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Miniaturized and Automated High-Throughput Verification of Proteins in the ISET Platform with MALDI MS

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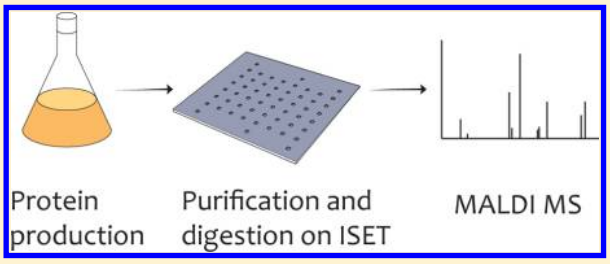
Supporting Information

ABSTRACT: A major bottleneck in high-throughput protein production is the validation step, which is why parallel and automated sample processing methods are highly desirable. Also, a miniaturized sample preparation format is preferred, as the reduction of reagent volumes significantly decreases the analysis cost per sample. We have developed an automated and miniaturized protein sequence verification protocol for recombinant proteins utilizing peptide mass fingerprinting and MS/MS analysis. The integrated selective enrichment target (ISET) platform, previously developed in our group, with its dual functionality, being both a sample preparation platform and a MALDI target plate, is employed. All steps including immobilized metal ion affinity chromatography of protein on cobalt-loaded beads, tryptic digestion, and MALDI MS analysis are performed in an array format, without any sample transfers, on the same ISET chip. The automated configuration reduced the sample preparation time significantly. Starting with crude lysate, a full plate of 48 purified, digested samples prepared for MALDI-MS can be generated in 4 h, with only 30 min of operator involvement. This paper demonstrates the utility of the method by parallel analysis of 45 His-tagged human recombinant proteins.



In large proteomics projects, where high-throughput is essential, protein production and purification play an important role.[1−3] Within the Human Protein Atlas (HPA) project antibody-based methods are used to characterize the human proteome.[4] The HPA project generates almost 300 antigens every week, and the process requires the use of standardized protocols optimized for maximal success rates.[1] Although the majority of the purified proteins (82%)[1] meet the established requirements of purity and amount, some antigens will be discarded due to either low expression level, too large proportions of contaminants, or failure in protein identification. Thus, characterization and verification are of great importance. However, as protein analysis is generally a late step in the protein production workflow, valuable resources can be lost on erroneously produced proteins. To decrease both the cost and effort spent on these proteins, a rapid protein characterization step, prior to scale-up for large-scale protein purification, is desirable.

Such protein characterization can be accomplished in a number of ways. Recombinant proteins are often produced as fusions to a polyhistidine (His) tag. The His-tag is commonly utilized in screening applications, where anti-His antibodies can be used for purification of protein products.[5−7] This tag also enables purification using immobilized metal ion affinity chromatography (IMAC). IMAC is a robust method with the advantage that it is selective also under denaturing conditions, which has contributed to its wide acceptance.[5−8] A small-scale method for screening of protein products using IMAC purification was recently developed within the HPA project, where the protein production, purification, and analysis setup was scaled down to a microplate format.[9]

Lately, chip-based solutions, which are scaled down even further, has been put in focus within the biotechnological field, and several microfluidic concepts have been developed for sample preparation applications[10,11] (reviewed by Lee et al.[12]). The ability to handle minute sample volumes, amenability to high-throughput analysis, and the increased reaction rates make miniaturization highly attractive (reviewed by Feng et al.[13] and Laurell and Marko-Varga[14]). A promising strategy for highly sensitive and accurate analysis of molecules in small sample volumes is sample preparation using microfluidic devices in combination with mass spectrometry (MS), which is an excellent method for protein product verification (reviewed by Wang and Chait[15] and Aebersold and Mann[16]). When analyzing continuously produced samples online, electrospray ionization (ESI) is usually preferred due to the potential of direct coupling of the fluidic device to the MS instrument.[17,18] However, when speed and high sample throughput are critical parameters, as in protein verification after microchip-based protein enrichment, matrix-assisted laser desorption/ionization (MALDI) MS is a better choice. Different miniaturized sample preparation protocols/methods have been presented in the

