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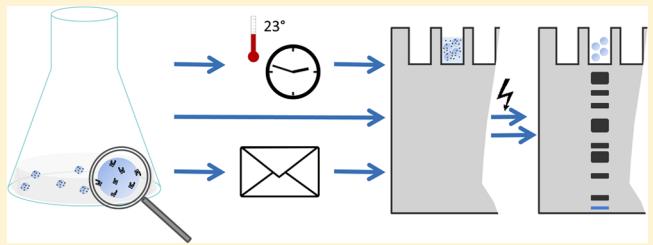
# Picking Vanished Proteins from the Void: How to Collect and Ship/Share Extremely Dilute Proteins in a Reproducible and Highly Efficient Manner

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## Supporting Information

**ABSTRACT:** Successful proteome analyses of highly dilute samples are strongly dependent on optimized workflows considering especially sample preparation prior to highly sensitive mass spectrometric analysis. Various methods are available for enrichment of proteome samples, each characterized by specific advantages and disadvantages limiting their general application as a method of choice. Here we suggest an optimized universal protocol ensuring reproducibility and effective enrichment of dilute samples by commercial affinity beads. By comparably assessing the performance of the new protocol with selected standard enrichment techniques, we show the seamless application of the enrichment in common mass spectrometry based proteomic workflows. Further, novel applications are suggested including a facile storage and shipping of desiccated, trapped proteome samples at ambient temperatures and usage of the affinity beads for gel-free proteomic approaches.



Analysis of proteins from highly dilute samples is a common technical task in different fields employing modern proteomics. Applications include basic and applied research in proteomics elucidating microbial protein secretion during stress and starvation, analysis of host–pathogen interactions mediated by secreted proteins, as well as enrichment of biomarkers from body fluids.<sup>1</sup> Further, protein cleanup and purification can be achieved by the same biochemical techniques allowing for a sensitive and comprehensive proteomic analysis. Here, several classical techniques of protein precipitation (trichloroacetic acid (TCA), acetone, chloroform/methanol, and salt precipitation), protocols relying on ultrafiltration with microfiltration units and workflows using various types of solid-phase extraction (SPE) are mostly applied.<sup>1,2</sup>

The selection of method is largely dependent on the techniques available and, more importantly, on specific advantages and disadvantages of the respective workflow. Therefore, a general method of choice can hardly be recognized. Protein precipitation workflows are feasible in most laboratories without specific instrumentation and are physically based on reduced solvation of proteins in aqueous solutions with following hydrophobic aggregation by salting out or by treatment with organic solvents, or by denaturing of proteins and subsequent precipitation by acids.<sup>3,4</sup> While high efficiencies of precipitation can be achieved, the downside of these techniques is resulting protein pellets that are difficult to resolubilize in a quantitative manner, the need for hazardous reagents like TCA, or the need for high volumes of volatile organic solvents (e.g., chloroform and methanol).<sup>1</sup>

Protein enrichment by ultrafiltration is independent of adding additional agents to the sample, and virtually unlimited

volumes of fluid containing the proteins might be filtered. Here, the widely experienced phenomena of theoretical size exclusion parameters that do not match actually passing protein species and proteins that stick to or aggregate on the membranes during the filtration process causing serious losses are severe obstacles for ultrafiltration to be used as a general method.<sup>5,6</sup>

The third possibility for protein enrichment is affinity enrichment or affinity chromatography, mostly performed as solid-phase extraction.<sup>7</sup> Contrary to protein precipitation, affinity enrichment provides minimal use of hazardous chemicals, little increase in sample volume, and one-pot and fast protocols yielding liquid chromatography–tandem mass spectrometry (LC–MS/MS) compatible samples. Adding to that, affinity-purified samples are compatible to most proteomic sample prefractionation methods, thus coupling efficient enrichment of proteins from highly dilute samples without introducing an additional bias for specific protein species. Various studies for unbiased protein enrichment by SPE and its application in modern proteomics have been published evaluating several affinity materials like diatomaceous earth,<sup>8</sup> surface-functionalized diamond nanocrystallites,<sup>9</sup> gold-coated nanoparticles,<sup>10</sup> or commercial affinity beads.<sup>11</sup> Whereas these workflows aim for unbiased enrichment of proteins in complex and/or dilute samples, novel approaches are designed for grading complex proteome features for biomarker discovery.<sup>12</sup> Diatomaceous earth is a relatively cost-effective method for protein enrichment: consisting of shells of diatoms, washed

