Absolute quantification of proteins in the fatty acid biosynthetic pathway using protein standard absolute quantification

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With worldwide attention on renewable energy and climate change, metabolic engineering of the fatty acid biosynthetic pathway has become an active area of research, with a view to enhance production of biofuels. Indeed, this pathway has already been extensively studied in Escherichia coli. Nevertheless, little is known about the absolute abundance of the enzymes involved, information that may be valuable for engineering, such as the optimal molar ratios of different proteins. In this study, we use protein standard absolute quantification (PSAQ) to measure the absolute abundance of proteins that catalyze fatty acid biosynthesis in E. coli. In addition, the changes of protein abundance were analyzed by comparing the differences between high-yield and the background strain. Our work highlights opportunities to enhance fatty acid production by measuring protein molar ratios and identifying catalytic and regulatory bottlenecks. More importantly, our results provide evidence that PSAQ is a generally valuable tool to investigate metabolic pathways.

1. Introduction

Growing global energy demand and environmental concerns have stimulated efforts to develop more sustainable and renewable transport fuels that can replace conventional fossil fuels. Metabolic engineering and synthetic biology provide promising solutions to produce such fuels directly from microorganisms. The fatty acid biosynthetic pathway is of particular interest, because fatty acids are precursors to alkanes, biosynthetic pathway is of particular interest, because fatty acids are precursors to alkanes,2 and fatty acids and their derivatives, particularly those with long hydrocarbon chains, are ideally suitable as fuel for today’s diesel engines, because they have the highest energy density per unit volume. Indeed, fatty acids and their derivatives, particularly those with long hydrocarbon chains, are ideally suitable as fuel for today’s diesel engines, because they have the highest energy density per unit volume.5,6 Thus, fatty acid synthase (FAS), the master enzyme of fatty acid biosynthesis, has become a popular engineering target. E. coli FAS, which catalyzes type II fatty acid biosynthesis, has been extensively studied, and is well understood. Its activity, regulation, and steady-state kinetic properties have been characterized.11–15 The first step in type II fatty acid biosynthesis is the formation of malonyl-CoA from acetyl-CoA, a reaction catalyzed by acetyl-CoA carboxylase (AccABCD) (Fig. 1). Subsequently, malonyl-CoA is transformed to acyl carrier protein (ACP) by malonyl-CoA:ACP transacylase (FabD).17 Cycles that elongate the fatty acid chain then begin when \( \beta \)-keto-acyl-ACP is genetated by \( \beta \)-keto-acyl-ACP synthase III (FabH) through Claissen condensation of acetyl-CoA and malonyl-ACP.16,18 Successive elongation reactions using acyl-ACP as substrates are catalyzed by the condensing enzymes FabB and FabF. In the elongation cycle, \( \beta \)-hydroxy-acyl-ACP is produced by \( \beta \)-keto-acyl-ACP reductase (FabG), and then converted to trans-\( \Delta^2 \)-enoyl-acyl-ACP by FabA and FabZ. \( \beta \)-Fab catalyzes the last step of the elongation cycle. Palmitoyl-ACP is produced after six additional cycles, and fatty acid chains are then released from ACP by the thioesterases TesA and TesB.

The fatty acid biosynthetic pathway has previously been engineered by genetic modification to enhance production. For example, overexpression of acetyl-CoA carboxylase has been...
shown to induce a 100-fold increase in the malonyl-CoA pool, but only a 6-fold increase in fatty acid production. Thus, while conversion of acetyl-CoA to malonyl-CoA has traditionally been thought to be rate-limiting, subsequent reactions in fatty acid biosynthesis may also limit fatty acid production. Genetic modification of the tesA gene in E. coli by deleting the leader sequence that was used to localize thioesterase I in the cytosol resulted in enhanced production. In addition, fatty acid production increased 20-fold, with 50% being in free form, when overexpression of acetyl-CoA carboxylase was combined with deletion of fadD and overexpression of two thioesterases. Furthermore, by cytosolic overexpression of “leaderless” version of TesA in a fadD and fadE strain, the free fatty acid titer reached 1.2 g/L. Finally, combinatorial optimization of three modules increased fatty acid titers to 8.6 g/L, the highest ever reported.

