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One-by-One Sample Preparation Method for Protein Network Analysis

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1. Introduction

Proteomics is the large-scale study of an organism's complete complement of proteins, and its relevant technologies have matured over recent years. Along with the development of mass spectrometry (MS), MS-based proteomics has emerged as an invaluable tool for large-scale identification and quantification of protein networks (Aebersold & Mann, 2003; Domon & Aebersold, 2006). Proteomic data is important for a wide range of research in basic and medical biology. In recent years, many large-scale projects have been performed and a huge amount of data has accumulated. However, because the data sets from individual projects often vary in quality, the value of proteomics for the wider scientific community is limited (Olsen & Mann, 2011).

One of the causes of this variation in proteomic data quality is thought to be the manual process of large-scale sample preparation. The sample preparation process for proteomic analysis consists of the several complicated steps. For example, sample preparation for protein interaction analysis using mammalian cells expressing a target protein typically requires 1×10^7-10^8 cells (one 10-cm or 15-cm tissue culture dish) (Blagoev et al., 2003; Burckstummer et al., 2006; Ewing et al., 2007). After cell recovery, steps such as cell lysis, purification of protein complexes, denaturation and modification of proteins, separation by gel electrophoresis, and enzymatic digestion are performed sequentially. In fact, many researchers and technicians are involved in laborious, repetitive work of large-scale sample preparation, in which they must handle tens of culture dishes at a time. In such a 'parallel sample preparation' process, during the preparation of a number of samples, the conditions undoubtedly differ between the first and last treated samples. Denaturation of the component proteins of complexes and proteolysis progress over time, and the denatured proteins are thought to be the cause of nonspecific binding. We came to realize that highly sensitive analysis could not be performed using the prevailing parallel sample preparation methods.

To optimize sample preparation conditions and improve sample quality, we considered that a 'one-by-one sample preparation' method would be useful. One-by-one sample preparation is the concept that one sample is finished at a time, followed by preparation of the next sample (Fig. 1). In this way, each sample can be prepared carefully under almost equal

conditions; however, this method is not realistic for large-scale analysis, because of the large amount of human time and work involved.

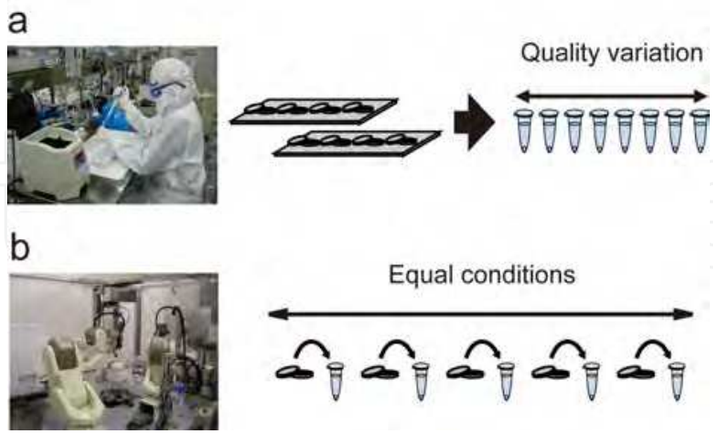

Fig. 1. Comparison of sample preparation processes. (a) Parallel preparation by the manual method. The quality of the samples was uneven. (b) One-by-one preparation. This method enables the preparation of samples under the same conditions.

To realize the one-by-one concept and perform a pilot feasibility study, a fully automated sample preparation system is required. However, in the proteomics field, partial automation for parallel preparation is usually only applied to save analysis time, to eliminate sample contamination, and to reduce human error (Alterovitz et al., 2006). Several semi-automated robots that are specialized in certain processes are commercially available, such as liquid dispenser robots, cell culture robots, and electrophoresis gel cutting robots. However, to develop a fully automated and highly precise system for sample preparation using commercial robots would be difficult, because these robots do not meet our specifications, or if they do, the integration of the robots from different vendors may prove difficult. Furthermore, robots for other multiple sample preparation processes have not yet been developed. To achieve a significant breakthrough, we need a versatile robotic system. Recently, high-performance and reliable multi-axis articulated vertical robots have been developed, and are used in various fields, such as the motor industry. The motion of these industrial robots is fast, precise, and flexible. Moreover, these robots are relatively easy to integrate with other robots and equipment. Although the robotic system requires considerable effort and patience to set up (Blow, 2008), once one of the designated conditions is determined, it becomes applicable in many other situations.

In this chapter, we assess the one-by-one sample preparation method compared with parallel preparation in protein network analysis, using an automated sample preparation system for liquid chromatography-tandem mass spectrometry (LC-MS/MS). This automated system is compatible with the single-step affinity purification technique using the Flag-tag system (Einhauer & Jungbauer, 2001), without sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) separation. Affinity-purification is a technique for purification of physiological protein complexes using target proteins (bait proteins) fused with affinity tags, such as short epitope peptides (e.g., Flag and Myc) or tandem-affinity purification (TAP) tags (Kocher & Superti-Furga, 2007). The bait proteins

are overexpressed in cells and are separated, together with the protein complexes, using affinity beads that bind to the tags. Finally, all component proteins are identified by LC-MS/MS. Using this system, we tested two Wnt signaling pathway (Rao & Kuhl, 2010) proteins, β-catenin and Axin1, as baits, and demonstrated that the one-by-one purification method using this system is highly sensitive and reproducible compared with the manual parallel purification method. The results indicate that gentle and equal preparation conditions are important for generating reliable data for large-scale protein-protein interaction network and for quantitative analysis.