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$\text{SiO}_2$  is used for protein enrichment from solution followed by elution with sample solubilization buffer for two-dimensional polyacrylamide gel electrophoresis (2D PAGE).<sup>8</sup> Despite initial successful application, it was shown that conventional diatomaceous earth is inferior for protein recovery compared to other methods like phenol extraction and TCA precipitation.<sup>1</sup> Nevertheless, modified  $\text{SiO}_2$  is available for protein cleanup and enrichment and commercially used for sample preparation.<sup>13</sup> Another option for SPE are nanoparticles, e.g., diamond nanocrystallites or gold-coated nanoparticles.<sup>14</sup> Surface-functionalized diamond nanocrystallites have excellent properties for enrichment of proteins from dilute samples.<sup>9</sup> Superior compatibility of the enrichment with subsequent 1D PAGE analysis, on-resin digest of proteins and even direct matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) on beads can be achieved.<sup>15</sup> The downside for this emerging technology with a potentially wide range of applications is the lack of commercial availability to date leaving the technology restricted to only few laboratories capable of controlling the chemistry of diamond nanocrystallites functionalization and their quality control. Further, gold-coated nanoparticles have successfully been applied to enrich proteins from biological samples including body fluids like urine.<sup>10</sup> Thus, besides the functionality of metal-based nanoparticles to allow for specific labeling of cells, aggregated gold nanoparticles can be used for sensitive protein enrichment.<sup>16,17</sup>

A third possibility for SPE is protein enrichment by the commercial affinity bead system "StrataClean".<sup>11</sup> It was originally introduced in the early 1990s for protein removal from polymerase chain reaction (PCR) samples<sup>18</sup> and is meanwhile found in a number of high-impact proteomics publications.<sup>19–21</sup> The beads are phenol-functionalized and rehydrated silica particles whose characteristics for protein interaction resembles those of the organic solvent phenol traditionally used in liquid phase for precipitation.<sup>18</sup> Despite the successful application in numerous proteomic studies, comparative studies assessing the performance and protein yields obtained by different methods including StrataClean beads were ambiguous. There is an obvious contradiction in literature on the one hand describing inferior efficacy in protein enrichment compared to other, classical methods, and on the other hand describing superior suitability for protein purification.<sup>8,22</sup>

In the present work, we have evaluated the suitability of StrataClean beads for an efficient, reliable, and reproducible sample preparation in comparison to classical methods focused on the need for a superior protocol for protein enrichment from highly dilute samples. We present an improved protocol ensuring the protein capture efficacy remaining constant within different batches of the beads, which was seriously fluctuating in past. Further, we have expanded the possibilities of using StrataClean beads in proteomics with novel applications including a facile storage/shipping of desiccated, trapped proteome samples at ambient temperature. Additionally, we have established a protocol for using StrataClean-enriched proteins directly without prefractionation on a one-dimensional gel.

## MATERIALS AND METHODS

**Optimized Protein Enrichment by Primed Affinity Bead Purification.** *Bead Priming:* An aliquot of 20  $\mu\text{L}$  of StrataClean beads (Agilent) (slurry of affinity beads in storage solution) was first spun down and washed twice with 500  $\mu\text{L}$  of

TE buffer (50 mM Tris, 10 mM EDTA, pH 7). For priming/preincubation, 200  $\mu\text{L}$  of 12 M HCl (Roth) was added and the suspension was incubated at 100 °C for at least 5 h to overnight (use qualified personnel for the process and ensure that all operations follow local bylaws and hazardous regulations). The beads were sedimented by centrifugation and washed twice with 1 mL of TE.

**Protein Binding:** For method evaluation of protein enrichment from dilute samples, cytosolic protein extract from *Bacillus subtilis* 168 and *Staphylococcus aureus* HG001 with a total amount of 20  $\mu\text{g}$  of protein was diluted to a final volume of 1, 2, or 200 mL with TE buffer, respectively. Primed StrataClean beads were added, and the mixture was incubated at 4 °C overnight with gentle shaking or overhead rotation. The beads were sedimented by centrifugation at 8000g: small sample volumes up to 2 mL for 5 min and larger volumes for 20 min. After removing of supernatants, the loaded beads were washed twice with 500  $\mu\text{L}$  of TE and transferred to a low binding reaction tube. For storage experiments the beads were evaporated to dryness under vacuum.