Moreover, potential metabolic bottlenecks have been examined by quantitatively investigating the substrates, intermediates, cofactors, and enzymes in an in vitro cell-free system. Indeed, in vitro reconstitution and steady-state analysis of the FASs also provide fundamental knowledge for engineering fatty acid biosynthetic pathway. The in vitro reconstitution of FASs indicated that the strain with protein molar ratios closing to optimal ratios appears to have a higher fatty acid production. Thus, measurements of protein molar ratios are critical for the rational engineering of strains from high yield production of fatty acids. In order to get the protein molar ratios, an absolute quantification strategy should be established to measure the absolute abundance of targeted proteins.

In this study, we measured, for the first time, the absolute abundance of proteins in the fatty acid biosynthetic pathway using PSAQ, which uses full-length isotope-labeled proteins as internal standards for MS-based quantification of target proteins in complex matrices. By comparing the differences between fatty acid high-yield strain and the background strain, we obtained insights valuable to biofuel production. Furthermore, our results indicate that PSAQ is accurate, and can be used to investigate a variety of metabolic pathways. Furthermore, this strategy is proven to be a promising solution for precisely determination at reduced cost and can be applied to other fields.

2. Results

2.1. Fed-batch fermentation of the fatty acid high-yield strain

To compare absolute protein abundance between the fatty acid high-yield strain and the background strain, we first investigated the scalability of growing the high-yield strain. TL101, a strain from which fadD has been deleted (Table 1), was transformed with plasmids containing E. coli acetyl-CoA carboxylase, medium-chain thioesterase from camphor, and variant of E. coli thioesterase.
(lacking the leader sequence for periplasmic secretion). Cells were cultivated by fed-batch fermentation at a 3-L scale using M9 media with glycerol as the only carbon source. Cultures were treated with isopropyl-β-D-thiogalactopyranoside (IPTG) to induce overexpression. Samples were extracted and analyzed by GC-MS. Total fatty acid titer reached 4.0 g/L 26 h after induction.

2.2. Protein identification using nanoLC-MS/MS

Total proteins were extracted from fermentation samples, and digested with trypsin using published methods. Proteins in three technical replicates were identified using an ekspert nanoLC 400 system (Eksigent) coupled to an AB SCIEX Triple TOF™ 5600+ System (AB SCIEX, Foster City, CA, USA) in Information Dependent Acquisition (IDA) mode. By matching tryptic peptides with the UniProt database, 979 proteins (Table 2) and 9343 peptides (Table 3) were identified with false discovery rate (FDR) < 1%. All proteins of interest, namely FabA/B/D/F/G/H/I/Z, ACP, TesA' (‘leaderless’ version of TesA), and AccABCD, were detected based on at least two peptides from each.

Peptides for Multiple Reaction Monitoring (MRM) were selected according to published criteria. Specifically, these peptides were 7–20 amino acids long with neither modifications to arginine nor missed cleavages, and were detected with high intensity at 99% confidence level. The MIDAS (MRM-initiated detection and sequencing) workflow was used to validate and confirm transitions (including the parent mass in Q1 and fragment mass in Q3) to be used for quantification. In order to confirm that the detected peptide is the peptide of interest and can be used to develop MRM method for quantification, in this workflow, the third quadrupole switched to ion-trap mode when a specific MRM transition was detected, and captured full-scan tandem mass spectrum to allow database matching. After transitions were verified, the data set was imported to Skyline software (MacCoss Lab Software; Seattle, WA, USA) to generate a scheduled MRM method to quantify proteins with unlabeled and 15N-labeled transitions and optimal declustering potential (DP) and collision energy (CE) (Table S1).

2.3. Preparation of unlabeled protein standards and isotope-labeled internal standards

We used PSAQ to measure absolute protein abundance (Fig. 3). In this approach, the internal standard should ideally behave exactly like the target protein throughout pre-analytical sample treatments and LC-MS/MS analysis. Thus, a full-length isotope-labeled target protein appears to constitute the internal standard of choice. Unlabeled protein standards were prepared as previously described. In addition, the full-length 15N-labeled proteins except FabZ were obtained from cultures growing in M9 media with 15NH4Cl as the only nitrogen source. Proteins were purified, analyzed on SDS-PAGE (Fig. S1), and quantified by Pierce BCA protein assay kit (Thermo Scientific). Because cells overexpressing FabZ barely grew in M9 media, an isotope-labeled FabZ peptide was synthesized, with sequence Val-Val-Cys-Glu-Ala-Thr-Met-Met-Cys-Ala[U13C3,15N]-Arg (Chinese Peptide Company, Hangzhou, China) and used as the isotope-labeled internal peptide of FabZ protein in the absolute quantitative analysis.

