Technical bulletin
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Enrichment and identification of phosphoprotein using PhosPro™

Introduction

PhosPro™, phosphoprotein enrichment kit, was developed to fractionate phosphorylated proteins from protein mixtures, such as cell lysate or body fluids and it is efficient to isolate and concentrate low copy phosphorylated proteins in cells. This kit utilized proprietary phosphoprotein precipitation method instead using column or bead such as IMAC column or immobilized anti-phophoprotein antibody beads. Then in addition to its specificity for isolation of phosphoproteins, it provides simple and convenient method for phosphoprotein fractionation and all processes are to be done with multi parallel samples in each one tube. This kit was designed to use denaturant and detergent solution as the starting material of protein extraction in order phosphoprotein isolation not to be prevented by possible steric hindrance of phosphorylated moiety of proteins and not to be omitted by difficulty in solubilization of phosphoproteins embedded in membrane fraction or cell debris.

Strategy

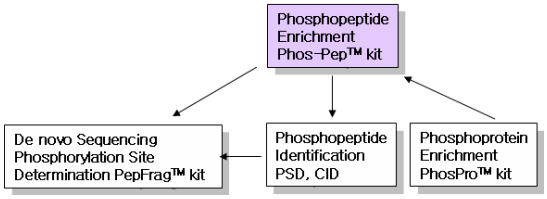


Fig.1. Phosphoprotein enrichment using PhosPro™. Protein phosphorylation could be identified by phosphoprotein specific enrichment in conjunction with phosphoprotein specific staining or MS-based phosphopeptide identification and phosphorylation site determination.

Materials & Methods

Materials

PhosPro™ kit contents

LYSIS BUFFER

DILUTION BUFFER

NATIVE HOMOGENATION BUFFER

SOLUTION A

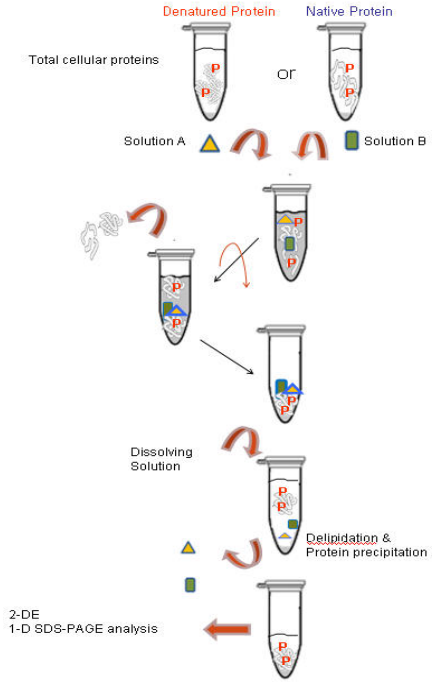
SOLUTION B

DISSOLVING SOLUTION

DELIPIDATION SOLUTION

Procedure Summary

1. Native & Denatured Protein extraction
2. Phosphoprotein specific complex forming
3. Precipitation of phosphoprotein complex
4. Delipidation and recovering of phosphoprotein



Additional Materials Required

. Methanol
. Ultrapure water

Detection of Phosphorylated Proteins

This phosphoprotein enrichment kit was optimized for the protein solution in denatured condition, for example, the samples prepared for 2-DE and can be adapted to native proteins. Enriched phosphorylated proteins could be detected by staining commercially available staining method using fluorescent dye[1] or by probing with antibodies specific for phosphorylated proteins.

Results and Discussion

Phosphoprotein enrichment from Saccharomyces cerevisiae protein extract

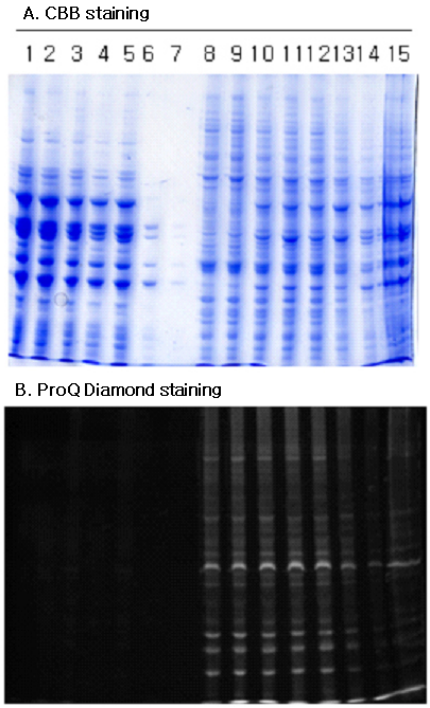


Fig.2. SDS-PAGE analysis of enriched phosphorylated proteins by PhosPro™. A: Coomassie Brillant Blue(SIGMA) staing, B:ProQ Diamond(Invitrogen) staining. Lane 1~7 ; The supernatant containing unphosphorylated proteins discarded in step3(See Procedure Summary above), Lane 8~15 ; enriched fraction containing phosphorylated proteins. Independent seven trials for phosphoprotein enrichment were performed and analysed by SDS-PAGE.