**Other Protein Precipitation and Enrichment Methods.** The precipitations were carried out as described before by Antelmann et al.,<sup>23</sup> Wessel and Flügge,<sup>24</sup> or by the manufacturer's instructions. Further details are available in the Supporting Information.

**Protein Elution (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis).** 20  $\mu\text{L}$  of gel loading buffer (125 mM Tris-HCl pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS, 3.75% (v/v)  $\beta$ -mercaptoethanol, 20 mM DTT, 0.04% (w/v) bromophenol blue) was added to the loaded and washed affinity beads. The resulting slurry was incubated for 5 min at 95 °C, cooled on ice, spun down by centrifugation, and loaded onto a Laemmli gel. Protein pellets from the precipitation approaches were solubilized in gel loading buffer and treated accordingly. Electrophoresis was carried out at 150 V. The gel was fixed with 10% (v/v) acetic acid in 40% (v/v) ethanol for 15 min and stained with colloidal Coomassie overnight.

**GeLC-MS Analysis.** After staining, the gel was washed twice with water to remove excessive Coomassie stain. The stacking gel was removed, and all gel lanes of interest were excised. The lanes were cut into 10 equidistant pieces. Each gel piece was further chopped up into small cubes of approximately 1 mm<sup>3</sup> and transferred into a low binding tube.

The gel pieces were washed/destained at least three times for 15 min with 700  $\mu\text{L}$  of gel wash buffer (0.2 mol ammonium bicarbonate in 30% (v/v) acetonitrile) at 37 °C under vigorous shaking. The destained gel pieces were desiccated in a vacuum centrifuge at 30 °C and rehydrated with trypsin solution (2  $\mu\text{g}$  of modified trypsin (Promega) in 1 mL of water) for 15 min. Excessive trypsin solution was removed, and the digest was performed overnight at 37 °C.

The gel pieces were covered with water, and the peptides were eluted from the gel matrix by immersion of the reaction tube in an ultrasonic bath for 15 min. The supernatant containing the peptides was removed, transferred to a glass vial, and concentrated to a final volume of 10  $\mu\text{L}$  in a vacuum centrifuge.

For LC-MS/MS analyses of 1D gel samples, in-house self-packed columns were prepared and used with an EASY-nLC II system (Thermo). In brief, fused-silica emitter tips with an inner diameter of 100  $\mu\text{m}$  and an outer diameter of 360  $\mu\text{m}$  were prepared by using a P-2000 laser puller (Sutter Instruments). The resulting emitter tip was then packed with

Aeris C18 reversed-phase material (3.6  $\mu\text{m}$  particles) in a custom-built pressure bomb to obtain a 20 cm nano-LC column.

The peptides were loaded onto the column by the LC system with 10  $\mu\text{L}$  of buffer A (0.1% (v/v) acetic acid) at a constant flow rate of 500 nL/min without trapping. The peptides were subsequently eluted using a nonlinear 85 min gradient from 1 to 99% buffer B (0.1% (v/v) acetic acid in acetonitrile) with a constant flow rate of 300 nL/min and injected online into the mass spectrometer. The gradient is described in Supporting Information Table 1.

MS and MS/MS data were acquired with a LTQ Orbitrap (Thermo). After a survey scan at a resolution of 30 000 in the Orbitrap with activated lockmass correction, the five most abundant precursor ions were selected for fragmentation. Singly charged ions as well as ions without detected charge states were not selected for MS/MS analysis. Collision-induced dissociation (CID) fragmentation was performed for 30 ms with normalized collision energy of 35, and the fragment ions were recorded in the linear ion trap.

**Gel-Free Workflow.** The gel-free workflow was performed as described in the Supporting Information. In brief, the proteins were digested on the beads with trypsin for 6 h with a second addition of trypsin after 3 h. The samples were analyzed on a Thermo LTQFT mass spectrometer coupled to a nanoAcuity (Waters) UPLC running reversed-phase separations with a 5 h nonlinear gradient.

**Data Analysis.** Database searching was done with Sorcerer-SEQUENT 4 (SageN). After extraction from the raw files, \*.dta files were searched with Sequest against a target-decoy database with a set of common laboratory contaminants. The target database was the Uniprot reference database of *B. subtilis* 168 (downloaded May 21, 2014). The resulting \*.out files were compiled with Scaffold 4. Proteins were only considered as identified if at least two unique peptides matching solid quality criteria ( $\Delta\text{cN} > 0.1$  and  $\text{XCorr} > 2.2; 3.3; 3.7$  for doubly, triply, or higher charged peptides) have been assigned, resulting in a false positive rate (FPR) below 0.2% on protein level.