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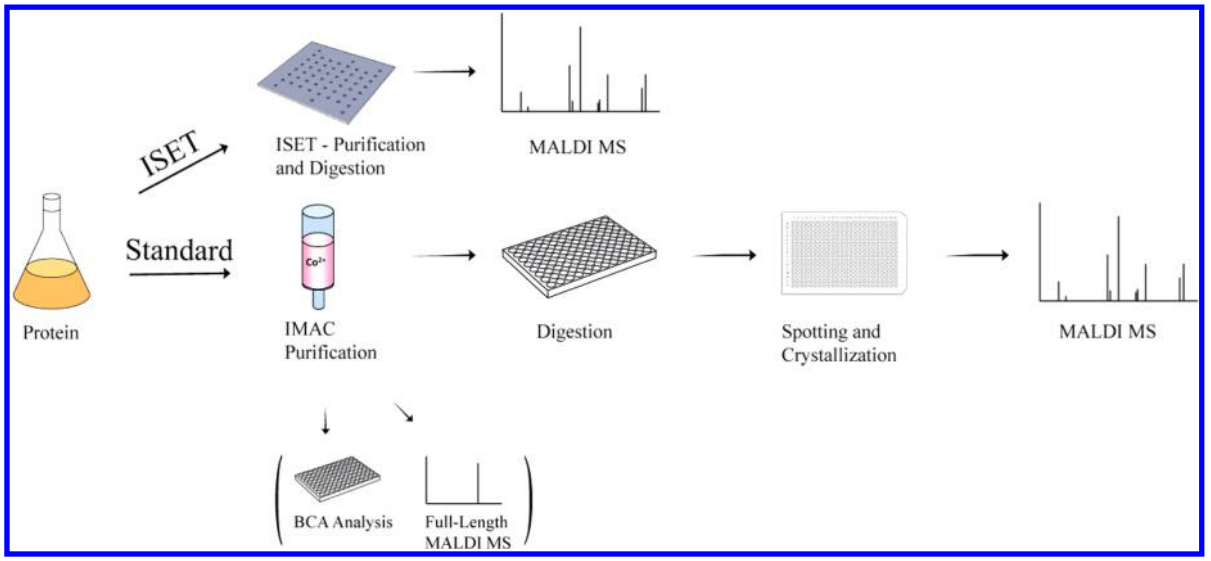

Figure 1. Workflow for the entire experimental setup. Recombinant proteins from the protein production are verified utilizing the ISET platform (top row). The proteins were also purified and verified in the same way but with a standard method (second row). The protein analyses that are performed on the purified proteins within the HPA project are concentration analysis (BCA) and MALDI analysis of the full-length proteins (bottom).

literature, e.g. compact disk based[19,20] and on-chip based.[21,22] A generic alternative is the integrated selective enrichment target (ISET),[23−25] which is a MALDI-target with integrated nanocolumns in a 48- or 96-array format. The ISET platform is compatible with standard liquid handlers and allows the user to load any type of chromatographic resin. Furthermore, it is compatible with any MALDI MS instrumentation.

In this paper, we have developed an automated, high-throughput protein validation method based on the ISET platform. The method is demonstrated by triplicate verification of 45 human recombinant proteins from the HPA project. These 45 proteins include human protein motifs and are used as antigens for antibody generation within the HPA project. In the protocol, IMAC purification and on-bead tryptic digestion was performed directly in the ISET nanocolumns, enabling peptide mass fingerprinting (PMF) and MS/MS analysis of the generated peptides. The automated configuration reduced the sample preparation time significantly compared to the reported standard method.

■ EXPERIMENTAL SECTION

Two methods were compared: the microfluidic ISET and a standard protocol, where the same steps were performed using standard labware. The complete experimental work-flow is depicted in Figure 1.

Protein Production. Strains of DNA sequences coding for 25−100 amino acid fragments of human proteins, fused to an N-terminal His and albumin binding protein (ABP) tag, were cloned into the plasmid expression vector pAff8c[26] (see Supporting Information for protein sequences). The vectors were transformed into *Escherichia coli* Rosetta (DE3) cells (Novagen, Merck, Darmstadt, Germany) for protein production.