In order to evaluate the performance of PhosPro™ , yeast protein extract was used. The resulting enriched phosphorylated protein was analysed with 1-D(Fig.2) or two-dimensional gel electrophoresis(2-DE, Fig.3)

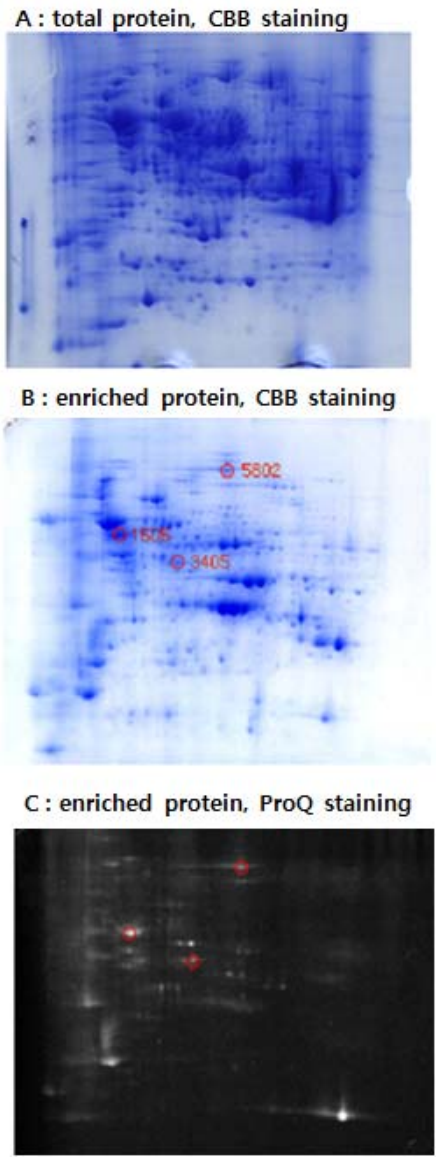


Fig.3. 2-DE analysis of total protein(A), enriched phosphoprotein fraction stained with CBB(B), and stained with ProQ Diamond staining(C)

As shown in Fig.2. and Fig.3. most of the proteins stained with ProQ Diamond was detected in enriched phosphoprotein fraction. This result represent that the PhosPro™ is highly specific for phosphoprotein fractionation.

Phosphoprotein enrichment from mouse brain native protein extract

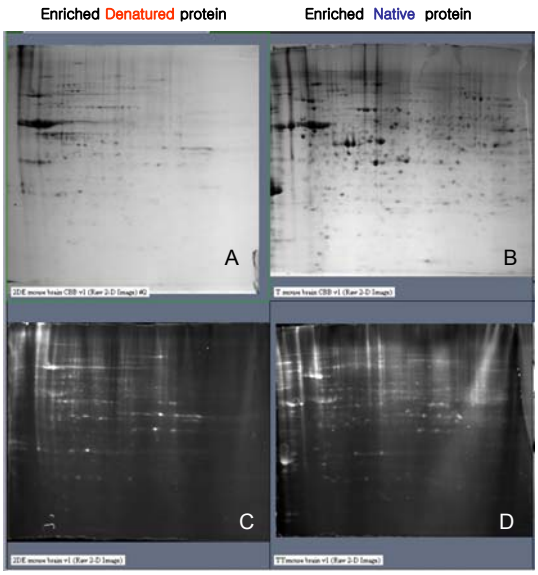


Fig.4. 2-DE analysis of enriched mouse brain phosphoprotein fraction from total denatured(A,C) & native protein(B,D) stained with CBB(A,B), and stained with ProQ Diamond staining(C,D)

In order to evaluate the performance of PhosPro™, mouse brain denatured(A,C) and native protein(B,D) extract was used as a starting material. The resulting enriched native phosphorylated protein was analysed using 2-DE (Fig.4)
As shown in Fig.4.D most of the proteins stained with ProQ Diamond in denatured protein fraction was detected in enriched phosphoprotein fraction from total native protein. But native phosphoprotein fraction contains more non-phosphorylated proteins(not stained with ProQ Diamond), which is assumed as subunit or proteins interacting with phosphorylated proteins than denatured phosphoprotein fraction.
As a result, PhosPro™ is applicable to both denatured rather pure phosphoprotein fraction and active phosphylated protein fractionation.

