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TITLE:

Evaluation of Protein–Protein Interactions using an On-Membrane Digestion Technique

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KEYWORDS:

Immunoprecipitation; LC/MS/MS; proteomics; protein interaction; PVDF membrane; screening

SUMMARY:

Here, we present a protocol for an on-membrane digestion technique for the preparation of samples for mass spectrometry. This technique facilitates the convenient analysis of protein–protein interactions.

ABSTRACT:

Numerous intracellular proteins physically interact in accordance with their intracellular and extracellular circumstances. Indeed, cellular functions largely depend on intracellular protein–protein interactions. Therefore, research regarding these interactions is indispensable to facilitating the understanding of physiologic processes. Co-precipitation of associated proteins, followed by mass spectrometry (MS) analysis, enables the identification of novel protein interactions. In this study, we have provided details of the novel technique of immunoprecipitation-liquid chromatography (LC)-MS/MS analysis combined with on-membrane digestion for the analysis of protein–protein interactions. This technique is suitable for crude immunoprecipitants and can improve the throughput of proteomic analyses. Tagged recombinant proteins were precipitated using specific antibodies; next, immunoprecipitants blotted onto polyvinylidene difluoride membrane pieces were subjected to reductive alkylation. Following trypsinization, the digested protein residues were analyzed using LC-MS/MS. Using this technique, we were able to identify several candidate associated proteins. Thus, this

method is convenient and useful for the characterization of novel protein–protein interactions.

INTRODUCTION:

Although proteins play constitutive roles in living organisms, they are continually synthesized, processed, and degraded in the intracellular environment. Furthermore, intracellular proteins frequently physically and biochemically interact, which affects the function of one or both[1-3]. For example, the direct binding of spliceosome-associated protein homolog CWC22 with eukaryotic translation initiation factor 4A3 (eIF4A3) is necessary for the assembly of the exon junction complex[4]. Consistent with this, an eIF4A3 mutant that lacks affinity for CWC22 fails to facilitate exon junction complex–driven mRNA splicing[4]. Thus, the study of protein interactions is crucial for the precise understanding of physiologic regulation as well as of cellular functions.

Recent advances in mass spectrometry (MS) have been applied to the comprehensive analysis of protein–protein interactions. For instance, the co-precipitation of endogenous proteins or exogenously introduced tagged proteins with their associated proteins, followed by MS analysis, enables the identification of novel protein interactions[5]. However, one major bottleneck of MS/MS analysis is poor recovery from tryptic digests of protein samples. For conducting proteomic analyses on cell lysates, in-gel and on-membrane digestion techniques are generally employed to prepare MS/MS samples. We have previously compared an in-gel digestion procedure with an on-membrane digestion technique[6], and showed that the latter was associated with better sequence coverage. Polyvinylidene difluoride (PVDF) membrane may be suitable for this purpose because it is mechanically robust and resistant to high concentrations of organic solvents[7,8], permitting the enzymatic digestion of immobilized proteins in the presence of 80% acetonitrile[9]. Furthermore, immobilization on a membrane can induce conformational changes in target proteins, leading to improvements in tryptic digestion efficiency[10]. Accordingly, in this article, we have described the use of immunoprecipitation-LC/MS/MS analysis of protein interactions using an on-membrane digestion technique. This simple method facilitates the convenient analysis of protein–protein interactions even in non-specialist laboratories.

PROTOCOL:

1. Immunoprecipitation

NOTE: We used non-sodium dodecyl sulfate (SDS) lysis buffer and citrate elution, as described in the following sections. However, the use of an alternative in-house immunoprecipitation technique may be also applicable for preparing LC-MS/MS samples.

1.1. Transfect cultured cells with vectors encoding an epitope tag alone or a fusion protein. For acquiring representative data, transfer J774 cells (1×10^6) with vectors encoding green fluorescent protein (GFP) alone or GFP-fused Capn6 (calpain-6 gene) using cationic liposomes.

1.2. Conjugate antibody to magnetic beads.

1.2.1. For this purpose, mix anti-GFP antibody (2 µL/reaction) with magnetic beads (25 µL/reaction) in 500 µL of citrate phosphate buffer (24.5 mM citric acid, 51.7 mM dibasic sodium phosphate; pH 5.0) in a 1.5 mL test tube.

1.2.2. Rotate the mixture at 50 rpm for 1 h at room temperature. Then, wash the IgG-conjugated beads three times with citrate phosphate buffer containing 0.1% Polyoxyethylene (20) sorbitan monolaurate.

1.3. Lyse the transfected cells using an appropriate lysis buffer. For acquiring representative data, lyse the transfected cells for 30 min on ice in 400 µL of lysis buffer (50 mM Tris-HCl; pH 7.5, 120 mM NaCl, 0.5% poly(oxyethelene) octylphenyl ether) containing 40 µmol/L phenylmethylsulfonyl fluoride, 50 µg/mL leupeptin, 50 µg/mL aprotinin, 200 µmol/L sodium orthovanadate, and 1 mM ethylene glycol tetraacetic acid (EGTA) in 1.5 mL test tubes 24 h after transfection.

1.4. Clear the lysate by centrifuging at 15,000 x *g* for 5 min and collect the supernatant.

1.5. Preclear the lysate. Add unlabeled magnetic beads (25 µL/reaction) in a 1.5 mL-test tube. Wash the beads three times with 500 µL of citrate phosphate buffer. After the removal of citrate phosphate buffer in final wash, add the cell lysate over the magnetic beads. Rotate the mixture using rotator at 50 rpm for 30 min at room temperature.