2. Experimental procedures

2.1 Design and development of a robotic system for one-by-one sample preparation

The robotic system was manufactured using four 6-axis robots, FC03N (Kawasaki Heavy Industries, Ltd., Hyogo, Japan) and a 3-axis robot comprising three single-axis robots (IAI corporation, Shizuoka, Japan), with help from the Japan Support System, Co., Ltd. (Ibaraki, Japan) and Nikkyo Technos, Co., Ltd. (Tokyo, Japan). In low femtomole level analysis, the key to obtaining reliable data quickly is to minimize contaminants, such as chemicals, airborne particles, and keratin proteins. Chemicals cause background noise, which limit the sensitivity of MS by decreasing the signal to noise ratio (S/N). Airborne particles, including dust, cause the blockage of the flow path and the nano LC column. Keratin proteins also cause background noise, which disturbs the detection of low abundance of proteins. Therefore, because we needed to perform sample preparation in a super clean room, our automated robotic system was designed for clean room specification (ISO class 4).

2.2 Immobilization of Anti-Flag antibodies to magnetic beads

Anti-Flag M2 antibodies (Sigma-Aldrich, St. Louis, MO) were immobilized via covalent binding of the primary amine group with 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC; Thermo Fisher Scientific, Waltham, MA)–modified Magnosphere MS300 magnetic beads (JSR, Tokyo, Japan). The beads (10 mg) suspension was transferred into a 1.5 ml-microtube. The beads were washed twice with 1 ml of activation buffer (0.1 M 2-[*N*-morpholino]ethane sulfonic acid (MES), pH 6.0, 0.5 M NaCl) and were resuspended in 1 ml of activation buffer. EDC and *N*-hydroxysulfosuccinimide (Sulfo-NHS; Thermo Fisher Scientific, Waltham, MA) were then added to the beads suspension. The final concentrations of EDC and sulfo-NHS were 2 and 5 mM, respectively. The mixture was incubated for 15 min at room temperature (RT), placed on the magnet, and the supernatant was discarded. The antibody (100 μg/ml) in conjugation buffer (50 mM sodium phosphate, pH 7.4, 0.15 M NaCl) was added to the beads and the mixture was incubated for 3 hr at 4 °C. After incubation, the supernatant was discarded and quenching buffer (20 mM HEPES-NaOH, pH 7.5, 0.15 M NaCl, 50 mM ethanolamine) was added. After quenching for 2 hr at 4 °C, the beads were washed three times with 1 ml of washing buffer (50 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 0.1% Triton X-100) and twice with storage buffer (20 mM HEPES-NaOH, pH 7.5, 0.15 M NaCl, 0.5% digitonin). The antibody-immobilized beads were stored in 1 ml of storage buffer at 4 °C.

2.3 Cell culture and transfection

HEK293T cells (approximately 5.0×10^6 cells per 10-cm dish) were seeded in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, San Diego, CA) containing 10% heat-inactivated fetal bovine serum (FBS; Invitrogen) the day before transfection. The cells were transfected with human β-catenin or human Axin1 cDNA, using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The cells were collected 24 h after transfection.

2.4 Cell collection and lysis

The culture medium was discarded from the 10-cm dish, and the HEK293T cells expressing a bait protein were scraped into 1 ml of cold phosphate buffered saline (PBS) and transferred into a 1.5 ml-microtube. After centrifugation at low speed (3,000 rpm) for 1 min at 4 °C, the supernatant was discarded, and 1.0 ml of lysis buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 50 mM NaF, 1 mM Na_3VO_4, 0.5% digitonin, 1 mM $MgCl_2$, 1 mM PMSF, 5 μg/ml leupeptin, 5 μg/ml aprotinin and 3 μg/ml pepstatin A) was added. The cells were lysed by gently mixing for a short time with a vortex mixer (parallel method) or with a pipette tip (one-by-one method). In this step, we chose the vortexing in the parallel method because we thought, in reality, this way had to be adopted in large-scale sample treatment. The lysate was centrifuged at high speed (15,000 rpm) for 10 min at 4 °C, and the cleared lysate was transferred into a microtube containing the anti-Flag antibody immobilized magnetic beads.

2.5 Immunoprecipitation

The supernatant was incubated with the magnetic beads at 4 °C for 10 min with a rotator (parallel method) or the 6-axis robot (one-by-one method; 10 times mixing → interval: 4 min at 4 °C → 10 times mixing → interval: 4 min at 4 °C). After incubation, the beads were washed twice with 1 ml of wash buffer (10 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% Triton X-100). The protein complexes containing the bait protein were then mixed with 100 μl of Flag peptide (0.5 mg/ml, SIGMA) in wash buffer for 5 min at 4 °C using a mixer (parallel method) or a 'protein complexes elution device' (Fig. 2a) (one-by-one method). The eluted fraction was transferred to a new microtube.

2.6 Limited proteolysis with lysyl endopeptidase C (Lys-C)

To concentrate the purified proteins and to exchange the buffer, trichloroacetic acid (TCA) precipitation was performed. Sodium deoxycholate (DOC) was added to a final concentration of 0.1%. After mixing, TCA was added to a final 10% concentration and the solution was precipitated at 0 °C for 30 min. The protein precipitate was collected by centrifugation (15,000 rpm for 10 min at 4 °C). The supernatant was carefully removed, 1 ml of acetone (precooled at -30 °C) was added to the pellet, and vortexing was carried out until the pellet became unstuck from the bottom of the tube. The proteins were collected by centrifugation (15,000 rpm for 5 min at 4 °C) and the supernatant was removed. The pellet was redissolved in 10 μl extraction buffer (0.1 M Tris-HCl, pH 8.8, 0.05% n-octyl glucopyranoside, 7M guanidine hydrochloride) using the microtube mixer. After the proteins were dissolved almost completely, 40 μl of digestion buffer (0.1 M Tris-HCl, pH 8.8,

0.05% n-octyl glucopyranoside) was added and mixed. Finally, 0.1 μg of lysyl endopeptidase (Lys-C; Wako, Osaka, Japan) was added and the mixture was incubated over night at 37 °C.