Table 1

| Name                  | Descriptions                                                                 | References |
|-----------------------|------------------------------------------------------------------------------|------------|
| BL21(DE3) E. coli     | E. coli BL21(DE3); ΔfadE                                                     | Yu et al.  |
| pXY-FabA pET28a       | pET7-FabA                                                                     | Yu et al.  |
| pXY-FabD pET28a       | pET7-FabD                                                                     | Yu et al.  |
| pXY-FabF pET28a       | pET7-FabF                                                                     | Yu et al.  |
| pXY-FabG pET28a       | pET7-FabG                                                                     | Yu et al.  |
| pXY-FabH pET28a       | pET7-FabH                                                                     | Yu et al.  |
| pXY-Fabl pET28a       | pET7-Fabl                                                                     | Yu et al.  |
| pXY-FabZ pET28a       | pET7-FabZ                                                                     | Yu et al.  |
| pTL14 pET28a          | pET7-ACP                                                                      | Liu et al. |
| pTL30 pET28a          | pET7-TesA’ (without the leader sequence)                                     | Liu et al. |
| pXL001 pET28a         | pET7-accA                                                                     | Li et al.  |
| pXL002 pET28a         | pET7-accB                                                                     | Li et al.  |
| pXL004 pET28a         | pET7-accC                                                                     | Li et al.  |
| pXL005 pET28a         | pET7-accD                                                                     | Li et al.  |
| pMSD8 pT7-accC, D, A  | Replication origin: pSC101                                                   | Davis      |
| pTL58 pBAD-TesA’      | (without the leader sequence) and Cinnamomum camphorum thioesterase; Replication origin: p15A | Liu et al.  |

Dependent Acquisition (IDA) mode. By matching tryptic peptides with the UniProt database, 979 proteins (Table 2) and 9343 peptides (Table 3) were identified with false discovery rate (FDR) < 1%. All proteins of interest, namely FabA/B/D/F/G/H/I/Z, ACP, TesA’ (‘leaderless’ version of TesA), and AccABCD, were detected based on at least two peptides from each.

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2.4. Construction of standard curves

Samples of unlabeled protein standards were prepared with final concentrations 0.005–5 μg per 100 μL. 15N-labeled internal standards were mixed and a defined quantity was spiked into samples, high- and low-concentration standards. As before, samples digested with trypsin, desalted, and analyzed in triplicate using an ekspert nanoLC 400 system coupled to an AB SCIEX QTRAP® 4500 System (AB SCIEX, Foster City, CA, USA) in scheduled MRM mode (Fig. 3). In this mode, transitions of a specific peptide are acquired only around the expected elution of the peptide, and dwell time for each transition depended on the number of targets eluting in a given time window. Data were then imported into Skyline software, and peak areas were calculated using MultiQuant™ version 2.1 (AB Sciex). We constructed standard curves based on the relationship between peak area ratio and concentration ratio of unlabeled protein standard and the corresponding 15N-labeled internal standard. These curves are displayed in Fig. S2, where the x-axis represents peak area ratio and the y-axis represents concentration ratio. We were able to obtain standard curves with R² > 0.97 for all proteins of interest except FabB and FabI, which were not detected in the allotted time window.

2.5. Absolute quantification of proteins in fatty acid biosynthetic pathway

We measured the absolute abundance of proteins in the fatty acid biosynthetic pathway. Using PSAQ, we were able to compare not only abundance between different samples, but also within each sample. The XIC of unlabeled and 15N-labeled transitions are shown in Fig. S3. The absolute abundance of 12 proteins was obtained using standard curves (Fig. 4). As expected, proteins were more abundant in the high-yield strain than in the background strain BL21(DE3) especially TesA' and AccABC, which were over-expressed. ACP, FabF and FabA were up-regulated while FabH was down-regulated. Surprisingly, the abundance of FabZ was about 4.5-fold higher in the high-yield strain.