For the cultivation, deep-well plates with 1 mL of culture media [TSB+Y/CmKm: 30 g/L tryptic soy broth (Merck) supplemented with 5 g/L yeast extract (Merck), 20 μg/mL chloramphenicol (Sigma-Aldrich, St. Louis, MO), and 50 μg/mL kanamycin (Duchefa, Haarlem, Netherlands)] per well were inoculated with 10 μL of a bacterial culture and incubated overnight at 37 °C at 150 rpm. The following cultivation was performed in shake flasks as previously described.[1] A total of 45 proteins were produced and used in both the standard and miniaturized method.

Standard Protein Verification and HPA Method. Standard protein verification was performed on the 45 bacterial lysates with overexpressed recombinant proteins using IMAC purification on columns and MALDI-MS detection. The IMAC purification was performed on an automated liquid-handling system.[27] Briefly, columns were manually packed with 1 mL of HisPur cobalt resin (Thermo Scientific, Rockford, IL) and equilibrated with 20 mL of washing buffer (6 M guanidinium chloride, 47 mM Na_2HPO_4, 3.4 mM NaH_2PO_4, 300 mM NaCl, pH 8.0−8.2). Lysates were added to the columns and washed with 30 mL of washing buffer. Finally, elution with 2.5 mL elution buffer (6 M urea, 50 mM NaH_2PO_4, 100 mM NaCl, 30 mM acetic acid, 70 mM sodiumacetate, pH 5.0) was performed.

To verify protein identity, the purified proteins were digested and analyzed using MALDI-MS. Of each sample, 1 μL was diluted in ammonium bicarbonate (AmBic, Sigma-Aldrich, 50 mM NH_4HCO_3, pH 7−8) to a total volume of 23 μL and digested with 2 pmol trypsin (Promega) for 16 h at 37 °C. To end the digestion and create a suitable environment for the matrix, 20 μL of 5% TFA was added to the samples. The digested samples were spotted (1 μL/spot) in triplicate on a standard-steel target MALDI plate, and the equal volume of matrix (8 mg/μL α-cyano-4-hydroxycinnamic acid, diluted in 60% ACN, 0.5% TFA) was added.

In the HPA method, the protein concentration of the eluates was determined with Thermo Scientific's bicinchoninic acid (BCA) protein assay kit in a microwell format according to the manufacturer's instructions. Molecular weights of the purified proteins in linear mode were verified using a MALDI-time of flight (TOF) LaserToF LT3 Plus instrument (SAI, Manchester, UK).

Miniaturized Automated Protein Verification Using the ISET Platform. The miniaturized automated protein verification was performed using an ISET silicon chip. The chip contains nanovials, which can be filled with chromatographic

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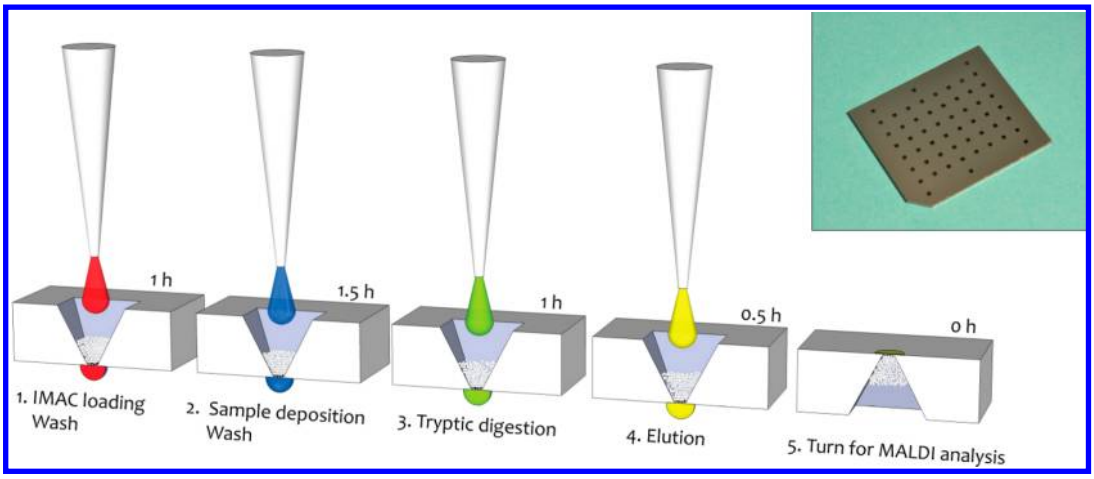