2.7 Western blotting

HEK293T cells were transfected with human β-catenin or human Axin1 cDNA, or pcDNA3 vector (as a negative control) as described in section 2.3. The purified proteins (from the immunoprecipitation step, section 2.5) were separated by electrophoresis on 10% SDS-PAGE and transferred onto Polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 2% BSA in TBS-T for 1 h at RT, followed by incubation with each primary antibody for 1 h at RT. After incubation with the secondary antibody for 1 h at RT, protein bands were detected with an ECL detection kit.

2.8 Direct nanoflow liquid chromatography tandem mass spectrometry system (DNLC-MS/MS)

All samples were diluted 10-fold with 0.1% formic acid and analyzed (2 μl) by DNLC system (Natsume et al., 2002) coupled to a QSTAR XL (AB Sciex, Foster City, CA). Peptides were separated on a C18 reversed-phase column packed with Mightysil C18 (particle size 3 μm; Kanto Chemical, Tokyo, Japan) at a flow rate of 100 nl/min by a 40-min linear gradient from 5% to 40% acetonitrile in 0.1% formic acid, and were sprayed on-line to the mass spectrometer. MS and MS/MS spectra were obtained in an Information Dependent Acquisition (IDA) mode. Up to two precursor ions above the intensity threshold of 50 counts with a charge state from 2 to 3 were selected for MS/MS analyses (1.0 sec) from each survey scan (0.5 sec). The MS and MS/MS scan ranges were *m/z* 400-1500 and 100-1500, respectively.

2.9 Data analysis

Peak lists were created by scripts of Analyst QS 1.1 Software (AB Sciex) using the following parameters: 0.1 amu Mass tolerance for combining MS/MS spectra, 2 cps MS/MS export threshold, 5 Minimum number of MS/MS ions for export, 50% Centroid height percentage, and 0.05 amu Centroid merge distance. All MS/MS spectra were queried against the National Center for Biotechnology Information (NCBI) non-redundant database (human; January 25, 2011; 137,349 sequences) using an in-house Mascot server (version 2.2.1; Matrix Science, London, UK). Search parameters were as follows: MS and MS/MS tolerance of 250 ppm and 0.5 Da, respectively; enzymatic specificity allowing for 1 missed cleavage site and K cleavage (enzyme: Lys-C/P); no fixed modification; and variable modification of N-acetyl (protein N terminus) and phosphorylations (Ser, Thr, and Tyr). Proteins that were identified by two or more peptides with a peptide expectation value of $p < 0.05$ were considered as reliable identifications.

3. Results

3.1 Automated robotic system for one-by-one sample preparation

To perform precise one-by-one sample purification for protein network analysis, we designed and developed a robotic system for fully automated sample preparation from cell

collection to limited proteolysis with Lys-C. This system consists of four 6-axis industrial robots, one 3-axis robot, high- and low-speed centrifuges, a CO_2 incubator, and other components, as illustrated in detail in Fig. 2.