Phosphoprotein identification by mass spectrometry

The protein spot enriched and stained with ProQ Diamond and identified as a phosphoprotein(spot N. 3405 in Fig.3. B and C) was further confirmed by mass spectrometry. Protein was identified by MALDI-TOF-based peptide mass fingerprinting. Its phosphopeptide was enriched by PhosPep™ and identified with MALDI-PSD by detecting the loss of phosphoprous group(98Da) from mother phosphopeptide(m/z 2900.392).

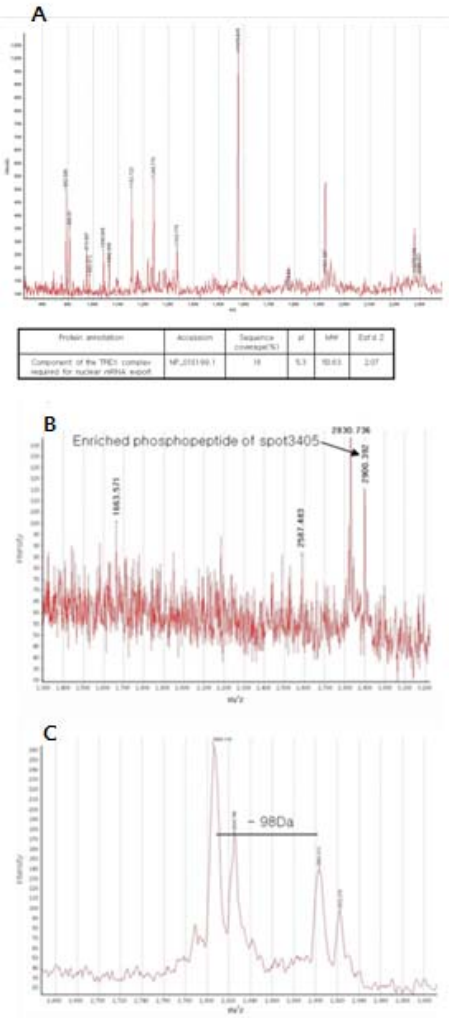


Fig.5. Identification of enriched phosphoprotein by mass spectrometry.

Phosphoprotein identification by dephosphorylation using λPPase

The phosphoproteins were enriched by PhosPro™ from cell lysate of h460 lung cancer cell line. The enriched protein fraction which was stained with phosphoprotein staining and presumed to be the phosphoproteins, was confirmed whether the staining was derived from the phosphate moiety on the proteins by examining the

changes after treatment of phosphatase. The λPPase was used as a phosphatase as previously described[2].

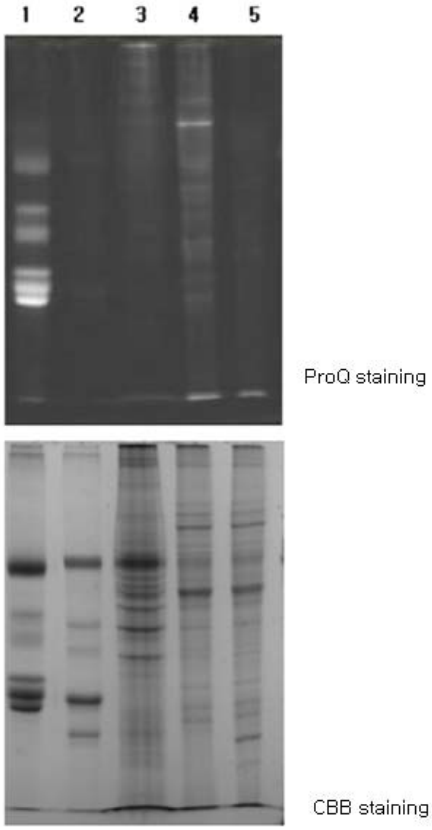


Fig.6. Phosphoprotein staining of enriched phosphoproteins and dephosphorylated proteins. Lane1: standard phosphoproteins, lane2, standard phosphoprotein treated with λPPase, lane3: supernatant fraction remained from phosphoprotein enrichment, lane4: phosphoprotein fraction enriched by PhosPro™, lane5:phosphoprotein fraction treated with λPPase

As shown in Fig.5 the proteins in enriched phosphoprotein fraction was stained with phosphoprotein staining whereas the same proteins treated with phosphatase, λPPase, was not stained with phosphoprotein staining but only with CBB staining. This result represent that the proteins stained with phosphoprotein staining was phosphate group specific and the PhosPro™ isolate the phosphoproteins from cell extract in a specific manner.