For label-free quantification the normalized exclusive spectral counts were compared.

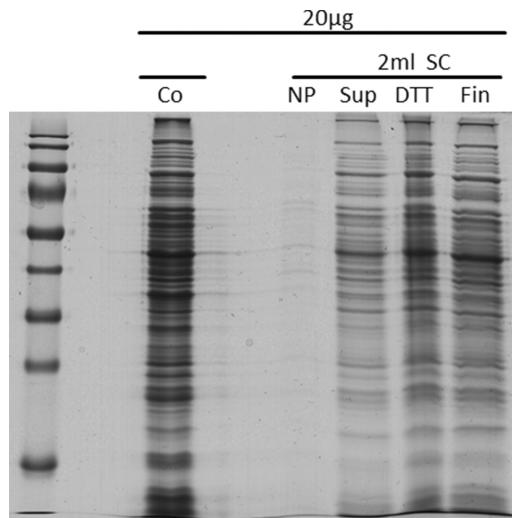
## RESULTS AND DISCUSSION

### Optimization of Protein Enrichment by Affinity Beads.

An optimized protocol to reproducibly enrich complex protein samples by affinity beads has been established, applicable for proteomic approaches based on 1D sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) coupled to gel-free mass spectrometry.

Protein enrichment by StrataClean beads was described as early as 1997 by Ziegler and co-workers and has been used in numerous studies. Despite its acceptance, use of StrataClean beads has been reviewed as being inferior to other methods. We have experienced in our laboratory that the commercially available beads are subject to batch to batch variances with respect to unwanted and nonspecific protein loads, and these contaminations are interfering with highly sensitive mass spectrometry. Therefore, we sought to establish an optimized protocol for interference-free and reliable protein enrichment of complex protein samples by StrataClean beads. The different optimization steps that are described in the following are displayed in Figure 1.

The first step was related to variations in protein yield and corresponding contaminations. We have discovered that

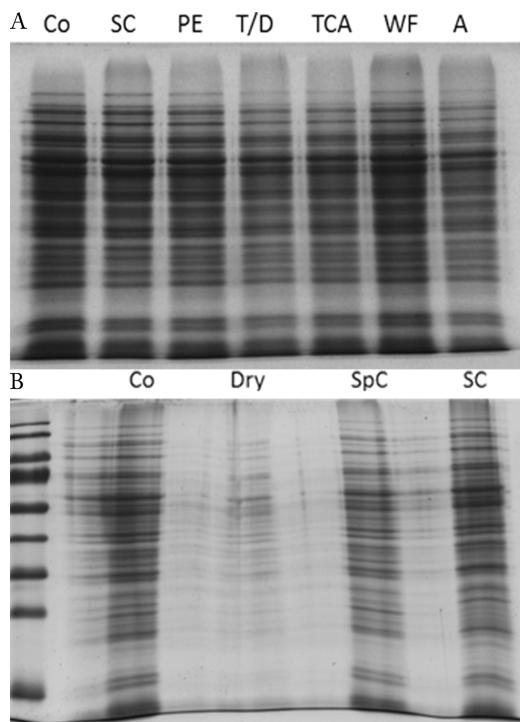


**Figure 1.** Contribution of optimization steps. An amount of 20  $\mu\text{g}$  of soluble protein extract of exponentially grown *B. subtilis* 168 was dissolved in 2 mL of TE buffer and enriched with four different versions of the protocol. Lanes 1–5 were loaded as follows: control (Co) 20  $\mu\text{g}$  of protein extract without dilution, loaded beads without preincubation with HCl (NP), supernatant (Sup) of chemically eluted preincubated beads (no electroelution), preincubated beads and supernatant of StrataClean sample eluted with DTT as sole reducing agent (DTT), final protocol (Fin) preincubated beads and supernatant eluted with mercaptoethanol-containing buffer.