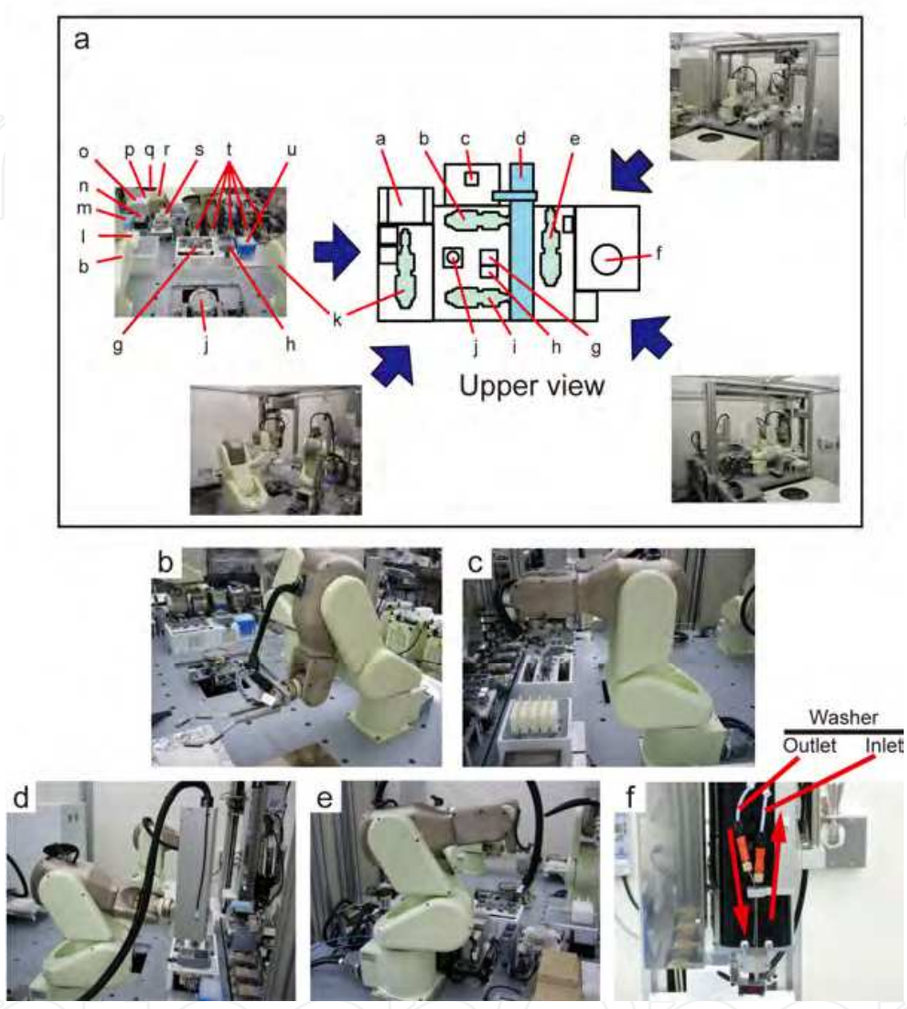

Fig. 2. Layout of the automated robotics for one-by-one sample preparation system.
(a) A schematic upper view diagram of the system and four photographs showing different views indicated by arrows. a: CO_2 incubator; b: 6-axis robot No. 2; c: low-speed centrifuge; d: 3-axis robot; e: 6-axis robot No. 4; f: high-speed centrifuge; g: microtube carriers for low-speed centrifuge; h: buffers position (lysis buffer and phosphate-buffered saline); i: 6-axis robot No. 3; j: culture dish stage; k: 6-axis robot No. 1; l: cell scrapers specialized for this system; m: pipette tips (2-200 µl); n: pipette tips (0.1-10 µl); o: protein complexes elution device; p: incubator (4 °C); q: microtube rack; r: incubator (37 °C); s: reagents rack (elution buffer, TCA, etc.); t: microtube capper/decapper (temperature-controlled); u: pipette tip (200-1,000 µl). (b) 6-axis robot No. 1: culture dish-carrying robot. (c) 6-axis robot No. 2: scraping and tube-carrying robot. (d) 6-axis robot No. 3: dispenser robot. (e) 6-axis robot No. 4: microtube-carrying robot. (f) 3-axis robot: micro-dispenser robot. A washer is attached to this robot.

The features of this system are: (i) The system is optimized for sample preparation from 10-cm culture dishes, and the process operates under gentle conditions to decrease protein denaturation and degradation compared to manual operation. The scraping robot (6-axis robot No. 2) can collect cells gently in a single scraping motion (Fig. 3a and 3b). In addition, a microtube delivery robot (6-axis robot No. 4) can mix the magnetic beads immobilized on the anti-Flag M2 antibody with cell extracts at intervals that will not over-mix or create a foam. Moreover, the elution of the protein complexes in the 'protein complexes elution device' (Fig. 2a) is performed by moving the beads backwards and forwards in the elution buffer between two magnets (Fig. 3c-e). The solution is not mixed vigorously; therefore, this procedure is expected to prevent the denaturation of the eluted protein. (ii) This system allows rapid purification of the protein complexes. One sample, from cell scraping to elution of protein complexes, can be prepared in 40 min. The manual parallel treatment of 20 samples takes more than 120 min. (iii) The one-by-one system can operate 24 hours a day, automatically, generating approximately 500 samples per month.

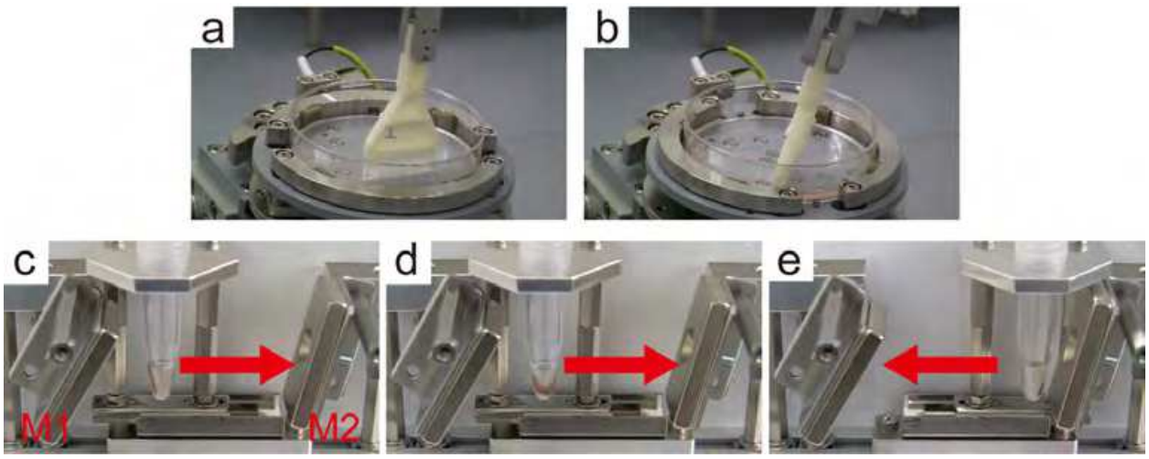

Fig. 3. Automated one-by-one sample preparation system. (a and b) Cell collection on the dish stage. (c-e) Process for elution of the protein complexes in the 'protein complexes elution device' (Fig. 1a). M1 and M2: magnets.

3.2 Comparison of parallel and one-by-one methods for the sample preparation by western blot analysis

To evaluate one-by-one sample preparation, we chose β-catenin and Axin1 as bait proteins because they are well-studied proteins that play key roles in the Wnt signaling pathway, and because, to date, many partners that interact with them have been identified (Daugherty & Gottardi, 2007; H. Huang & He, 2008; S.M. Huang et al., 2009). Furthermore, it is difficult to analyze β-catenin and Axin1-interacting proteins using affinity purification and LC-MS/MS, because these bait proteins are likely to be degraded, not only by the ubiquitin-proteasome system, but also nonspecifically by various proteases during the purification steps, even if protease inhibitors are added. Therefore, we expected that the gentle one-by-one purification method would allow these proteins to remain intact to the greatest extent possible, and would permit the identification of more interacting partner proteins.