3. Discussion

Immunoassays like western blotting and ELISA have traditionally been used to measure protein abundance. However, these techniques are limited by 1) the ability to produce antibodies; 2) narrow dynamic range, which for ELISA is 2 logs; and 3) cost of antibody production and assay optimization.35,37 Thus, MS-based quantification strategies have gained increasing popularity over the years, especially since these methods may have dynamic range of 4–5 logs. Indeed, strategies such as SILAC, ICAT, iTRAQ and label-free approaches have been developed to provide a comparison of relative protein abundance between samples.35

On the other hand, absolute protein abundance in a sample is also meaningful and can provide valuable information, particularly in metabolic engineering. Fortunately, absolute quantification has become possible by AQUA,38–41 QconCAT42 and PSAQ. In AQUA, chemically synthesized isotope-labeled peptides are used as...
internal standards. In contrast, the internal standard in QconCAT is an artificial, labeled polypeptide produced by concatenating many peptides from different proteins. In any case, these techniques enable comparison not only of proteins within a sample, but also between laboratories, because measurements are absolute.35,43 These strategies rely on isotope-labeled standards with the same analytical properties as the target protein, but are distinguishable from it by a difference in mass.44 Previous studies show that PSAQ is more accurate and precise than AQUA and QconCAT, because PSAQ does not suffer from potential differences between the efficiency of digestion of internal standards and target proteins.27

Indeed, relative changes in protein abundance were determined by targeted proteomics to investigate the mechanism by which FadR enhances fatty acid production.29 However, quantification by targeted proteomics is relative, and can only measure differences between samples.45 An absolute approach, QconCAT, was recently used to quantify proteins involved in major metabolic pathways, including fatty acid biosynthesis.36 While this strategy is high throughput, it is obvious that the artificial internal standard does not behave exactly like the target proteins throughout the analysis, as it should.35 To avoid these issues, we used PSAQ to measure the abundance of proteins that catalyze fatty acid biosynthesis in E. coli. Thus, we believe our results are more accurate, and our strategy enables us to compare protein abundance not only between samples, but also within one sample.

We found that up-regulation of acetyl-CoA carboxylase and thioesterase (without leader sequence) improves fatty acid production, as has been shown in many studies.21–23 Perhaps most interestingly, the abundance of FabZ in the high-yield strain was 4.5-fold higher than in the background strain. This implies that FabZ is key to improving fatty acid production. Similarly, Yu et al.15 found that the highest yield is achieved when FabZ is at least 10-fold more abundant than other Fab enzymes.

In contrast, FabH was down-regulated in the high-yield strain, perhaps indicating that this enzyme normally inhibits fatty acid production even in very low concentrations. This result is consistent with a previous study, which demonstrated that FabF and FabH inhibit fatty acid production significantly at concentrations higher than 1 μM.45 However, FabF was up-regulated in the high-yield strain, and we believe that down-regulation of this enzyme will further improve yield. Finally, we found that, in comparison to the background strain, high-yield strain contains FabA, FabD, FabF, FabG, FabH, FabZ, ACP and TesA at a molar ratio (Table 4) closer to 1:1:1:1:10:30:30, the ratio found to be optimal by Yu et al.15 in in vitro reconstitution experiments. This result indicates that we can engineer target strains that express enzymes at the optimal ratio to maximize biofuel production.

### 4. Conclusion

In this study, we characterized 14 proteins in the fatty acid biosynthetic pathway using nanoLC-MS/MS in IDA mode, and selected and verified transitions using MIDAS workflow. Subsequently, MRM was used to quantify these proteins. Since we can use PSAQ to obtain the molar ratios of proteins in vivo, we can further engineer strains based on the quantification results and enhance biofuel production. This approach appears to be more accurate compared with other techniques, and is a promising tool to investigate metabolic inefficiencies, as well as to fill in data gaps left by existing tools. A current limitation of the PSAQ strategy is the challenge and cost to produce a large amount of labeled protein standards. However, this issue may soon be resolved, as quite a few recombinant proteins have been produced and purified in structural genomics efforts.47

### 5. Materials and methods

#### 5.1. Materials

Sequencing-grade modified Trypsin was purchased from Promega Corporation (Madison, WI, USA). Ammonium chloride (15N, 99%) was obtained from Cambridge Isotope Laboratories (Andover, MA, USA), and other reagents were procured from Sigma-Aldrich (St. Louis, MO, USA). *Escherichia coli* BL21(DE3) was

![Fig. 4. Absolute abundance of 12 proteins in the fatty acid biosynthetic pathway. Data were collected from triplicate experiments, and SD is reported.](image-url)

| Table 4 |
|---------|
| Protein molar ratios | FabA | FabD | FabF | FabG | FabH | FabZ | ACP | TesA | AccA | AccB | AccC | AccD |
| Background strain | 3 | 6 | 1 | 3 | 7 | 7 | 30 | 2 | 1 | 11 | 3 | 4 |
| High-yield strain | 2 | 2 | 4 | 1 | 1 | 11 | 9 | 17 | 3 | 16 | 5 | 1 |
used as background strain. *E. coli* strain TL101 was constructed by deleting *fadE* from BL21(DE3) as previously described. Strains and plasmids are listed in Table 1.