relatively harsh preincubation washing steps are necessary to achieve reproducible and contamination-free protein binding capacity. For effective washing and priming, the beads were incubated for at least 5 h in 12 M HCl. Without priming (results as shown in gel lane 2 of Figure 1) only a small fraction of proteins could be recovered. As we have experienced, this result may vary with the manufacturing batches used. The second step to optimize the protocol for GeLC-MS analyses included conditions for protein elution prior to and at SDS-PAGE. It is described in literature that there are two ways to elute the loaded and washed proteins from the affinity beads: First, SDS-containing sample buffers or high-molar urea buffers are used to incubate the loaded beads to elute the proteins separately for subsequent SDS-PAGE or further use, e.g., in 2D gel electrophoresis.<sup>22</sup> Second, loaded beads incubated with SDS-PAGE sample buffer are directly applied to the stacking gel in discontinuous SDS-PAGE. We examined both possibilities. Our experiments showed that for complete elution a combined process, based on detergents and electroelution, is necessary to yield a maximum of proteins in GeLC-MS analyses (see lanes SUP vs DTT and Fin in Figure 1). The third step in optimization is related to protein aggregation in the enrichment phase and electroelution during SDS-PAGE. Frequently, we observed a band at the interphase of stacking and resolving gel, most probably caused by aggregated proteins. Stable conditions to avoid aggregation and loss of proteins are sufficient concentrations of strong reducing agents like mercaptoethanol in the elution buffer, with DTT as common mild reducing agent in Laemmli sample buffers not being sufficient. As a result, reproducible and maximum yields of protein enriched by StrataClean beads and resolved by SDS-PAGE are ensured by (i) priming StrataClean beads by acid hydrolysis, (ii) detergent-aided electroelution of on-bead bound

proteins into the SDS-PAGE, and (iii) use of strong reducing conditions in the Laemmli loading buffer.

**Comparison with Common Protein Precipitation and Enrichment Methods.** Though protein purification by affinity enrichment is widely accepted as being theoretically superior to protein precipitation by acids and organic solvents, available literature is ambiguous in evaluation of the performance of StrataClean beads. Therefore, we have compared our optimized protocol for protein enrichment with standard procedures according to protein yield. We have compared protein enrichment with StrataClean to the following established protocols for protein precipitation: TCA precipitation, detergent-assisted TCA precipitation, acetone precipitation, precipitation according to Wessel and Flügge, and the commercially available ProteoExtract kit. As seen in Figure 2A, all precipitation and enrichment protocols gave similar

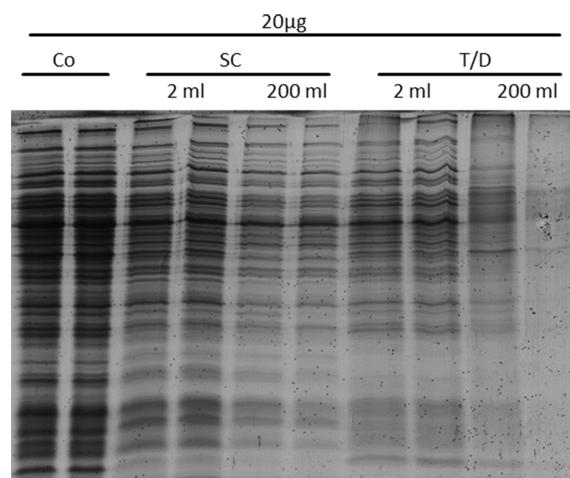


**Figure 2.** Comparison of classical protein enrichment and precipitation methods with StrataClean. An amount of 20  $\mu\text{g}$  of soluble protein extract of exponentially grown *Staphylococcus aureus* HG001 was dissolved in 1 mL of TE buffer and subjected to eight different enrichment/precipitation methods and analyzed via 1D PAGE. (Gel 1, A) Lanes 1–7 were loaded as follows: control (Co) 20  $\mu\text{g}$  of untreated protein extract without dilution, StrataClean (SC), ProteoExtract (PE), TCA/Deoxycholat (T/D), TCA precipitation (TCA), Wessel–Flügge precipitation (WF), acetone precipitation (A). (Gel 2, B) Lanes 1–4 were loaded as follows: control (Co) 20  $\mu\text{g}$  of protein extract without dilution, desiccation (Dry), SpinColumn Enrichment (SpC), StrataClean (SC).

results if compared to the unchanged protein extract. Therefore, we conclude that affinity enrichment by StrataClean beads is comparable to classical methods in concentrated solutions of proteins. Additionally, we compared StrataClean purification with protein enrichment by desiccation of the solvent and via SpinColumns. As shown in Figure 2B, desiccation led to concentrated samples with a buildup of high amount of salts in the sample. This either calls for a second desalting step of the concentrated sample leading to