We first compared the bait proteins (β-catenin and Axin1) from parallel preparation with those of one-by-one preparation. Flag-tagged β-catenin or Axin1 was expressed in HEK293T cells, purified by the parallel and the one-by-one method, and analyzed by western blotting (Fig. 4). In the case of parallel preparation, both β-catenin and Axin1 were found to be degraded. In particular, Axin1 degradation tended to be fast, and a protein band of approximately 120 kDa, corresponding to the intact form, was almost absent in some cases. On the other hand, in samples prepared by the one-by-one method, degradation of the bait proteins was significantly reduced. Interestingly, for Axin1, only one prominent band of the size of the intact protein was detected in most cases. These data indicated that the one-by-one method minimizes protein denaturation and degradation during sample preparation compared to the parallel method.

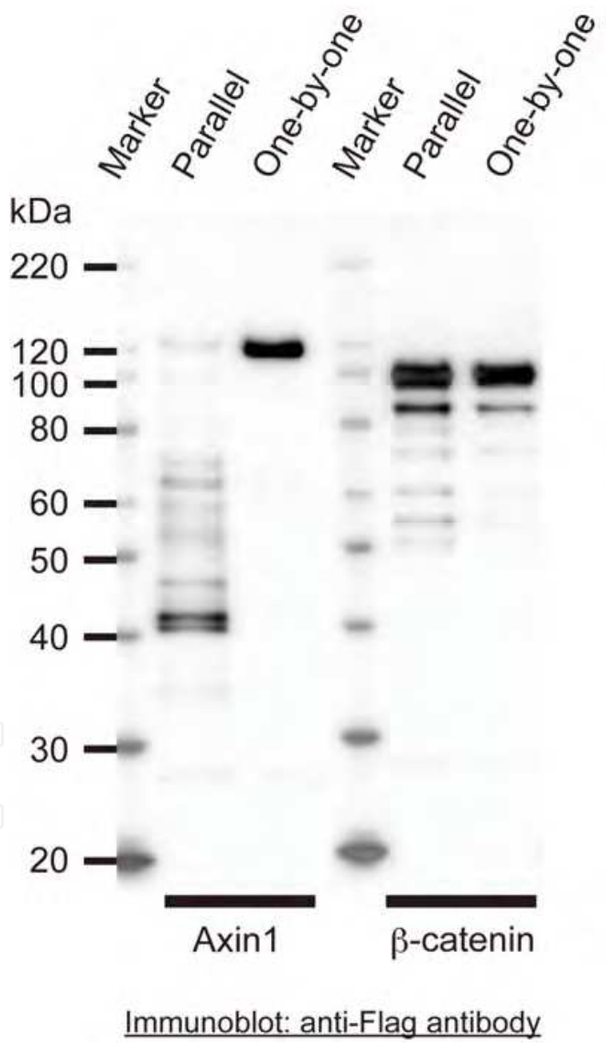

Fig. 4. Comparison of bait protein (β-catenin and Axin1) purification quality. Flag-tagged β-catenin or Axin1 proteins were expressed in HEK293T cells, purified by the parallel or one-by-one methods until the elution steps, and analyzed by western blot analysis. One-by-one: automated one-by-one method; Parallel: manual parallel method.

3.3 Comparison of parallel and one-by-one methods for the sample preparation by protein network analysis

Next, we compared the component proteins interacting with the bait proteins (β-catenin and Axin1) prepared by the parallel and one-by-one methods. Each bait protein was expressed in HEK293T cells and purified with its binding partner proteins. These proteins were then digested with Lys-C and analyzed by a DNLC-MS/MS system (Natsume et al., 2002). The identified proteins that interact with β-catenin and Axin1, excluding nonspecific binding, are listed in Table 1. As expected, the one-by-one preparation method showed better detection sensitivity and reproducibility compared with the parallel method.

Bait: β-catenin

Name[a]	Symbol[a]	Parallel[b] reproducibility (n = 10)	One-by-one[b] reproducibility (n = 10)
Adenomatous polyposis coli	APC	10 (100%)	10 (100%)
Adenomatosis polyposis coli 2	APC2	0	8 (80%)
Axin 1	AXIN1	10 (100%)	10 (100%)
Axin 2	AXIN2	1 (10%)	9 (90%)
Beta-transducin repeat containing	BTRC|FBXW11	1 (10%)	10 (100%)
Cadherin 1, type 1	CDH1	1 (10%)	9 (90%)
Cadherin 2, type 1	CDH2	2 (20%)	10 (100%)
Casein kinase 1, alpha 1	CSNK1A1	10 (100%)	10 (100%)
Catenin, alpha 1	CTNNA1	10 (100%)	10 (100%)
Catenin, alpha; 1 or 2	CTNNA1|CTNNA2	10 (100%)	10 (100%)
Catenin, alpha; 1 or 3	CTNNA1|CTNNA3	10 (100%)	10 (100%)
Catenin, beta interacting protein 1	CTNNBIP1	8 (80%)	10 (100%)
Catenin, delta 1; isoform 1B	CTNND1	0	10 (100%)
Cathepsin A	CTSA	9 (90%)	10 (100%)
Cullin 1	CUL1	2 (20%)	10 (100%)
Ezrin	EZR	0	8 (80%)
Family with sequence similarity 123B	FAM123B	10 (100%)	10 (100%)
F-box and WD repeat domain containing 11	FBXW11	1 (10%)	10 (100%)
Galactosidase, beta 1	GLB1	8 (80%)	10 (100%)
Glycogen synthase kinase 3 alpha	GSK3A	2 (20%)	10 (100%)
Glycogen synthase kinase 3; alpha or beta	GSK3A|GSK3B	6 (60%)	10 (100%)
Glycogen synthase kinase 3 beta	GSK3B	4 (40%)	10 (100%)
HMG-box transcription factor TCF-3	TCF7L1	7 (70%)	10 (100%)
Lymphoid enhancer-binding factor 1	LEF1	8 (80%)	10 (100%)