5.2. Fed-batch fermentation of the fatty acid high-yield strain

Fed-batch fermentation of fatty acid high-yield strain was performed in a 5-L bioreactor (Sartorius BIOSTAT B, Germany) according to published methods. Briefly, single colonies were inoculated into 10 mL LB, cultured overnight at 30 °C in a rotary shaker at 220 rpm, and subcultured at 30 °C for 20 h in 300 mL M9 minimal media with glycerol. Cultures were then harvested and resuspended in approximately 50 mL M9 media, and inoculated into 3 L modified M9 media, which contains 30 g (NH4)2SO4, 25.5 g KH2PO4, 3 g MgSO4-7H2O, 1.5 g sodium citrate, 120 g glycerol, 300 mg thiamine, 210 mg CaCl2 and 12.5 mL each of vitamins and metals solutions referred to earlier. Fermentation was kept at 30 °C and pH 7.0, using sodium hydroxide as required. Antifoam 204 was used to control foaming. At OD600 5.0–6.0, the culture was fed a sterile glycerol feed solution at a constant rate of 0.3 mL/min. Overnight cultures were inoculated at 1% v/v into LB media containing 50 μg/mL kanamycin, and several single colonies were pre-cultivated in 10 mL LB media. Cultures were then cooled to 18 °C, and induced with IPTG at a final concentration of 0.1 mM. After another 16–18 h, cells were harvested by centrifugation at 5420 × g, and resuspended in Buffer A (50 mM Tris, 300 mM NaCl, and 4 mM β-mercaptoethanol, pH 7.6) containing 30 mM imidazole. Cells were lysed by sonication, and centrifuged at 12,000 × g for 10 min. The supernatant was further cleared by centrifugation at 20,000 × g for 1 h, and then loaded on to a Ni-NTA column (Bio-Rad). The system was initially held at 100 °C for 5 min, heated to 240 °C at a rate of 10 °C/min and held for 8 min.

5.3. Analysis of total fatty acids

Fatty acids were extracted from fermentation samples in 1:1 v/v chloroform: methanol using published methods, and quantified by GC-MS (Thermo Scientific TSQ Quantum XLS Triple Quadrupole GC/MS, USA) using 200 mg/L pentadecanoic acid as internal standard. The system was initially held at 100 °C for 5 min, heated to 80 °C at a rate of 10 °C/min and held for 8 min.

5.4. Protein identification using nanoLC-MS/MS in Information Dependent Acquisition (IDA) mode

After induction for 26 h, cultures were harvested by centrifugation at 5420 × g for 5 min. To extract total proteins, cells were resuspended in 100 mM ammonium bicarbonate, lysed by sonication, and centrifuged at 12,000 × g for 30 min. The lysate was further cleared by centrifugation at 20,000 × g for 2 h. The protein concentration in the final supernatant was determined with Pierce BCA protein assay kit. Sequencing-grade modified trypsin was added at a ratio of 1:10 to samples containing 100 μg total proteins in 100 mM ammonium bicarbonate, as previously described. Digestion products were desalted using Sep-Pak C18 Vac 35 cc cartridge (Waters, Milford, MA, USA) before analyzed by nanoLC-MS/MS.

Samples were separated on an ekspert nanoLC 400 system at a flow rate of 300 nL/min. The column was equilibrated for 1 min in Buffer A (2% acetonitrile, 0.1% formic acid) supplemented with 5% buffer B (98% acetonitrile, 0.1% formic acid), and a gradient to 80% Buffer B was applied over 75 min to separate peptides. The solvent composition was held for 4 min, and quickly ramped back to 5% Buffer B, and held for 10 min to re-equilibrate the system for the next sample.