1.6. Place the test tube on a magnetic stand for 5 min for magnetic separation, and collect the cell lysate. Use of the magnetic stand designated by the manufacturer is recommended.

1.7. Bind the target proteins to the conjugated beads. Place the immunoglobulin (Ig)G-conjugated beads on a magnetic stand for 5 min for magnetic separation, discard the citrate buffer, and then add the cell lysate to the separated beads.

1.8. Rotate the mixture at 50 rpm for 1 h at room temperature.

1.9. Separate the target proteins from free non-target proteins. Wash the beads three times using 500 µL of citrate phosphate buffer containing 0.1% Polyoxyethylene (20) sorbitan monolaurate. After the final wash, add 30 µL of citrate buffer (pH 2–3), and incubate for 5 min at room temperature to elute the target proteins.

NOTE: If recovery from the immunoprecipitation is insufficient, the use of alternative epitope tags, such as FLAG or HIS, may improve the efficiency. If the efficiency of the elution is insufficient, the use of other eluants, such as an SDS-based solution, may improve the efficiency.

2. On-membrane digestion of proteins

NOTE: Using protein-free materials and equipment is necessary to avoid contamination with

exogenous proteins. In addition, it is recommended that the operator wear a surgical mask and gloves to avoid contamination by human proteins.

2.1. Cut PVDF membranes into 3 mm x 3 mm pieces using surgical scissors that have been wiped with methanol and dried immediately prior to use.

2.2. Add 2–5 µL of ethanol on the pieces of PVDF membrane on clean aluminum foil.

2.3. Before they dry completely, add the eluant (2–5 µL each, 4–6 pieces per sample) onto the hydrophilic PVDF membranes, and then air-dry the membranes until the membrane surface becomes matte. Dried membranes can be stored at 4 °C.

2.4. Transfer the all membranes into 1.5 mL plastic tubes, add 20–30 µL of ethanol to make the membranes hydrophilic, and then remove the ethanol using a pipette.

2.5. Before the membrane dries out completely, add 200 µL of dithiothreitol (DTT)-based reaction solution (80 mM NH_4HCO_3, 10 mM DTT, and 20% acetonitrile) and incubate it at 56 °C for 1 h.

2.6. Replace the reaction solution with 300 µL of iodoacetamide solution (80 mM NH_4HCO_3, 55 mM iodoacetamide, and 20% acetonitrile), and incubate for 45 min at room temperature in the dark.

2.7. Wash the membranes twice with 1 mL of distilled water and once with 1 mL of 2% acetonitrile by vortex mixing for >10 s.

2.8. Dissolve lyophilized MS-grade trypsin (**Table of Materials**) directly in the reaction solution (30 mM NH_4HCO_3 containing 70% acetonitrile). Add 100 µL of the reaction solution containing 1 µg of trypsin (**Table of Materials**) (30 mM NH_4HCO_3 containing 70% acetonitrile) and incubate at 37 °C overnight.

2.9. Transfer the reaction solution containing the tryptic digests into a clean 1.5 mL test tube using a pipette.

2.10. Add 100 µL of wash solution (70% acetonitrile/1% trifluoroacetic acid) to the membrane and incubate it at 60 °C for 2 h. Collect the wash solution and mix it with the reaction solution. Repeat this process twice.

2.11. Dry the solution using a vacuum concentrator.

2.12. Dissolve the residue in 10 µL of 0.2% formic acid. After centrifugation (12,000 × *g*, 3 min at room temperature), transfer the supernatant into a sample tube.

NOTE: The washes are the critical steps of this section. During on-membrane protein digestion,

the washing of the membranes containing the immobilized proteins after reduction and alkylation with distilled water, followed by 2% acetonitrile, for more than 10 sec each, is critical for the removal of the reagents.

3. LC–electrospray ionization (ESI)-MS/MS analysis

3.1. Activate an ESI-MS/MS instrument (**Table of Materials**) coupled with a nano-LC HPLC system (**Table of Materials**). Link a pre-column (**Table of Materials**) and an analytical column (**Table of Materials**).

3.2. Prior to the analysis, calibrate the mass spectrometer using tryptic digests of bovine serum albumin dissolved in 0.2% formic acid, which provides standard peptides.

3.3. Analyze the samples using positive ion mode and a mass range of 400–1,250 m/z; then acquire up to 10 MS/MS spectra (100 ms each) with a mass range of 100–1,600 m/z and a linear gradient of 2%–80% acetonitrile and 0.2% formic acid for 80 min at a flow rate of 300 nL/min.

3.4. Analyze the output data using software for protein identification (**Table of Materials**) to identify the candidate associated proteins. For the positive identification of a candidate protein, detection of at least one high-fidelity peptide fragment (> 95% fidelity) is required.

3.5. Omit the proteins that are similarly precipitated in GFP-expressing lysates (transfection of vector expressing a GFP tag alone) from the list of candidate proteins.

NOTE: If few proteins are identified in the database analysis, modification of the MS/MS acquisition conditions (e.g., changing the time from 100 ms each to 50 ms each) may increase the number of proteins identified.