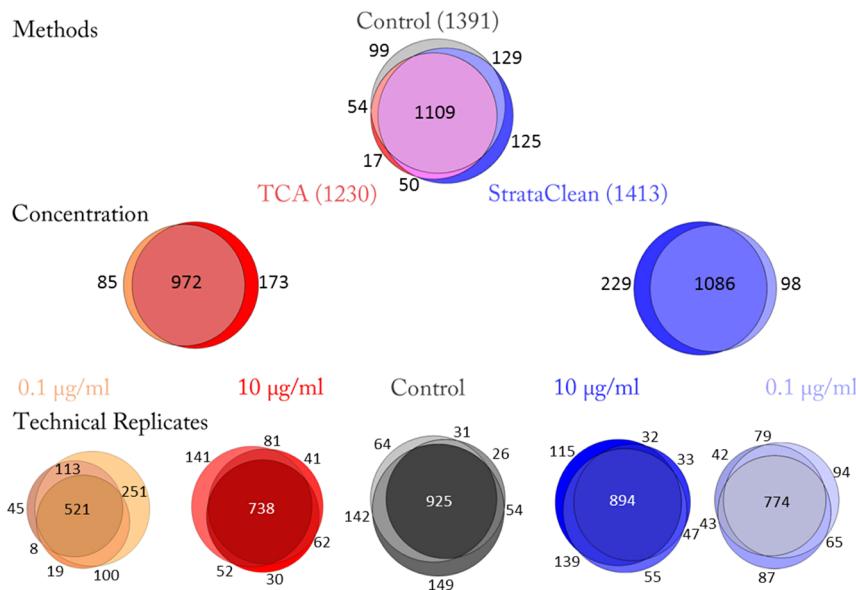
sample losses or leads to poor performance in 1D SDS-PAGE separation properties. Protein enrichment via SpinColumns seems to be comparable to classical precipitation approaches according to the gel image.

**Protein Recovery from Highly Diluted Samples.** The most important advantage of affinity-based approaches for protein enrichment is the lack of organic solvents, the unaffected sample volume, and the possibility to obtain enrichment in one-pot reactions with almost no limits in sample volume. The initially propagated key feature of StrataClean beads for enrichment of highly dilute samples is hardly found in literature and has not been evaluated by modern techniques of mass spectrometry based proteomics. The protein concentration chosen in the experiment above (20  $\mu\text{g}/\text{mL}$ ) is exemplary for a typical situation with the strong need to concentrate proteins prior to proteomic analysis. However, certain samples might be even lower concentrated or interfering substances could require additional dilution. Consequently, we have compared the performance of StrataClean enrichment and detergent-assisted TCA precipitation to recover 20  $\mu\text{g}$  of protein (soluble protein extract of *B. subtilis* 168) from highly diluted samples with concentrations of complex protein mixtures as low as 100 ng/mL. Both methods were performing equally in recovering 20  $\mu\text{g}$  of proteins dissolved in 2 mL of TE at a concentration of 10  $\mu\text{g}/\text{mL}$ , as seen in Figure 3. In the same experiment, we have gone one



**Figure 3.** Protein precipitation and enrichment of highly diluted protein samples. Amounts of 20  $\mu\text{g}$  of soluble protein extract of exponentially grown *B. subtilis* 168 were dissolved in either 2 or 200 mL of TE buffer and subjected to TCA/Deoxycholat precipitation or StrataClean enrichment. Lanes 1–10 were loaded in pairs as follows: control (Co) 20  $\mu\text{g}$  of untreated protein extract without dilution, first dilution of proteins in TE (10  $\mu\text{g}/\text{mL}$ ) and second dilution (100 ng/ $\text{mL}$ ) enriched with StrataClean, the respective dilutions precipitated with detergent-assisted trichloroacetic acid (T/D).

step further to recover 20  $\mu\text{g}$  protein dissolved in 200 mL of TE (100 ng/mL), with hardly reproducible protein recovery for the detergent-aided TCA approach. On the contrary, though less intense than the original protein extract in the control, the StrataClean lanes reveal reasonable and reproducible recovery of proteins from highly diluted solutions. The experiment was performed in triplicate; the gel image from replicate 3 is shown in the Supporting Information. To reinforce the visual results, we have subjected both the resolved control and the different enrichment and precipitation lanes to GeLC–MS analyses to