Figure 2. Sample preparation workflow using the ISET chip. Vacuum is used to facilitate liquid transport. From the left to the right: HisPur cobalt beads are deposited and subsequent washing is performed (1), the crude lysate is added and washing is carried out to remove contaminants (2), tryptic digestion is performed while buffer is added to keep the beads wet (3), the peptides are eluted onto the backside of the chip (4), and the chip is turned upside down for MALDI analysis (5). The ISET chip is shown in the top right corner.

media. The membrane in the bottom of each vial, consisting of a 3 × 3 square array of microholes, each 22 × 22 μm, holds the resin in place. The ISET plate, 53 × 41 mm in size, containing 48 nanovials, was positioned in a vacuum device using an in-house-made adapter. The ISET chip was placed in a Biomek 3000 Laboratory Automation Workstation (Beckman Coulter, Brea, CA) to enable automated sample handling with an external vacuum pump. During a fixed vacuum of 8−10 inHg, the nanovials were each packed with 500 nL of HisPur cobalt resin, and 2 × 5 μL of wash buffer was subsequently pulled through the vials. To each nanovial, 6 μL of *E. coli* lysate with overexpressed recombinant proteins (see Protein Production) was provided in triplicate. The beads were washed with 4 × 5 μL of wash buffer and 4 × 5 μL of AmBic. The vacuum adaptor together with the chip was transferred to a custom-built eight-channel solenoid system (Seyonic SA, Neuchâtel, Switzerland) for the tryptic digestion. The beads were wetted with 250 nL of AmBic before addition of 2 × 250 nL of 4 pmol/μL porcine trypsin (Promega, Madison, WI). Tryptic digestion was performed without vacuum pressure at room temperature for 1 h. AmBic was continuously added to prevent the beads from drying, which would inactivate the enzyme. For elution, 2 × 250 nL of α-cyano-4-hydroxycinnamic acid (Fluka Analytical, Sigma-Aldrich) [3 mg/mL, diluted in 60% acetonitrile (ACN, Sigma-Aldrich) and 0.5% trifluoroacetic acid (TFA, Sigma-Aldrich)] was used. During elution, the vacuum pressure was again turned on, but lowered to 2−3 inHg. This made the eluted liquid form an analyte droplet on the backside of the chip, which was allowed to crystallize around the outlet hole.

MALDI-MS Analysis and Data Evaluation. After crystallization, the ISET chip was turned upside down, loaded in an adapted target holder, and like the standard-steel target for the standard method inserted into a MALDI-Orbitrap XL (Thermo Scientific). Spectra were acquired using a mass range of m/z 600−4000 at a resolution of 60 000. The MALDI method allowed for one Fourier transform (FT) MS scan event followed by ion trap (IT) MS/MS on the 50 highest peaks reiterated with a time limit of 3 min per spot. MS/MS was performed using a normalized collision energy of 50% during an activation time of 30 ms and activation Q 0.250. An inclusion list with all the theoretical tryptic peptides (two missed cleavages) was used for MS/MS detection. A FASTA file of the produced target protein sequences (Table S-1, Supporting Information), *E. coli* proteins, and common laboratory contaminants was produced for the data analysis. For the PMF analysis, the monoisotopic masses were extracted with Xcalibur (Thermo Scientific) and analyzed with MassSorter 3.1 (Harald Barsnes, University of Bergen, Bergen, Norway). The database search allowed for 10 ppm mass accuracy and two missed cleavages. Thermo Proteome Discoverer 3.1 was used for the MS/MS data analysis, Sequest was used as the search engine with two missed cleavages, and the mass tolerance was set to 10 ppm for the precursor and 0.8 Da for the fragment ions.