REPRESENTATIVE RESULTS:

By means of the above-described procedure, immunoprecipitants were analyzed using LC-MS/MS (**Figure 1**). After the exclusion of exogenously derived proteins (proteins from other species and IgGs), 17 proteins were identified in calpain-6-associated immunoprecipitants (**Table 1**) and 15 proteins were identified in GFP-associated immunoprecipitants (**Table 2**). Of the calpain-6 and GFP-associated proteins, 11 were identified in both immunoprecipitants (**Figure 2**). Once these and calpain-6 itself were excluded, five candidate calpain-6-associated proteins remained: complement C1q subcomponent subunit C, keratin type II cytoskeletal 8, IgE-binding protein, ADP/ATP translocase 1, and ubiquitin.

Figure 1. Scheme for the on-membrane digestion technique

Cell lysates were precipitated using magnetic beads conjugated with specific antibodies. The eluant from the immunoprecipitation was blotted onto pieces of PVDF membrane. Subsequently, the membranes were treated with reagents for reductive alkylation, and then incubated with trypsin. The reaction solution was then analyzed using LC-MS/MS.

Figure 2. Overview of the representative data
Seventeen proteins were identified in the calpain-6-associated immunoprecipitant and 15 proteins were detected in the GFP-associated immunoprecipitant. Of these, 11 proteins were identified in both immunoprecipitants.

Table 1. Calpain-6-associated proteins

Table 2. GFP-associated proteins

DISCUSSION:
We have previously described an analysis of the oxidative modifications of apolipoprotein B-100 in oxidized low-density lipoprotein using LC-MS/MS preceded by an on-membrane digestion technique[6]. In the present study, we combined this technique with immunoprecipitation and have identified several calpain-6-associated proteins. This novel technique represents a convenient method of screening for candidate associated proteins. Calpain-6 is a non-proteolytic member of the calpain proteolytic family[11] that has reportedly modify cellular functions through its protein–protein interactions[12,13]. Using an on-membrane digestion technique, we have previously identified other calpain-6-associated proteins employing a different MS set-up[12]. Therefore, we recommend testing several MS conditions for maximizing the number of candidates identified. For the same reason, the evaluation of the immunoprecipitation conditions, such as the types of epitope tag, detergent, and elution solution used, is important for obtaining optimal outputs.

In the present study, we identified 11 proteins in both calpain-6- and GFP-associated immunoprecipitants. The majority of the overlapping proteins were actin isotypes, and contamination with actin cytoskeletal proteins is common in the analyses of cellular protein–protein interactions because the expression levels of these proteins are very high. These candidates should therefore be omitted from further analysis. In addition, elongation factors were detected in both immunoprecipitants. They regulate the speed and fidelity of protein synthesis and affect protein-folding[14], and, therefore, the co-immunoprecipitation of elongation factors is not surprising. These proteins should also be omitted from further evaluation.

Immunoprecipitants can be subjected to reductive alkylation and enzymatic digestion directly in the elution solution[15], and this in-solution digestion method may also be applied for the preparation of samples for MS/MS. However, we consider the use of on-membrane digestion to have considerable advantages over in-solution digestion. PVDF membrane serves as a scaffold for the subsequent reductive alkylation and enzymatic digestion, meaning that the solvents required for these processes can be replaced easily. Consequently, it is possible to use a variety of elution solutions for immunoprecipitation. Conversely, for in-solution digestion, it may be challenging to use SDS-based or low pH elution solutions because protease activity may be limited under such conditions. Furthermore, immobilization of the target proteins can make the subsequent washing procedure easier. Hence, on-membrane digestion is highly suitable for the preparation of immunoprecipitants for MS/MS analysis. A limited number of proteases may be

appropriate for this protocol. Thus far, only Lysyl-C, other than trypsin, is reportedly active in the presence of up to 80% acetonitrile[6,9,10,15].

Our on-membrane digestion technique is suitable for the identification of proteins in a small quantity of immunoprecipitant with a highly sensitive detection limit. However, it should be remembered that the detection efficiency for a target protein depends on the amount present. Proteins that are present in larger quantities are preferentially detected and they may prevent other proteins present in smaller amounts being detected by MS. Nevertheless, under normal circumstances numerous proteins are detected using LC-MS/MS analysis, and other assays must be used for clarify which of the candidate proteins are of appropriate biologic significance.

In this study, we have evaluated an on-membrane digestion technique for the analysis of immunoprecipitants. Such a convenient and comprehensive method for the analysis of protein–protein interactions should be widely applicable to improve the throughput of future proteomic analyses.

DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

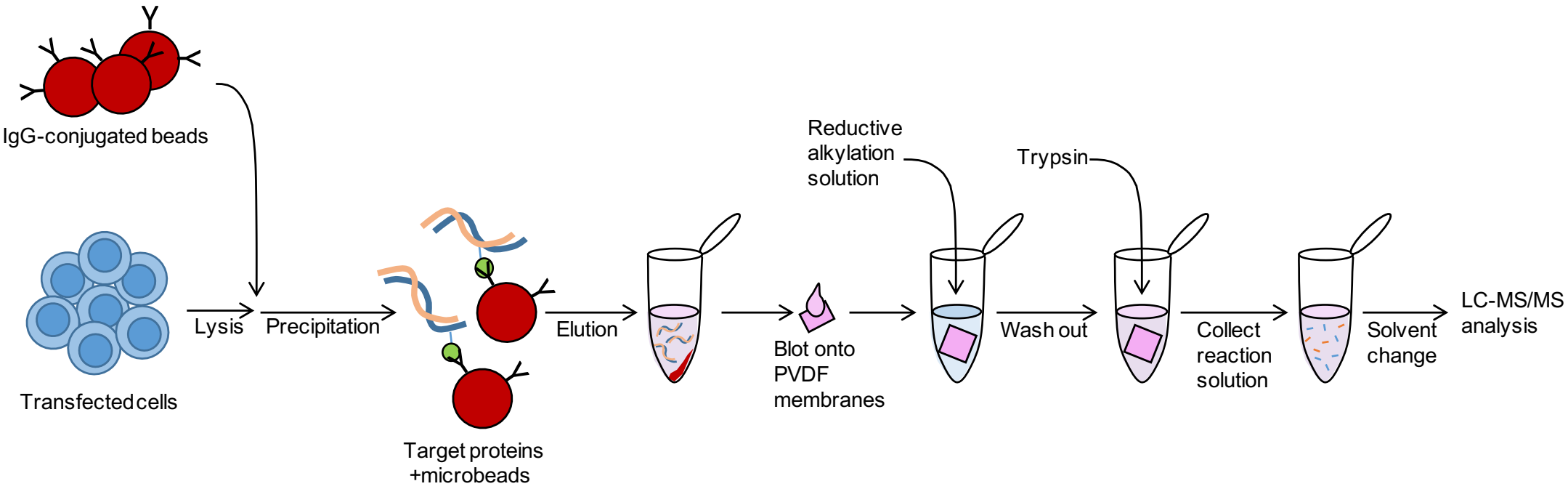
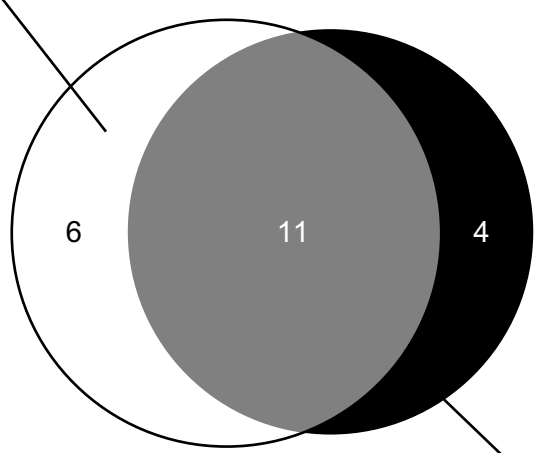


Figure 2

Calpain-6-associated proteins
- Complement C1q subcomponent subunit C
- Keratin, type II cytoskeletal 8
- IgE-binding protein
- ADP/ATP translocase 1
- Ubiquitin
- (calpain-6)



GFP-associated proteins
- rRNA 2'-O-methyltransferase fibrillarin
- Prohibitin
- Heterogeneous nuclear ribonucleoprotein U
- Elongation factor 1-gamma

Table 1. Calpain-6-associated proteins.

Name	Accession	Peptides(95%)	%Cov
Actin, cytoplasmic 2	sp|P63260|ACTG	5	18.4
Actin, aortic smooth muscle	sp|P62737|ACTA	5	18.57
Actin, cytoplasmic 1	sp|P60710|ACTB	5	18.41
Actin, alpha skeletal muscle	sp|P68134|ACTS	5	13.53
Actin, alpha cardiac muscle 1	sp|P68033|ACTC	5	13.53
Actin, gamma-enteric smooth muscle	sp|P63268|ACTH	5	13.56
Elongation factor 1-alpha 1	sp|P10126|EF1A1	3	33.33
Elongation factor 1-alpha 2	sp|P62631|EF1A2	3	16.2
Keratin, type II cytoskeletal 8	sp|P11679|K2C8	3	22.65
Complement C1q subcomponent subunit C	sp|Q02105|C1QC	2	8.94
IgE-binding protein	sp|P03975|IGEB	2	21.72
Calpain-6	sp|O35646|CAN6	1	15.91
ADP/ATP translocase 2	sp|P51881|ADT2	1	27.52
ADP/ATP translocase 1	sp|P48962|ADT1	1	19.13
Ubiquitin	sp|P62991|UBIQ	1	40.79
Runt-related transcription factor 3	sp|Q64131|RUNX3	1	15.89
Isoform 2 of Runt-related transcription factor 3	sp|Q64131-2|RUNX3	1	13

“Peptides (95%)” indicates the number of peptides identified with a fidelity score > 95% in the MS/MS data. “%Cov” refers to the percentage of the amino acid residues identified in all the peptides (with > 95% fidelity) relative to the total number of amino acid residues constituting the corresponding protein. To improve clarity, exogenous proteins (proteins from other species and IgGs), ribosomal proteins, and

Table 2. GFP-associated proteins.