Bait: β-catenin

Name[a]	Symbol[a]	Parallel[b] reproducibility (n = 10)	One-by-one[b] reproducibility (n = 10)
S-phase kinase-associated protein 1	SKP1	2 (20%)	10 (100%)
Transcription factor 7 (T-cell-specific, HMG-box); isoform 1	TCF7	3 (30%)	10 (100%)
Transcription factor 7-like 2	TCF7L2	10 (100%)	10 (100%)

Bait: Axin1

Name[a]	Symbol[a]	Parallel[b] reproducibility (n = 10)	One-by-one[b] reproducibility (n = 10)
Adenomatous polyposis coli	APC	0	10 (100%)
Beta-catenin	CTNNB1	0	10 (100%)
Casein kinase 1, alpha 1	CSNK1A1	1 (10%)	10 (100%)
Glycogen synthase kinase 3 beta	GSK3B	2 (20%)	10 (100%)
Macrophage erythroblast attacher	MAEA	0	10 (100%)
WD repeat domain 26; isoform b	WDR26	0	8 (80%)

Table 1. Comparison of identified proteins and their reproducibility from samples prepared by parallel and one-by-one methods (analyzed by MS). [a]Protein names and Symbols refer to the Entrez Gene database. The proteins identified by a common peptide sequence are indicated by 'or' in the Name column, and ' | ' in the Symbol column. The identified proteins exclude nonspecific proteins (Table 2). [b]The samples were prepared independently by the parallel or the one-by-one method and analyzed by the DNLS-MS/MS system.

In the analysis of the one-by-one preparation β-catenin, we identified membrane proteins (Cadherins 1 and 2), peripheral membrane proteins (δ-catenin and Ezrin), the Skp1- Cullin-F-box-protein (SCF) E3 ubiquitin ligase complex (BTRC/FBXW11, Skp1, and Cullin1) and other component proteins (Adenomatosis polyposis coli 2 (APC2) and Axin2) using the one-by-one method, whereas some of these proteins were not identified by the parallel method. The reproducibility increased from below 20% (parallel preparation, n = 10) to above 80% (one-by-one preparation, n = 10). In the analysis of Axin1, the one-by-one method dramatically increased the precision of the identification of well-known interaction partners, such as Adenomatous polyposis coli (APC), δ-catenin, Glycogen synthase kinase 3β (GSK3β), and Casein kinase 1, whereas no specific interactions were identified using the parallel method (Table 1). This improvement is probably the result of the minimal degradation of Axin1 (Fig. 4). Furthermore, we found two new interacting partners: MAEA and WDR26. To confirm these interactions, Flag-tagged Axin1 was expressed in HEK293T cells and the cell extracts were subjected to immunoprecipitation with anti-Flag antibody, followed by western blotting with anti-MEAE or anti-WDR26 antibody. As shown in Fig. 5, both MAEA and WDR26 were found to form a complex with Axin1. Further work is required to determine the biological relevance of these interactions.

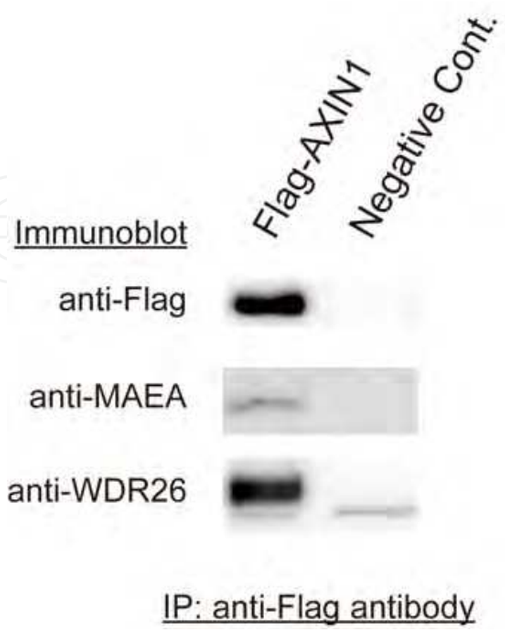

Fig. 5. Interaction of Axin1 with MAEA and WDR26. HEK293T cells were transfected with Flag-Axin1 or an empty vector (pcDNA3) as a negative control (Negative cont.). Expressed protein complexes were purified by the automated one-by-one methods until the elution step and analyzed by western blot analysis.

4. Discussion

Sample preparation is one of the most important processes for MS-based proteomics, such as large-scale protein-protein interaction networks and quantitative analyses. In affinity purification, although the single Flag-tag purification MS approach is useful and raises the possibility of identification of low abundant and transient interacting proteins, the problem is that this approach leads to a high false positive rate (Chen & Gingras, 2007). To overcome this problem, several protocols have been devised (Burckstummer et al., 2006; Selbach & Mann, 2006), and computational data processing to remove nonspecific proteins is performed during large-scale analysis (Ewing et al., 2007; Ho et al., 2002; Gavin et al., 2002). However, because it is possible to reliably identify low amounts of true interacting proteins by improving the signal-to-noise ratio in LC-MS/MS, we considered that reproducibly decreasing the level of nonspecific noise proteins in single-step purification samples would be a valid approach. Therefore, we empirically developed and optimized the conditions for sample preparation, and using this methodology, found more than fifty significant protein-protein interactions (Hirano et al., 2005; Kitajima et al., 2006; Iioka et al., 2007; Komatsu et al., 2007; Nishiyama et al., 2009; Kaneko et al., 2009; Komatsu et al., 2010). In spite of this useful methodology, we realized the limitations of the existing preparation method in large-scale analysis, because we found that the amount of true interactors, as well as nonspecific proteins, in manually parallel-

prepared samples varied. The ultimate solution for this problem was to use a one-by-one purification method. In addition, because this preparation process needs to be automated to prepare samples under precisely equal conditions, we designed and developed a fully automated robotic sample preparation system for LC-MS/MS.