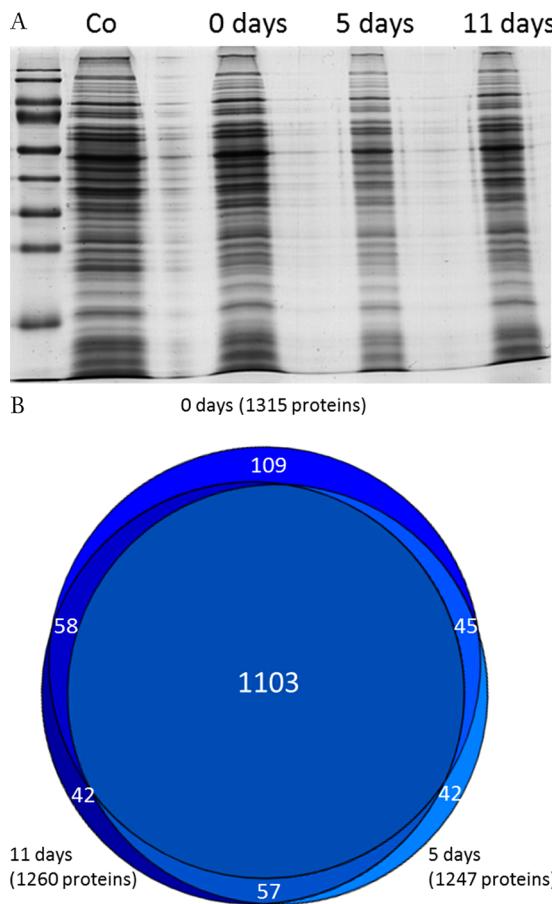
**Figure 4.** Protein precipitation and enrichment of highly diluted protein samples. Amounts of 20 µg of soluble protein extract of exponentially grown *B. subtilis* 168 were dissolved in either 2 or 200 mL of TE buffer and subjected to TCA/Deoxycholate precipitation or StrataClean enrichment. All samples were subjected to GeLC–MS analysis, and the identifications were compared both on the methods as well as dilution level. Adding to that, technical replicates were compared.

allow for a more accurate comparison based on the number of protein identifications. Altogether, we identified 1600 proteins from the samples, of which 17 were common laboratory contaminants. In two samples a single decoy hit has been identified, which results in a very low FPR of below 0.2% in all samples. As shown in Figure 4 and Supporting Information Table S1, the total identification rates are comparable between the samples, whereas in the StrataClean samples slightly more proteins could be identified than in the corresponding TCA samples. Obviously, the striking difference lies in the reproducibility as investigated by technical replicates. Less than 50% of the 1057 target proteins identified in the 0.1 µg/mL TCA approach could be found in all three replicates, in contrast the amount in relative and absolute numbers was higher in the StrataClean sample. Here we identified 1184 target proteins in total of which more than 65% could be found in all samples. This proves a superior reproducibility of StrataClean enrichment compared to precipitation approaches, likely because of the more stable pellets and the reduced number of washing steps. This combination of both higher absolute numbers and superior reproducibility leads to almost 50% more highly reproducible identifications that can be found in all replicates. Besides this clear result pointing out the unique performance of StrataClean beads for enrichment of proteins from highly dilute samples, it has to be additionally stressed that particularly protocols relying on organic solvents are not applicable for this kind of setting due to unmanageable increase in sample volume encountered, e.g., for analyses of body fluids like urine or secreted proteins.

**Protein Storage on StrataClean Beads.** To expand the scope of application of StrataClean even further, we have tested the affinity bead system in a setting that is common to modern proteomic laboratories or proteomics core facilities frequently managing external collaborations. Here, shipping of protein samples is feasible only at the cost of elaborate logistics. In this light, we hypothesized if it is possible to “store” proteins bound on StrataClean beads to be able to ship and share those

proteins at ambient temperature. To test our hypothesis, again 20 µg of protein was recovered from 2 mL TE with StrataClean. Hereafter the loaded beads were dried completely and “stored” for 0, 5, respectively, 11 days at room temperature, mimicking conditions of shipping samples without cooling. The experiment was performed in triplicate. As seen in Figure 5A, the protein yield of affinity bead stored protein extract is surprisingly similar to the control sample. For further investigation the stored proteins were analyzed with LC–MS/MS and the identification rates were compared with the 2 mL samples from the dilution experiment as shown in Figure 5B and Supporting Information Table S1. In both storage approaches more than 1200 proteins could be identified of which more than 1000 were identified in two or more replicates each. This proves that complex proteome samples can be stored on StrataClean Beads without obvious protein degradation. Thus, a new application for affinity enrichment by StrataClean beads is a facilitated shipping of complex proteome samples. Taken the immanent feature of solid-phase extraction for effective cleanup of samples, usage of StrataClean beads therefore allows cost-effective sample processing with limited influence of the respective biological matrices and a well-suited logistic interface of laboratories with a strong emphasis on wet-lab work and their respective analytical counterparts.