Name	Accession	Peptides(95%)	%Cov
Elongation factor 1-alpha 1	sp|P10126|EF1A1	3	24.24
Elongation factor 1-alpha 2	sp|P62631|EF1A2	3	24.41
Actin, alpha skeletal muscle	sp|P68134|ACTS	3	13.53
Actin, alpha cardiac muscle 1	sp|P68033|ACTC	3	13.53
Actin, gamma-enteric smooth muscle	sp|P63268|ACTH	3	13.56
Actin, cytoplasmic 2	sp|P63260|ACTG	3	13.6
Actin, aortic smooth muscle	sp|P62737|ACTA	3	13.53
Actin, cytoplasmic 1	sp|P60710|ACTB	3	13.6
ADP/ATP translocase 2	sp|P51881|ADT2	2	25.5
rRNA 2'-O-methyltransferase fibrillarin	sp|P35550|FBRL	2	14.37
Prohibitin	sp|P67778|PHB	1	9.19
Heterogeneous nuclear ribonucleoprotein U	sp|Q8VEK3|HNRPU	1	10.63
Runt-related transcription factor 3	sp|Q64131|RUNX3	1	39.12
Isoform 2 of Runt-related transcription factor 3	sp|Q64131-2|RUNX3	1	26
Elongation factor 1-gamma	sp|Q9D8N0|EF1G	1	12.36

“Peptides (95%)” indicates the number of peptides identified with a fidelity score > 95% in the MS/MS data. “%Cov” refers to the percentage of the amino acid residues identified in all the peptides (with > 95% fidelity) relative to the total number of amino acid residues constituting the corresponding protein. To improve clarity, exogenous proteins (proteins from other species and IgGs), ribosomal proteins, and

Name of Material/ Equipment	Company
Acetonitrile	Wako
Citric acid	Wako
DiNA	KYA Tech Co.
DiNa AI	KYA Tech Co.
DTT	Nacalai tesque
Dynabeads protein G	Thermo Fisher Scientific
Formic acid	Wako
HiQ Sil C18W-3	KYA Tech Co.
Iodoacetamide	Wako
Lipofectamine 3000	Thermo Fisher Scientific
Living Colors A.v. Monoclonal Antibody (JL-8)	Clontech
NaCl	Wako
NH_4HCO_3	Wako
Nonidet P-40	Sigma
peptide standard	KYA Tech Co.
PP vial	KYA Tech Co.
Protease inhibitor cooctail	Sigma
ProteinPilot software	Sciex
Sequencing Grade Modified Trypsin	Promega
Sodium orthovanadate	Sigma
Sodium phosphate dibasic dihydrate	Sigma

TFA	Wako
trap column	KYA Tech Co.
TripleTOF 5600 system	Sciex
Tris	Wako
Tween-20	Wako

Catalog Number	Comments/Description
014-00386	
030-05525	
	nanoflow high-performance liquid chr
	nanoflow high-performance liquid chr
14112-94	
10003D	
066-00461	
E03-100-100	0.10mmID * 100mmL
095-02151	
L3000008	
632380	
191-01665	
018-21742	
N6507	poly(oxyethelene) octylphenyl ether (n=9
tBSA-04	tryptic digests of bovine serum album
03100S	plastic sample tube
P8465	
5034057	software for protein identification
V5111	trypsin
S6508	
71643	

206-10731
A03-05-001 0.5mmID * 1mmL
4466015 Hybrid quadrupole time-of-flight tanc

207-06275
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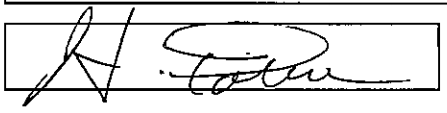
Signature:	 Date: Jan. 23, 2019

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Xiaoyan Cao, Ph.D.
Review Editor
JoVE

Dear Dr. Cao,

Thank you very much for your valuable comments and suggestions. Here is the point-by-point response to the reviewing comments.

Sincerely,

Hiroyuki Itabe, PhD.
Division of Biological Chemistry, Department of Molecular Biology, Showa University School of Pharmacy, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8555, Japan

Point-by-point answers to the Editor

Editorial comments:

Changes to be made by the author(s) regarding the manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

[Our response] The revised manuscript was carefully checked before submitting to the editorial office.

2. Authors and affiliations: Please provide an email address for each author.

[Our response] Email addresses for each author were added.

3. Please add a Summary section before the Abstract section to clearly describe the protocol and its applications in complete sentences between 10−50 words: “Here, we present a protocol to …”

[Our response] A summary section was added.

4. Please define all abbreviations before use.

[Our response] Abbreviations in the text were defined, and a list of abbreviations is added.

5. Introduction: Please expand to include the advantages of the presented method over alternative techniques with applicable references to previous studies, description of the context of the technique in the wider body of literature and information that can help readers to determine if the method is appropriate for their application.

[Our response] The advantage of presented method over alternative techniques was added as below. (line 61 - 71)

——However, one major bottleneck of the MS/MS analysis is poor recovery from tryptic digests of the protein samples. For conducting proteomic analyses on cell lysates, in-gel e and on-membrane digestion techniques are generally employed to prepare MS/MS samples. We have previously compared an in-gel digestion procedure with an on-membrane digestion technique, and showed that the latter was associated with better sequence coverage. Polyvinylidene difluoride (PVDF) membrane may be suitable for this purpose because it is mechanically robust and resistant to high concentration of organic solvents, permitting the enzymatic digestion of immobilized proteins in the presence of 80% acetonitrile. Furthermore, immobilization on a membrane can induces conformational changes in target proteins, leading to improvements in tryptic digestion efficiency.

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[Our response] All commercial languages were replaced with the generic terms.

7. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.

[Our response] The numbering of the protocol was adjusted.

8. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

[Our response] Protocol section was extensively revised to answer all the comments raised by the editor and the reviewers. Accordingly, the number of steps increased to 26 from 22 in total.