RESULTS AND DISCUSSION

In this study, a high-throughput, miniaturized screening method for validation of recombinant proteins has been developed. The method utilizes the ISET platform, which enables integrated sample preparation and MALDI MS analysis on the same chip without any sample transfers. Purification of His-tagged proteins from crude lysate, tryptic digestion, and analysis using PMF and MS/MS were performed on-chip. The method is presented in three main sections: the automated protocol, MS analysis, and subsequently a comparison with the standard method.

Automated and Miniaturized ISET Protocol. Enrichment of target proteins from crude lysate of a bacterial cultivation, tryptic digestion, and MALDI sample preparation was performed for a full ISET chip with 48 positions in only 4 h. Apart from the short processing time, the developed method has a number of advantages. It should be noted that due to the miniaturized format of the ISET platform, only a minute amount of sample (6 μL) is required for the analysis, and due to the new configuration of the outlet holes, undiluted crude lysate can be applied directly without dilution.[28] In addition, since the ISET chip acts both as a sample preparation platform and MALDI target plate and as there are no sample transfers, sample losses and contamination risks in this system are minimized. The ISET sample preparation, which consists of five steps, is presented in Figure 2.

In the first step, the ISET chip is placed in the vacuum manifold of the Beckman robot, where cobalt resin is loaded. This requires 1 h for an ISET chip with 48 positions, using

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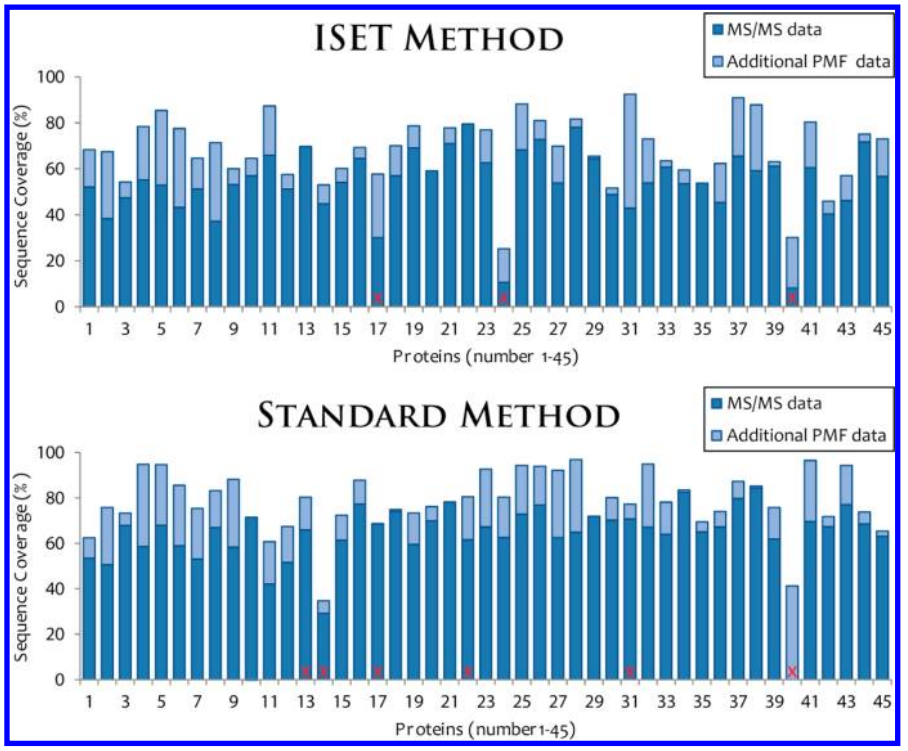

Figure 3. Sequence coverage for PMF and MS/MS data for the ISET and standard method. The sequence coverage in the PMF analysis is shown in light blue, and in front of those bars, the additional data from MS/MS are visualized in dark blue. The red crosses indicate proteins that only have sequence coverage from the common tag and therefore are not considered as identified.