Enrichment of low abundant phosphoproteins from lung cancer cell lines and lung cancer tissues

In 2-DE gel analysed using 300ug proteins of total cell lysate of lung cancer cell lines(h460), about 35 protein spots was stained with phosphoprotein staining. When the phosphoproteins(300ug) were enriched from total protein extract of lung cancer cell line, 198 protein spots were detected as a phosphoproteins.(Fig.6.B).

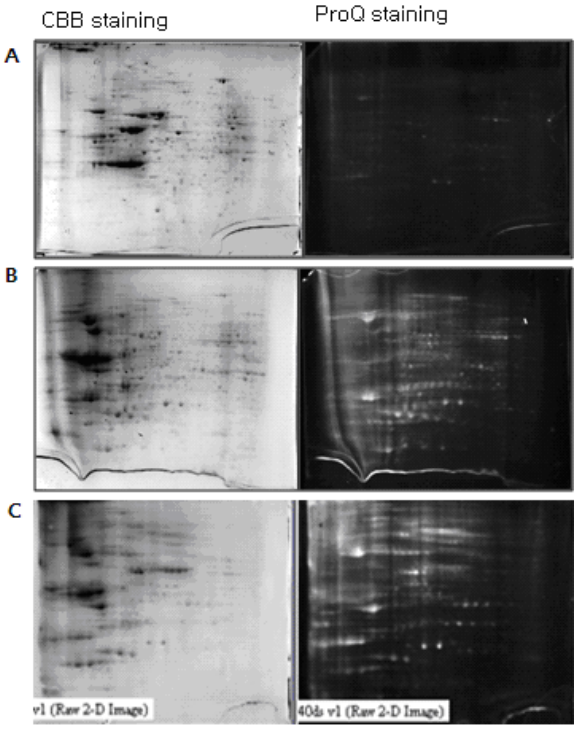


Fig.7. 2-DE gel analysis of total extract of lung cancer cell line(A), enriched phosphoprotein pool from the extract of human lung cancer cell line(B) and enriched phosphoprotein pool from the extract of human lung cancer tissues(c)

This result was represented in human lung cancer tissues which is composed more complex composition of cell types and body fluids.

As a result from the evaluation with phosphoprotein standards, α-casein, β-casein, pepsin, ovalbumin and phosvitin, PhosPro™ technology showed phosphoprotein specific, sensitive and high yield effective fractionation capabilities.(Table.1)

Table.1 Phosphoprotein fractionation capability of PhosPro™

Evaluation category	Specification
Specificity	100%
Selectivity	>87%
Sensitivity	>93% / 50ng
Yield	>93%

Large format phosphoprotein enrichment

Phosphoproteins were enriched from 10 mg of total proteins from SKOV ovarian cancer cell by using PhosPro™. 2-DE gel electrophoresis was performed using 24cm long IPG strip for isoelectric focusing and 12% of 24X20cm-sized gel for SDS-PAGE.

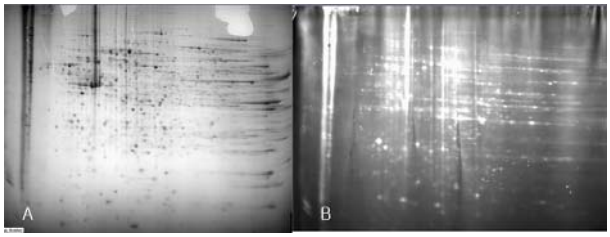


Fig.8. 2DE analysis of phosphoprotein enrichment from SKOV ovarian cancer cell stained with CBB(A), and stained with ProQ Diamond staining(B)

The results show that about 1200 spots from the cell can be detected in a single gel with CBB staining (Fig.8.A) and about 700 spots with ProQ Diamond, Phosphoprotein specific staining reagent (Fig.8.B).

These results indicate that PhosPro™ is a useful kit to enrich phosphoproteins efficiently from a large quantity of total proteins.

References

1. Alein, L. *et al*. Proteomics, 6, 2157–2173 (2006).
2. Akira Yamagata, et al., Proteomics, 2, 1267–1276 (2002)