9. Line 59: What container is used for the transfected cells? For how long are the cells lysed?

[Our response] Text was modified as below. (Line 90 - 95)

—— Lyse the transfected cells using an appropriate lysis buffer. For acquiring representative data, the transfected cells were lysed for 30 min on ice with 400 µL of lysis buffer (50 mmol/L Tris-HCl; pH7.5, 120 mmol/L NaCl, 0.5% poly(oxyethelene) octylphenyl ether) containing 40 µmol/L phenylmethylsulfonyl fluoride, 50 µg/mL leupeptin, 50 µg/mL aprotinin, 200 µmol/L sodium orthovanadate, 1 mmol/L ethylene glycol tetraacetic acid (EGTA) in 1.5mL-test tube at 24 h after transfection.

10. Line 63: How to rotate the antibody in a tube? What is the rotating speed?

[Our response] Text was modified as below. (line 84 - 89)

—— Conjugate antibody to magnetic beads. For this purpose, anti-GFP antibody (2 µL/reaction) was mixed with magnetic beads (25 µL/reaction) in 500 µL citrate phosphate buffer (24.5 mmol/L citric acid, 51.7 mmol/L dibasic sodium phosphate; pH 5.0) in a 1.5 mL test tube. Next, the mixture was rotated at 50 rpm for 1 h at room temperature. Afterward, wash the IgG-conjugated beads three times with citrate phosphate buffer containing 0.1% Polyoxyethylene (20) sorbitan monolaurate.

11. Line 68: What magnetic fields? Do you mean a magnetic stand?

[Our response] Magnetic field was corrected to magnetic stand. (line 101-102)

——Place the test tube on a magnetic stand for 5 min for magnetic separation, and collect the cell lysate. Use of the magnetic stand designated by the manufacturer is recommended.

12. Line 70: Please specify the rotating speed?

[Our response] Text was modified as below. (line 106)

——Rotate the mixture at 50 rpm for 1h at room temperature.

13. Line 71: What volume of citrate phosphate buffer is used to wash?

[Our response] The volume used for washing step was 500 µL. (line 107 - 110)

——Separate the target proteins from free non-target proteins. Wash the beads three times using 500 µL of citrate phosphate buffer containing 0.1% Polyoxyethylene (20) sorbitan monolaurate. After the final wash, add 30 µL citrate buffer (pH 2–3), and incubate for 5 min at room temperature to elute the target proteins.

14. Line 72: Please describe how elution is done.

[Our response] Text was modified as below. (line 107 - 110)

——Separate the target proteins from free non-target proteins. Wash the beads three times using 500 µL of citrate phosphate buffer containing 0.1% Polyoxyethylene (20) sorbitan monolaurate. After the final wash, add 30 µL citrate buffer (pH 2–3), and incubate for 5 min at room temperature to elute the target proteins.

15. Line 77: What is used to cut?

[Our response] Text was modified as below. (line 119 - 120)

—— Cut PVDF membranes into 3x3 mm pieces using surgical scissors that have been wiped with methanol and dried immediately prior to use.

16. Line 81: How to dry out the membranes?

[Our response] Text was modified as below. (line 122 - 124)

——Before they dry completely, add the eluent (2-5 µL each, 4 – 6 pieces per sample) onto the hydrophilic PVDF membranes, and then air-dry the membranes until the membrane surface becomes matte. Dried membranes can be stored at 4°C.

17. Line 82: What container is used to hold the membranes? How many membranes per container? What volume of the reaction solution is used?

[Our response] Text was modified as below. (line 124 - 125)

——Transfer the membranes into 1.5 mL plastic tubes, add 20-30 of ethanol to make the membranes hydrophilic, and then remove the ethanol using pipette.

18. Line 88: Please specify the incubation temperature. What volume of trypsin solution is used?

[Our response] Text was modified as below. (line 135 - 138)

——Dissolve lyophilized MS-grade trypsin (Table of Materials) directly in the reaction solution (30 mmol/L NH_4HCO_3 containing 70% acetonitrile). Add 100 µL of the reaction solution containing 1 µg of trypsin (Table of Materials) (30 mmol/L NH_4HCO_3 containing 70% acetonitrile) and incubate at 37°C overnight.

19. Line 91: How to collect the reaction solution? Using a pipette?

[Our response] Text was modified as below. (line 139 – 140)

——Transfer the reaction solution containing the tryptic digests into a clean 1.5 mL test tube using a pipette.

20. Line 97: What volume of formic acid is used?

[Our response] The volume of formic acid used was 10 µL. (line 145-146)

——Dissolve the residue in 10 µL of 0.2% formic acid. After centrifugation (12,000 ×g, 3 min at room temperature), transfer the supernatant into a sample tube.

21. Line 113: How to identify proteins that are precipitated similarly in GFP-expressing lysates?

[Our response] Text was modified as below. (line 162 - 166)

——Analyze the output data using a software for protein identification (Table of Materials) to identify a candidate associated proteins. For the positive identification of a candidate protein, detection of at least one high-fidelity peptide fragment (> 95% fidelity) is required.

——Omit the proteins that are similarly precipitated in GFP-expressing lysates (transfection of vector expressing a GFP tag alone) from the list of candidate proteins.

22. Please apply single line spacing throughout the manuscript, and include single-line spaces between all paragraphs, headings, steps, etc.

[Our response] Text style was modified throughout the manuscript.