**Gel-Free Quantification.** Another application of protein enrichment by StrataClean beads is its application in gel-free proteomic approaches. It has been shown, e.g., for affinity enrichment by nanoparticles, that on-bead digestion is a useful feature circumventing SDS–PAGE prior to LC–MS/MS analysis. Hence, we have evaluated the general feasibility of gel-free approaches with an experiment using label-free protein quantification relying on spectral counting. We compared a protein extract enriched from TE buffer with StrataClean and subsequent tryptic digestion with a control extract, which was directly subjected to tryptic digestion. Both samples were prepared in three technical replicates.

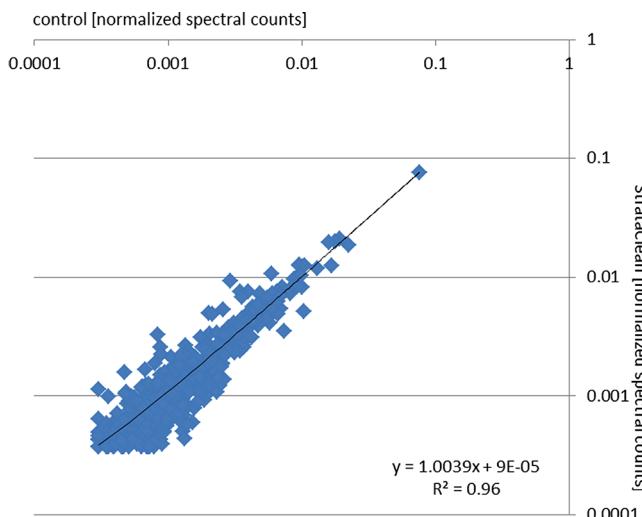


**Figure 5.** Storage of StrataClean trapped proteome samples. Amounts of 20  $\mu$ g of soluble protein extract of *B. subtilis* were either loaded directly onto the gel (Co) or enriched from 2 mL of TE with StrataClean. The loaded beads were subsequently desiccated and loaded either onto the gel (0 days) or—to simulate transport at ambient temperature—stored for 5 as well as 11 days at room temperature prior to GeLC–MS analysis. The gel image is shown in panel A, and the identification results are displayed in panel B.

In the control samples slightly more proteins could be identified (638 proteins in total, 539 in two or more replicates) than in the StrataClean approach (505, respectively, 416). The different identification rates are visualized with Venn diagrams in Supporting Information Figure 3 and listed in Supporting Information Table S1. For label-free quantification, the normalized spectral counts were compared for all proteins which were identified both in control and StrataClean-enriched samples as well as in 3/3 samples either in control or StrataClean-enriched samples. As shown in Figure 6, the spectral counts correlate very well, with a  $R^2$  value of 0.96. A comparison of the different replicates is shown in the Supporting Information. The unbiased enrichment and digestion makes StrataClean enrichment suitable for relative label-free quantification.

## CONCLUSIONS

In this work, an optimized protocol for reproducible and exceedingly sensitive enrichment of highly dilute complex protein samples is proposed. Protein enrichment by the commercially available beads is improved by (i) priming StrataClean beads by acid hydrolysis, (ii) detergent-aided electroelution, and (iii) use of strong reducing conditions in the



**Figure 6.** Label-free quantification. For evaluation of the gel-free digest, StrataClean-enriched, and on-bead digested proteins were compared with nonenriched control samples. The normalized spectral counts were compared. All proteins had to be identified both in control and StrataClean-enriched samples as well as in 3/3 samples either in control or StrataClean-enriched samples.

Laemmli loading buffer in applications aiming to combine affinity enrichment and GeLC–MS analyses. The performance of affinity purification by our optimized protocol is evaluated compared to common protein precipitation and enrichment methods. Besides proven applicability of the protocol, the unsurpassed performance of StrataClean enrichment is demonstrated with highly diluted samples of complex protein extracts. Finally, novel applications of StrataClean are proposed including a facile storage and shipping of desiccated, trapped proteome samples at ambient temperatures and usage of the affinity beads for gel-free approaches.

## ASSOCIATED CONTENT

### Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Notes

The authors declare no competing financial interest.

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