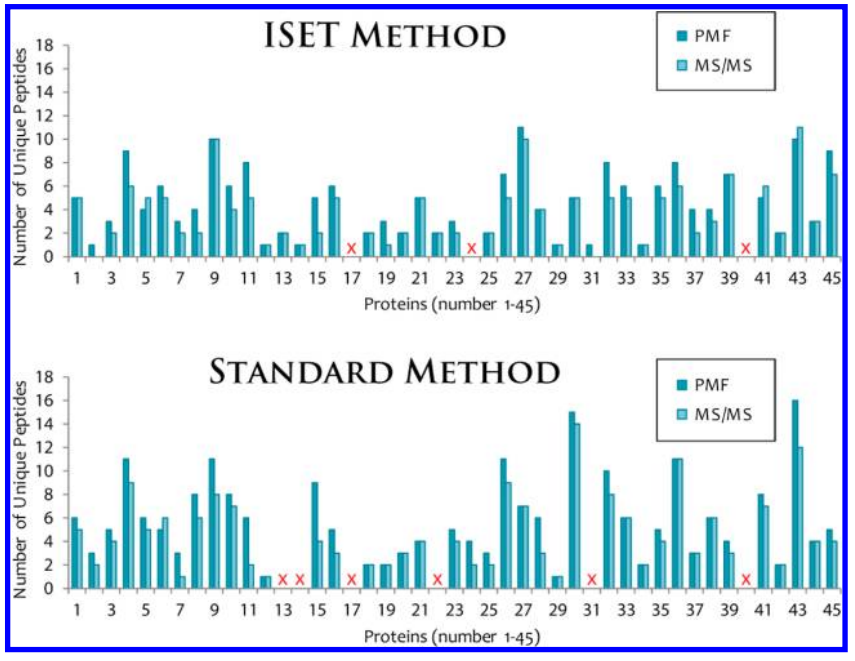

Figure 4. The unique peptides found when using the ISET and standard method. On the top, the PMF and MS/MS results for the ISET method are shown and underneath the corresponding results for the standard method. Proteins with no identified unique peptides are indicated with a red cross. Note that the different proteins have an unequal amount of theoretical tryptic peptides and are therefore not comparable.

single channel liquid handling, including setup of the robotic systems with samples and buffers.

The second step, which consists of affinity capture of His-tagged proteins in the nanovials and subsequent washing to remove cell debris and unspecific binding, is carried out in 1.5 h.

Tryptic digestion (third step) is performed for 1 h without vacuum, during which time the noncontact nanoliter volume dispensing robotic system is programmed to continuously add buffer to keep the nanovials from drying. Protein digestion directly on a MALDI target after desalting or target enrichment has been presented previously.[29,30] Also, digestion of purified proteins on the ISET has been reported.[31] However, for the

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first time purification of proteins using IMAC from a crude cultivation broth and tryptic digestion has been performed in an integrated and automated sequence on the ISET platform. As a result of the miniaturized format, the tryptic digestion step could be performed in only 1 h at room temperature.

In the fourth step, elution liquid passes through the vials and forms a droplet that evaporates to give crystallized MALDI spots on the backside of the chip. Finally, the chip is turned upside down and inserted into the mass spectrometer. A problem with MALDI MS is the heterogeneity of the sample spots, which can be diminished by decreasing the spot size. In our method the spot size is reduced by eluting the protein peptides with 250 nL twice, which leads to a spot size of around 1.5 mm in diameter. The total amount of time needed for the elution and crystallization step is 30 min.

Within the four sample preparation hours the operator time is approximately 30 min and includes setup of the robots with samples and buffers and transfer of the ISET chip between the Biomek 3000 and Seyonic robots. The total time of 4 h for 48 samples corresponds to 5 min per sample, excluding MS analysis, which can be compared to the work of Calderón-Santiago et al., where 12 min per sample was required for an automated regular solid-phase extraction protocol.[32] The short sample processing time makes the automated ISET method highly favorable in many screening applications.

PMF and MS/MS Analysis. To enable more accurate verification, compared to analysis of the intact proteins as previously reported within the HPA project,[9] PMF and MS/MS were performed. The analyzed proteins consist of an N-terminal HisABP tag and a target protein sequence of 25−100 amino acids at the C terminus (see the Supporting Information for protein sequences), which are to be used as antigens for polyclonal antibody production within the HPA project.