After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Notes

cannot usually be filmed and should be excluded from the highlighting. Please do not highlight any steps describing anesthetization and euthanasia.

Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

[Our response] Important steps in the protocol are highlighted according to the instructions

26. References: Please do not abbreviate journal titles.

[Our response] The style of the journal titles was re-examined and corrected.

27. Please upload each Figure individually to your Editorial Manager account as a .png, .tiff, .pdf, .svg, .eps, .psd, or .ai file.

[Our response] The figures are uploaded as PDF file.

28. Please upload each Table individually to your Editorial Manager account as an .xlsx file.

[Our response] The tables are uploaded as xlsx file.

29. Table 1: What does "%Cov" stand for?

[Our response] Peptides(95%) and %Cov were defined as below. (legends in tables)

——"Peptides (95%)" indicates the number of peptides identified with a fidelity score >95% in the MS/MS data. "%Cov" refers to the percentage of the amino acid residues identified in all peptides (>95% of fidelity) relative to the total number of amino acid residues constituting the corresponding protein.

30. Table of Materials: Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please remove trademark (™) and registered (®) symbols and sort the items in alphabetical order according to the name of material/equipment.

[Our response] Table of Materials was reevaluated and corrected according to the suggestions.

31. JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please revise the Discussion to explicitly cover the following in detail in 3-6

paragraphs with citations:

a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

d) The significance with respect to existing methods

e) Any future applications of the technique

[Our response] Discussion section was extensively revised. In addition, critical steps were indicated as "Note" at the end of Protocol section. (line111-113, 147-150, 167-169)

Reviewers' comments:

The language in the manuscript is not publication grade. Please employ professional copy-editing services.

[Our response] The manuscript was revised by the professional linguistic proof reader (Enago, Crimson Interactive Pvt. Ltd).

Please note that novelty is not a requirement for publication and reviewer comments questioning the novelty of the article can be disregarded.

Please note that the reviewers raised some significant concerns regarding your method and your manuscript. Please revise the manuscript to thoroughly address these concerns. Additionally, please describe the changes that have been made or provide explanations if the comment is not addressed in a rebuttal letter. We may send the revised manuscript and the rebuttal letter back to peer review.

Reviewer #1:

Major Concerns:

The method provided in the manuscript is a standard used by several laboratories to perform membrane digestion of proteins before MS analysis. They have not provided or highlighted any novel steps in their procedure. Additionally, the procedure is not through. They do not provide details on any cleaning, blocking or handling steps of the PVDF membrane before digestion. Nor do they provide any information on the challenges, troubleshooting steps. Considering these issues, the manuscript is not suitable for publication.

[Our response] The protocol was revised according to the editorial comments. On-membrane

tryptic digestion has been used to prepare LC-MS samples, however the unique point of this protocol is to apply protein samples directly onto PVDF membrane. Non-purified samples are applicable, so that it would be useful for crude samples with small volume such as immunoprecipitants. In addition, cleaning and blocking of the PVDF membrane is unnecessary, since the recovery, but not specificity, is important for the process after the antibody-based separation of the tag-associated proteins. These advantages of this protocol are described in Discussion section and some notification comments (including troubleshooting) are added in Protocol section. (line 111-113, 147-150, 167-169 201-211)

Reviewer #2:

Minor Concerns:

1. Protocol/ 1.Immunoprecipitation/2./line 62: It is not clear whether lysates need to be precleared by centrifugation before use in step 4. Classical IP procedures require a centrifugation step.

[Our response] It is thought that the preclear of the lysate by unlabeled beads may improve the specificity of the immunoprecipitation. This was added to the protocol. (line 97-102)

——Preclear the lysate. Add unlabeled magnetic beads (25 µL/reaction) in 1.5 mL-test tube. Wash the beads three times with 500 µL citrate phosphate buffer. After the removal of citrate phosphate buffer in final wash, add the cell lysate over the magnetic beads. Rotate the mixture using rotator at 50 rpm for 30 min at room temperature.

——Place the test tube on a magnetic stand for 5 min for magnetic separation, and collect the cell lysate. Use of the magnetic stand designated by the manufacturer is recommended.

2. Protocol/ 1.Immunoprecipitation/6./line 72: How long is the elution step? Is it at room temperature?

[Our response] Elution step was carried out for 5 min at room temperature as described in the revised version (line 107 - 110).

——Separate the target proteins from free non-target proteins. Wash the beads three times using 500 µL of citrate phosphate buffer containing 0.1% Polyoxyethylene (20) sorbitan monolaurate. After the final wash, add 30 µL citrate buffer (pH 2–3), and incubate for 5 min at room temperature to elute target proteins.

3. Protocol/2.On membrane digestion of proteins/4/line 83: Please explain what volume of DTT-based reaction solution to use per membrane or for all membranes.

[Our response] DTT treatment was defined as noted below (line 125 - 129).

——Transfer the all membranes into 1.5 mL plastic tubes, add 20–30 µL of ethanol to make the membranes hydrophilic, and then remove the ethanol using a pipette.
——Before the membrane dries out completely, add 200 µL of dithiothreitol (DTT)-based reaction solution (80 mmol/L NH4HCO3, 10 mmol/L DTT, and 20% acetonitrile) and incubate it at 56°C for 1 h.

