use CP27 (NEB #E6606S or #E6607S) for easy detection of ligation by gel shift.

Is Western blot analysis possible if the extent of ligation is not very good?

In Western Blot analysis, even a small amount of ligated product will give a positive signal (Figure 1, 27 ng of CP39 was loaded per well).

What are the causes of inefficient ligation?

The possibilities for ligation problems are:

1. Peptide does not possess an N-terminal cysteine or the sulfhydryl group is oxidized.
2. The peptide solution may be very acidic and cause the pH of the reaction to drop significantly. If the pH of your peptide solution is below 6 dissolve the peptide in 1 M Tris-HCl (pH 9.0).
3. Peptide preparation contains impurities. Purify the peptide.
4. Concentration of peptide is incorrect.
5. Peptide or ligation product is insoluble.
6. The carrier protein has lost its ligation capabilities due to repeated freeze-thaw cycles or long-term storage at –20°C. Use the control peptide, PB1, to test ligation.

What controls can be done for a Western blot?

Monitor the transfer of the proteins onto the nitrocellulose membrane using the Prestained Protein Marker, Broad Range (NEB #P7708). Always use the antibodies at the recommended concentrations. Check your ligation using a Coomassie blue stained SDS-PAGE gel.

Can an insoluble peptide be used?

Even if the peptide is not completely soluble, the amount of ligation may be sufficient for Western blot analysis. If necessary, dissolve the peptide first in dimethyl sulfoxide (DMSO), add water, and use in ligation. You can also add the solid peptide to the ligation reaction.

Does the peptide have to be purified?

We have found ligation to work with both crude and purified peptides. However, consistent results are obtained when peptide purity is above 30%. To ensure the greatest amount of ligated product we purify our peptide on a Vydac semi-preparative C18 column.

How do I calculate the concentration of the peptide solution?

If the molecular mass of the peptide is 2862 daltons then the calculation is as follows:

1 M = 2862 gm/l

1 mM = 2.862 mg/ml

Can a ligated peptide be used for (ELISA)?

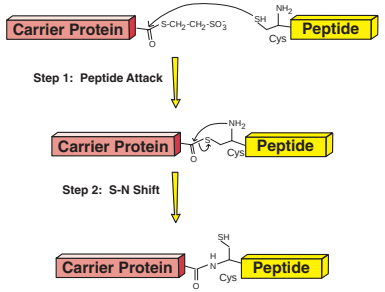
Yes. The use of ligated peptide (CP39-peptide) significantly increased the sensitivity of certain peptide substrates in ELISA.

Can I use crude antibody in the Western blot analysis?

Yes. We have tested crude mouse and crude rabbit antibodies and observed a single distinct band of the ligated product (Figure 1). CP39 is a recombinant protein from *Haemophilus haemolyticus*, which has low cross reactivity with mouse and rabbit antibodies. Various dilutions of antibodies should be tested.

Appendix: Chemical Mechanism of Intein Mediated Protein ligation (IPL)

The IPL reaction allows the ligation of a synthetic peptide with an N-terminal cysteine residue to a bacterially expressed protein with a C-terminal thioester through a native peptide bond (1,2). To create a C-terminal thioester the IMPACT-TWIN System (NEB #E6950S) may be used (1,3).



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Companion Products:

Carrier Protein 39 (Western)	
#E6602S	50 Reactions
Carrier Protein 39 (Array)	
#E6603S	25 Reactions
2X Control Peptide, PB1	
#S6605S	50 µl
10X Carrier Protein (CP) Reaction Buffer	
#B6604S	0.5 ml
Carrier Protein 27 (Western)	
#E6606S	50 Reactions
Carrier Protein 27 (Array)	
#E6607S	25 Reactions
Anti-PB1 Peptide Antibody	
#E6608S	0.05 ml