For this study, 45 proteins were randomly selected. An in-silico tryptic digestion was carried out to determine the number of unique peptides for each protein. As each protein contained a common HisABP tag, peptides corresponding to parts of this sequence would not contribute to identification of a specific protein. Peptides ranging between 600 and 4000 Da were generated in the in-silico digestion, and it was found that one of the proteins did not include any unique peptides in this size range. Protein identity was considered verified after identification of at least one unique peptide and a sequence coverage over 35%.

PMF was performed on all samples, resulting in an average protein sequence coverage of 68%. The obtained sequence coverage for all 45 proteins is shown in Figure 3 and listed in the Supporting Information (Table S-2). As evident in Figure 4, 42 out of the 45 studied proteins could be verified with the PMF data. For the remaining three proteins (17, 24, and 40), no unique peptide could be found. Protein 17 had a sequence that provided no theoretical peptides in the given mass range and could therefore not be verified. The lack of theoretical tryptic peptides could turn into a difficulty when the produced protein is too short or lacks cleaving sites for the used enzyme, as a solution different proteases could be used.[33,34] For the remaining two unassigned proteins, either ion suppression effects[35] could be the culprit or the proteins could have been incompletely produced from the *E. coli*.

As MS/MS will provide more detailed sequence data than PMF analysis, this analysis was also executed. Due to sequence-dependent fragmentation and scan time restrictions, a slightly lower mean sequence coverage of 54% was obtained (see Table S-2 of the Supporting Information and Figure 3). The MS/MS analysis was unsuccessful with the same three proteins as the PMF analysis and two additional proteins (2 and 31) (see Figure 4).

In conclusion, 42 out of 45 proteins were successfully verified with the ISET setup. Although protein 17 could not provide any tryptic peptides and protein 40 showed too low expression (see BCA value after standard purification; Table S-2, Supporting Information), only protein 24 failed for an unknown reason.

The background level of bacterial proteins did not interfere with the protein validation process described herein. In 26 of the 45 proteins, bacterial proteins from *E. coli* could be detected. The mean sequence coverage of the bacterial proteins reached only 11%, of which no more than four proteins were found for any triplicate. The observed bacterial proteins were found to be highly abundant housekeeping proteins.

Comparison to Standard and HPA Setup. To evaluate the accuracy of the ISET setup, a comparison to a standard method was carried out. The standard method includes the corresponding steps: protein capture on IMAC beads, wash, elution, digestion using a manual in-solution digestion protocol, and spotting and crystallization on a standard steel MALDI target (section Standard Protein Verification and HPA Method). Table S-2 of the Supporting Information shows data from the standard method for the 45 proteins: the unique peptides found, and the total sequence coverage. The mean sequence coverage from the PMF analysis was 68 and 78%, respectively, for the ISET and the standard method, and the corresponding standard deviations were calculated to 14 and 13%. For the MS/MS analysis, the average sequence coverage was 54 and 64% for the ISET and standard method, respectively, with the corresponding standard deviations of 16 and 14%. As can be seen in Figures 4 and 5, the difference is minor in terms of sequence coverage and number of identified unique peptides.

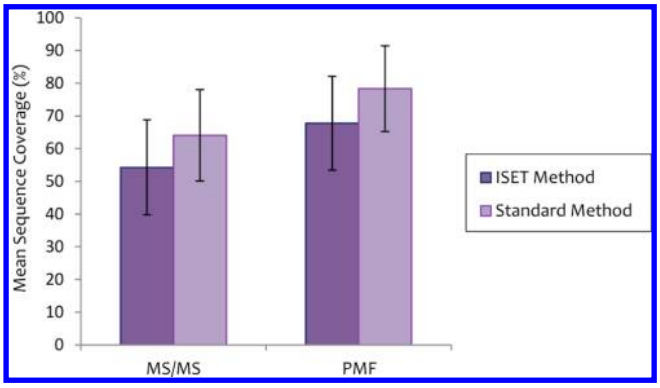

Figure 5. Comparison of mean sequence coverage between the ISET platform and the standard method. The ISET results are shown in dark purple and the standard method in light purple. Mean sequence coverage from left to right: 54, 64, 68, and 78%. Corresponding standard deviations from left to right: 15, 14, 14, and 13%.