4. Protocol/2.On membrane digestion of proteins/5/line 86: Please explain what volume of iodoacetamide solution to use per membrane or for all membranes.
[Our response] Iodoacetamide treatment was defined as noted below (line 130-132).
——Replace the reaction solution with 300 µL of iodoacetamide solution (80 mmol/L NH_4HCO_3, 55 mmol/L iodoacetamide, and 20% acetonitrile), and incubate at room temperature for 45 minutes in the dark.

Reviewer #3:
Major Concerns:
1. The authors do not have good command of the English language.
[Our response] The manuscript was revised by the professional linguistic proof reader (Enago, Crimson Interactive Pvt. Ltd) as answered to reviewer #1.

2. A more generic description of the procedure could be beneficial to a general audience. For example, the authors describe on Step 1 the transfection of J774 cells with vectors for Capn6, they also describe on Step 3 (LC-ESI-MS/MS analysis) a specific mass spectrometer, and a specific software for peptide/protein identification. All these steps should be described in a more generic way.
[Our response] Text was revised according to the editorial comment. Specific experimental conditions and instruments were omitted from the protocol section. In addition, key steps and troubleshooting were added to the protocol.

3. The authors should describe the advantages of this protocol over others, particularly with a standard direct digestion of the immunopurified proteins in solution. The discussion is weak
[Our response] Discussion was added as below. (line 201 - 213)
——Immunoprecipitants can be subjected to reductive alkylation and enzymatic digestion directly in the elution solution; and this in-solution digestion method may also be applied for preparation of samples for MS/MS. However, we consider the use of on-membrane digestion to have considerable advantages over in-solution digestion. PVDF membrane serves as a

scaffold for the subsequent reductive alkylation and enzymatic digestion, meaning that the solvent required for these processes can be replaced easily. Consequently, it is possible to use a variety of elution solution for immunoprecipitation. Conversely, for in-solution digestion, it may be challenging to use SDS-based or low pH elution solutions because protease activity may be limited under such conditions. Furthermore, immobilization of the target proteins can make the subsequent washing procedure easier. Hence, on-membrane digestion is highly suitable for the preparation of immunoprecipitants for MS/MS analysis. A limited number of proteases may be appropriate for this protocol. Thus far, only Lysyl-C, other than trypsin, is reportedly active in the presence of up to 80% acetonitrile.

Minor Concerns:
It should be mass spectrometry not mass spectroscopy.
[Our response] Thank you for pointing out the wrong word usage. Text was corrected.

Reviewer #4:

Major Concerns:
1. Using immunoprecipitation to demonstrate protein-protein interaction followed by identification of protein with mass spectrometry is a widely used method in the field. For example, Dr. Steve Gygi's group is a pioneer in this field.
[Our response] Importantly, it is noteworthy that novelty is not a requirement for publication (please refer editor's comment). As pointed out by the reviewer, Dr. Gygi's group reportedly conducted MS/MS analysis of immunoprecipitants on membrane, however, our procedure has some differences in comparison with theirs. First, our procedure does not employ electrophoresis. Second, another difference is procedure to transfer the target proteins onto the membrane. While Gygi's group employed electronic transfer of the target proteins from gels, our procedure employs direct immobilization of the target proteins onto the membrane. As a result, two time-consuming processes (electrophoresis and electronic transfer) can be skipped in our procedure that can avoid sample loss and improve recovery of tryptic digests.

2. Please clarify the advantage of digesting the immunoprecipitants on PVDF membrane. Why not make the reduction/alkylation/digestion for the eluent in a test tube directly, which seems to be a more convenient procedure? Does the PVDF membrane cause any non-specific binding problem for proteins/peptides?

[Our response] It is considered that the recovery, but not specificity, is important for the process after the antibody-based purification of the tag-associated proteins. Advantages of on-membrane digestion was discussed in Discussion. (201 - 213)

——Immunoprecipitants can be subjected to reductive alkylation and enzymatic digestion directly in the elution solution; and this in-solution digestion method may also be applied for preparation of samples for MS/MS. However, we consider the use of on-membrane digestion to have considerable advantages over in-solution digestion. PVDF membrane serves as a scaffold for the subsequent reductive alkylation and enzymatic digestion, meaning that the solvent required for these processes can be replaced easily. Consequently, it is possible to use a variety of elusion solution for immunoprecipitation. Conversely, for in-solution digestion, it may be challenging to use SDS-based or low pH elution solutions because protease activity may be limited under such conditions. Furthermore, immobilization of the target proteins can make the subsequent washing procedure easier. Hence, on-membrane digestion is highly suitable for the preparation of immunoprecipitants for MS/MS analysis. A limited number of proteases may be appropriate for this protocol. Thus far, only Lysyl-C, other than trypsin, is reportedly active in the presence of up to 80% acetonitrile.

3. For the proteomics search results, please state clearly the criteria for protein identification. Based on the results listed in table 1 and table 2, it seems like proteins with 1 and even 0 peptide (95%) identified are considered as hits. In the field, it's more acceptable that proteins with at least confidently identified peptides are considered as reliable outputs from proteomics search.

[Our response] Although there are a few or no peptide identified with high-fidelity, sometimes the protein can be identified from a number of peptides with slightly lower fidelity. In this manuscript, we simplified the tables to show only the proteins identified with at least one peptide with high fidelity (>95%). In addition, the explanation of “Peptide (95%)” was added in the legend of the table. According to the changes in the Tables, Figure 2 was corrected.