In a validation study using the Wnt signaling pathway proteins, β-catenin and Axin1, the rate of protein degradation was significantly higher in the parallel preparation compared with the one-by-one preparation. This higher protein degradation in parallel preparation is probably caused by the manual scraping of cells and increased preparation time. In parallel preparation, manual scraping of cells involves several rapid strokes, which may increase the cells' susceptibility to damage and increase the level of proteolytic enzymes released from subcellular compartments. The proteases, similarly to nonspecific binding proteins, are likely to attach to and degrade the purified protein complexes over time, and these degraded and denatured proteins are thought to cause nonspecific binding.

In contrast to manual parallel preparation, an important feature of the one-by-one system is the careful and brief sample preparation. The association rate of nonspecific proteins is thought to be slower than that of specific binding proteins; therefore, the careful and rapid one-by-one method reduces nonspecific protein associations. In fact, as shown in Table 2, the number of nonspecific proteins precipitated using the one-by-one method was significantly lower than that by the parallel method. Using the one-by-one method, this decrease was accompanied by a remarkable increase in known interactors, because the signal-to-noise ratio was increased in combination with the prevention of protein degradation. Although it was previously reported that single-affinity tag purifications brought an increase in nonspecific binding proteins (Chen & Gingras, 2007), we have found that the single-step one-by-one purification using anti-Flag antibody immobilized magnetic beads is valuable because of its considerable reduction in nonspecific binding proteins under optimized conditions.

Name[a]	Symbol[a]	Parallel[b]	One-by-one[b]
Actin, alpha 1, skeletal muscle | Actin, alpha 2, smooth muscle, aorta | Actin, beta | Actin, alpha, cardiac muscle 1 | Actin, gamma 1 | Actin, gamma 2, smooth muscle, enteric	ACTA1 | ACTA2 | ACTB | ACTC1 | ACTG1 | ACTG2	2	2
Actin, alpha 1, skeletal muscle | Actin, alpha 2, smooth muscle, aorta | Actin, beta | Actin, gamma 2, smooth muscle, enteric	ACTA1 | ACTA2 | ACTC1 | ACTG2	1	1
ATPase family AAA domain-containing protein 3A | ATPase family AAA domain-containing protein 3B	ATAD3A | ATAD3B	6	ND
Complement component 1, q subcomponent binding protein	C1QBP	2	ND

Name[a]	Symbol[a]	Parallel[b]	One-by-one[b]
DEAH (Asp-Glu-Ala-His) box polypeptide 9	DHX9	4	ND
Eukaryotic translation elongation factor 1 alpha 1|Eukaryotic translation elongation factor 1 alpha 2|Eukaryotic translation elongation factor 1 alpha 1 pseudogene 5	EEF1A1|EEF1A2|EEF1A1P5	5	ND
Eukaryotic translation initiation factor 4A1	EIF4A1	6	2
Eukaryotic translation initiation factor 4A1|Eukaryotic translation initiation factor 4A2	EIF4A1|EIF4A2	2	ND
Histone cluster 1, H1c|Histone cluster 1, H1d|Histone cluster 1, H1e	HIST1H1C|HIST1H1D|HIST1H1E	2	ND
Heat shock protein 90kDa alpha (cytosolic), class A member 1	HSP90AA1	7	2
Heat shock protein 90kDa alpha (cytosolic), class A member 1|Heat shock protein 90kDa alpha (cytosolic), class A member 2	HSP90AA1|HSP90AA2	3	1
Heat shock protein 90kDa alpha (cytosolic), class A member 1|Heat shock protein 90kDa alpha (cytosolic), class A member 2|Heat shock protein 90kDa alpha (cytosolic), class B member 1|heat shock protein 90kDa alpha (cytosolic), class B member 2 (pseudogene)	HSP90AA1|HSP90AA2|HSP90AB1|HSP90AB2P	2	2
Heat shock protein 90kDa alpha (cytosolic), class A member 1|Heat shock protein 90kDa alpha (cytosolic), class B member 1	HSP90AA1|HSP90AB1	2	2
Heat shock protein 90kDa alpha (cytosolic), class B member 1	HSP90AB1	4	1
Heat shock protein 90kDa alpha (cytosolic), class B member 1|Heat shock protein 90kDa alpha (cytosolic), class B member 3 (pseudogene)	HSP90AB1|HSP90AB3P	3	1
Heat shock 70kDa protein 1A|Heat shock 70kDa protein 1B	HSPA1A|HSPA1B	13	5
Heat shock 70kDa protein 1A|Heat shock 70kDa protein 1B|Heat shock 70kDa protein 1-like	HSPA1A|HSPA1B|HSPA1L	4	3