The verification criteria for the standard method were identical to those of the ISET method, as described above. Using the standard setup, 39 out of 45 proteins were successfully verified. It was not possible to identify the six remaining proteins (13, 14, 17, 22, 31, and 40) with either PMF or MS/MS. Protein 17 has, as described above, no theoretical unique peptides, and protein 40 showed a low expression level

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(see Supporting Information, Table S-2). To analyze 48 samples with the standard method 25 h are needed (8 h for IMAC purification, 16 h for digestion, and approximately 1 h of manual liquid handling).

Results from the HPA method, i.e. protein concentrations as determined by BCA and full-length MALDI-MS, are presented in Table S-2 (Supporting Information). With MALDI-MS analysis of the proteins in full-length in the HPA method, 40 out of 45 proteins could be detected (Supporting Information, Table S-2). Five proteins could not be verified using this method (7, 14, 40, 44, and 45); in contrast, we have here verified four of these with both PMF and MS/MS in the new chip-integrated ISET setup.

The new setup was shown to be highly suitable for protein validation and the data correlated well to the results obtained from the corresponding standard purification process. There is only a minor difference in the mean sequence coverage between the standard and the ISET setup, but the automated ISET method has other important features to be noted, such as a higher throughput, miniaturization, and less involvement of an operator. The ISET method lowers the time per sample significantly: i.e., 5 min per sample compared to 31 min for the standard method. Moreover, costs are reduced; e.g., in lieu of using columns with a large amount (1 mL) of solid phase only a minute amount of IMAC material (500 nL) is needed in the ISET chip.

CONCLUSIONS

Fast, parallel systems for high-throughput protein verification are needed to maintain quality while the speed of large-scale protein production is increased. We have demonstrated, for the first time, a method for automated, miniaturized screening of recombinant His-tagged protein products from the Human Protein Atlas using the ISET platform. In only 4 h, with a hands-on time of 30 min, we perform a microscale IMAC purification from a crude cultivation broth, tryptic digestion, and sample preparation for MALDI MS analysis of a total of 48 samples. The miniaturized format makes the procedure highly cost-efficient in terms of process time and chemicals. The tryptic digestion step further increases the specificity of the verification, compared to analysis of intact proteins and it also enables MS/MS analysis. Thus, the ISET platform could work as a high-speed microscale screening step prior to commencement of large-scale protein purification.

An important point is the fact that the ISET platform is a generic platform with great flexibility, which means that its use extends beyond IMAC purification. The nanovials of the ISET plate can in principle be filled with any kind of chromatographic resin, having bead size as the only limiting factor, which means that separation based on different chromatographic properties can be carried out. In this perspective, the ISET platform may become a viable strategy for validation, screening, and other high-throughput applications in protein production quality control. In the future, the approach could also allow for evaluation of purity and contaminations.

ASSOCIATED CONTENT

Supporting Information

Table S-1 shows the unique amino acid sequence for the recombinant proteins as well as the HisAPB-tag. Table S-2 displays the data for the protein and the peptide analysis, including protein concentration by BCA, MALDI-TOF full-length data, PMF, and MS/MS data (sequence coverage and unique peptides identified) for the ISET and standard method. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

[§]These authors contributed equally. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare the following competing financial interest(s): Conflict of interest: ISET AB holds the patent application for the ISET technology. Thomas Laurell is one of the inventors and is founder and shareholder of ISET AB.

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ABBREVIATIONS

HPA	Human Protein Atlas
His	polyhistidine
IMAC	immobilized metal ion affinity chromatography
MS	mass spectrometry
ESI	electrospray ionization
MALDI	matrix-assisted laser desorption/ionization
ISET	integrative selective enrichment target
PMF	peptide mass fingerprinting
APB	albumin binding protein
AmBic	ammonium bicarbonate
BCA	bicinchoninic acid
TOF	time of flight
ACN	acetonitrile
TFA	trifluoroacetic acid
FT	Fourier transform
IT	ion trap

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