Peptides from LC were ionized by a nanospray ion source and analyzed using an AB Sciex Triple TOF™ 5600+ system operating in IDA mode with Analyst TF 1.6 software. Scans were performed in positive ion mode. Peptide profiles were obtained over a mass range of 350–1500 Da, and analyzed by an MS/MS product ion scan over a mass range of 100–1500 Da with abundance threshold set at more than 120 cps. The accumulation time for ions was set at 50 ms. Target ions were excluded from scans for 15 s after being detected, and former ions were excluded after one repetition. Collision energy was automatically ramped up using the “Rolling Collision Energy” option. A maximum of 50 spectra were collected per cycle. Three technical replicates were analyzed in this manner. ProteinPilot (Version 4.5.0.0 by AB Sciex) was used to search the UniProt protein database for possible peptide matches. False discovery rate was analyzed, and set to <1%.

5.5. Preparation of unlabeled protein standards and isotope-labeled internal standards

Unlabeled protein standards, FabA/B/D/F/G/H/J/Z, ACP, TesA, AccA, AccB, AccC and AccD, were individually overexpressed and purified using published methods, with minor modifications. Briefly, plasmids (Table 1) were transformed into BL21(DE3) component cells. Transformants were selected on 50 μg/mL kanamycin, and several single colonies were pre-cultivated in 10 mL LB media. Overnight cultures were inoculated at 1% v/v into LB media containing 50 μg/mL kanamycin, and grown at 37 °C until OD600 reached 0.6–0.8. Cultures were then cooled to 18 °C, and induced with IPTG at a final concentration of 0.1 mM. After another 16–18 h, cells were harvested by centrifugation at 5420 × g, and resuspended in Buffer A’ (50 mM Tris, 300 mM NaCl, and 4 mM β-mercaptoethanol, pH 7.6) containing 30 mM imidazole. Cells were lysed by sonication, and centrifuged at 12,000 × g for 10 min. The supernatant was further cleared by centrifugation at 20,000 × g for 1 h, and then loaded on to a Ni-NTA column (Bio-Rad). The column was washed using Buffer A’ with 30 mM imidazole, and proteins were eluted using Buffer A’ with 300–500 mM imidazole (pH 7.5–8.5), depending upon the theoretical isoelectric point of the target protein. Fractions containing purified target protein were concentrated using Amicon Ultra-15 centrifugal filter devices (Millipore), analyzed on SDS-PAGE, and quantified.

We prepared 15N-labeled full-length internal proteins except FabZ in a similar manner, except that cells were cultivated in M9 media containing 20 g/L glycerol and 1 × trace metals. M9 media contains 6 g Na2HPO4, 3 g KH2PO4, 1 g 15NH4Cl, 0.5 g NaCl, 0.12 g MgSO4, and 0.11 g CaCl2 per liter. A 1000 × solution of trace metals contains 2.86 g H3BO3, 1.81 g MnCl2 • 4H2O, 0.222 g ZnSO4 • 7H2O, 0.39 g Na2MoO4 • 2H2O, and 0.079 g CuSO4 • 5H2O per liter.

5.6. Preparation of protein extracts and standard curves

Total proteins were extracted from fermentation samples as previously described. Known quantities of unlabeled protein standards were mixed in 100 mM ammonium bicarbonate as the stock solution in low-protein binding tubes (Thermo Fisher) that were pre-treated with 5 μg BSA to prevent standards from interacting with the tube. This solution was then used to prepare high- and low-concentration standards that tightly bracketed the high and low end of the estimated abundance of targeted proteins. In addition, a mixed internal standard was spiked into all samples, high- and low-concentration standards. Finally, samples were digested with trypsin and desalted.

5.7. Absolute protein quantification using nanoLC-MS/MS in scheduled MRM mode

Samples, high- and low-concentration standards were analyzed using an ekspert nanoLC 400 system coupled to an AB SCIEX QTRAP® 4500 System operating in scheduled MRM mode (Fig. 3). Mass scans were performed in positive ionization mode with curtain gas flow at 30 psi, collisionally activated dissociation set on
high, ion spray voltage at 2700 V, ion gas 1 at 20 psi, ion gas 2 at 1000 psi, interface heater temperature at 150 °C, ion spray voltage at 2700 V, ion gas 1 at 20 psi, ion gas 2 at 1000 psi, entrance potential at 15 V. Declustering potential was used to analyze data. As described previously,\textsuperscript{29} we selected 1–3 tryptic peptides per protein, and 1–3 y-fragment ions for each peptide to increase specificity and confidence (Table S1). Standard curves were constructed for each protein. Finally, protein abundance was determined by integrating the peak area of the transitions of each protein, and normalizing to corresponding \textsuperscript{15}N-labeled internal standards. As before, samples were analyzed in triplicate.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.synbio.2016.01.001.

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