Name[a]	Symbol[a]	Parallel[b]	One-by-one[b]
Heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)	HSPA5	19	8
Heat shock 70kDa protein 8	HSPA8	12	9
Heat shock 60kDa protein 1 (chaperonin)	HSPD1	21	10
Nucleolin	NCL	13	2
Nucleophosmin (nucleolar phosphoprotein B23, numatrin)	NPM1	3	1
Poly(A) binding protein, cytoplasmic 1	PABPC1	3	ND
Poly (ADP-ribose) polymerase 1	PARP1	15	4
Ribosomal protein L10a	RPL10A	3	ND
Ribosomal protein L11	RPL11	4	ND
Ribosomal protein L12	RPL12	4	ND
Ribosomal protein L13	RPL13	2	ND
Ribosomal protein L18	RPL18	3	1
Ribosomal protein L22	RPL22	2	1
Ribosomal protein L23	RPL23	2	1
Ribosomal protein L23a	RPL23A	5	ND
Ribosomal protein L24	RPL24	2	1
Ribosomal protein L28	RPL28	2	1
Ribosomal protein L29	RPL29	4	ND
Ribosomal protein L3	RPL3	5	ND
Ribosomal protein L30	RPL30	2	ND
Ribosomal protein L31	RPL31	3	ND
Ribosomal protein L35	RPL35	2	ND
Ribosomal protein L37a	RPL37A	2	ND
Ribosomal protein L38	RPL38	3	ND
Ribosomal protein L4	RPL4	5	2
Ribosomal protein L5 | Ribosomal protein, large, P0	RPL5 | RPLP0	5	2
Ribosomal protein L6	RPL6	6	ND
Ribosomal protein L7a	RPL7A	3	2
Ribosomal protein L8	RPL8	2	ND
Ribosomal protein L9	RPL9	3	1
Ribosomal protein, large, P0	RPLP0	2	ND
Ribosomal protein, large, P2	RPLP2	4	2
Ribosomal protein S11	RPS11	2	ND
Ribosomal protein S12	RPS12	2	ND
Ribosomal protein S13	RPS13	5	1
Ribosomal protein S15	RPS15	2	ND
Ribosomal protein S16	RPS16	4	ND
Ribosomal protein S19	RPS19	4	2
Ribosomal protein S20	RPS20	3	ND
Ribosomal protein S23	RPS23	2	ND

Name[a]	Symbol[a]	Parallel[b]	One-by-one[b]
Ribosomal protein S24	RPS24	2	ND
Ribosomal protein S25	RPS25	4	ND
Ribosomal protein S27a | Ubiquitin A-52 residue ribosomal protein fusion product 1 | Ubiquitin B | Ubiquitin C	RPS27A | UBA52 | UBB | UBC	3	2
Ribosomal protein S3	RPS3	5	2
Ribosomal protein S3A	RPS3A	7	2
Ribosomal protein S4, X-linked	RPS4X	4	1
Ribosomal protein S4, X-linked | Ribosomal protein S4, Y-linked 1 | Ribosomal protein S4, Y-linked 2	RPS4X | RPS4Y1 | RPS4Y2	3	ND
Ribosomal protein S5	RPS5	3	ND
Ribosomal protein S6	RPS6	4	ND
Ribosomal protein S7	RPS7	4	2
Ribosomal protein S8	RPS8	3	ND
Ribosomal protein S9	RPS9	4	1
Ubiquitin A-52 residue ribosomal protein fusion product 1	UBA52	3	1

Table 2. Comparison of nonspecific proteins identified from samples prepared by parallel and one-by-one methods (analyzed by MS). [a]The nonspecific proteins co-purified with β-catenin (n = 10) and Axin 1 (n = 10) using each method were categorized according to the criteria reported by Chen and Gingras. Protein Symbols and Names refer to the NCBI Gene database. Proteins identified by a common peptide sequence are indicated by ' | ' in the Name, Symbol columns. [b]Total number of the identified peptides. ND: not detected.

5. Conclusion

We have described a one-by-one sample preparation method for MS-based high-precision protein network analysis. To perform a pilot feasibility study of the one-by-one method, we designed and developed a fully automated robotic system. This system makes it possible to prepare samples under equally fast and gentle conditions. To clarify the importance of the one-by-one method, we compared protein complexes prepared by the automated one-by-one system with manual parallel preparation using β-catenin and Axin1 as baits, which are well-characterized Wnt signaling pathway proteins. One-by-one purification resulted in a sharp decrease in proteolytic degradation of purified proteins and in nonspecific binding proteins, allowing the reproducible identification of known interaction partners, as well as novel component proteins. These results suggest that one-by-one sample preparation by the automated system is useful for obtaining reliable data for high-precision analysis of protein identification and quantification for large-scale protein network analysis compared with manual parallel preparation.

We expect that this system will allow highly sensitive analyses of protein interactions using various types of cells, such as embryonic stem (ES), neuronal, and primary cells, which are

limited in supply. Furthermore, we envision that this system could be used for qualitative and quantitative protein interaction network studies including chemical proteomics (Rix & Superti-Furga, 2009).

In future work, we will develop a multi-purpose robotic system that can be flexibly customized. Finally, our goal is to develop an automated robotic system that can operate not only in affinity purification, but also in general proteomics.

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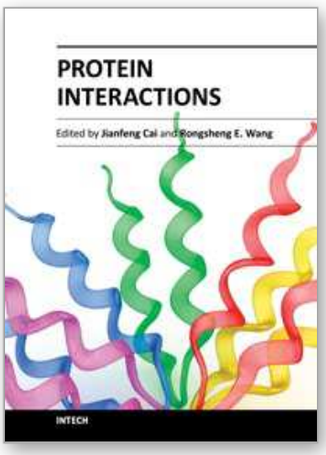

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Protein interactions, which include interactions between proteins and other biomolecules, are essential to all aspects of biological processes, such as cell growth, differentiation, and apoptosis. Therefore, investigation and modulation of protein interactions are of significance as it not only reveals the mechanism governing cellular activity, but also leads to potential agents for the treatment of various diseases. The objective of this book is to highlight some of the latest approaches in the study of protein interactions, including modulation of protein interactions, development of analytical techniques, etc. Collectively they demonstrate the importance and the possibility for the further investigation and modulation of protein interactions as technology is evolving.

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