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TITLE:

Absolute Quantification of Cell-Free Protein Synthesis Metabolism by Reversed-Phase Liquid Chromatography-Mass Spectrometry

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KEYWORDS:

cell-free protein synthesis, liquid chromatography-mass spectrometry, aniline tagging, central carbon, energy metabolism, internal standard, metabolic network, isotopic labeling

SUMMARY:

Here, we present a robust protocol to quantify 40 compounds involved in central carbon and energy metabolism in cell-free protein synthesis reactions. The cell-free synthesis mixture is derivatized with aniline for effective separation using reversed-phase liquid chromatography and then quantified by mass spectrometry using isotopically labelled internal standards.

ABSTRACT:

Cell-free protein synthesis (CFPS) is an emerging technology in systems and synthetic biology for the in vitro production of proteins. However, if CFPS is going to move beyond the laboratory and become a widespread and standard just in time manufacturing technology, we must understand the performance limits of these systems. Toward this question, we developed a robust protocol to quantify 40 compounds involved in glycolysis, the pentose phosphate pathway, the tricarboxylic acid cycle, energy metabolism and cofactor regeneration in CFPS reactions. The method uses internal standards tagged with ^{13}C-aniline, while compounds in the sample are derivatized with ^{12}C-aniline. The internal standards and sample were mixed and analyzed by reversed-phase liquid chromatography-mass spectrometry (LC/MS). The co-elution of compounds eliminated ion suppression, allowing the accurate quantification of metabolite concentrations over 2-3 orders of magnitude where the average correlation coefficient was 0.988. Five of the forty compounds were untagged with aniline, however, they were still detected in the CFPS sample and quantified with a standard curve method. The chromatographic run takes approximately 10 min to complete. Taken together, we developed a fast, robust method to

separate and accurately quantify 40 compounds involved in CFPS in a single LC/MS run. The method is a comprehensive and accurate approach to characterize cell-free metabolism, so that ultimately, we can understand and improve the yield, productivity and energy efficiency of cell-free systems.

INTRODUCTION:

Cell-free protein synthesis (CFPS) is a promising platform for manufacturing of proteins and chemicals, an application that has traditionally been reserved for living cells. Cell-free systems are derived from crude cell extracts and eliminate the complications associated with cell growth[1]. In addition, CFPS allows for direct access to metabolites and the biosynthetic machinery without the interference of a cell wall. However, a fundamental understanding of the performance limits of cell-free processes has been lacking. High-throughput methods for metabolite quantification are valuable for the characterization of metabolism and are critical for the construction of metabolic computational models[2-4]. Common methods used to determine metabolite concentrations include nuclear magnetic resonance (NMR), Fourier transform-infrared spectroscopy (FT-IR), enzyme-based assays, and mass spectrometry (MS)[5-8]. However, these methods are often limited by their inability to efficiently measure multiple compounds at once and often require a sample size greater than typical cell-free reactions. For example, enzyme-based assays can often only be used to quantify a single compound in a run, and are limited when the sample size is small, such as in cell-free protein synthesis reactions (typically run on a 10-15 μL scale). Meanwhile, NMR requires a high abundance of metabolites for detection and quantification[5]. Toward these shortcomings, chromatography methods in tandem with mass spectrometry (LC/MS) provide several advantages, including high sensitivity and the capability of measuring multiple species simultaneously[9]; however, the analytical complexity increases considerably with the number and diversity of species being measured. It is important, therefore, to develop methods that fully realize the high-throughput potential of LC/MS systems. Compounds in a sample are separated by liquid chromatography and identified through mass spectrometry. The signal of the compound depends on its concentration and ionization efficiency, where the ionization can vary between compounds and may also depend on the sample matrix.

Achieving the same ionization efficiency between the sample and standards is a challenge when using LC/MS to quantify analytes. Further, quantification becomes more challenging with metabolite diversity due to signal splitting and heterogeneity in proton affinity and polarity[10]. Lastly, the co-eluting matrix of the sample can also affect the ionization efficiencies of the compounds. To address these issues, metabolites can be chemically derivatized, increasing the separation resolution and sensitivity by LC/MS systems, while simultaneously decreasing signal splitting in some cases[10,11]. Chemical derivatization works by tagging specific functional groups of metabolites to adjust their physical properties like charge or hydrophobicity to increase ionization efficiency[11]. Various tagging agents can be used to target different functional groups (e.g., amines, hydroxyls, phosphates, carboxylic acids, etc.). Aniline, one such derivatization agent, targets multiple functional groups at once, and adds a hydrophobic component into hydrophilic molecules, thereby increasing their separation resolution and signal[12]. To address the co-eluting matrix ion suppression effect, Yang and coworkers developed a technique based on

Group Specific Internal Standard Technology (GSIST) labeling where standards are tagged with ^{13}C aniline isotopes and mixed with the sample[12,13]. The metabolite and corresponding internal standard have the same ionization efficiency since they co-elute, and their intensity ratio can be used to quantify the concentration in the experimental sample.

In this study, we developed a protocol to detect and quantify 40 compounds involved in glycolysis, the pentose phosphate pathway, the tricarboxylic acid cycle, energy metabolism and cofactor regeneration in CFPS reactions. The method is based on the GSIST approach, where we used ^{12}C-aniline and ^{13}C-aniline to tag, detect, and quantify metabolites using reversed-phase LC/MS. The linear range of all compounds spanned 2-3 orders of magnitude with an average correlation coefficient of 0.988. Thus, the method is a robust and accurate approach to interrogate cell-free metabolism, and possibly whole-cell extracts.

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PROTOCOL:

1. Preparation of reagents for aniline tagging

1.1. Prepare a 6 M aniline solution at pH 4.5. Working in a hood, combine 550 µL of aniline with 337.5 µL of LCMS grade water and 112.5 µL of 12 M hydrochloric acid (HCl) in a centrifuge tube. Vortex well and store at 4 °C.

NOTE: Aniline can be stored at 4 °C for 2 months.

CAUTION: Aniline is highly toxic and should be worked with in a fume hood. Hydrochloric acid is highly corrosive

1.2. Prepare a 6 M ^{13}C aniline solution at pH 4.5. Combine 250 mg of $^{13}C_6$-aniline with 132 µL of water and 44 µL of 12 M HCl. Vortex well and store at 4 °C.

1.3. Prepare 200 mg/mL N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC) solution. Dissolve 2 mg of EDC in 10 µL of water for every sample to be tagged and vortex well.

NOTE: EDC solution should be prepared the same day as the reaction. EDC acts as a catalyst for the derivatization of compounds with aniline[12].

2. Preparation of standards

2.1. Make separate stock solutions of all compounds dissolved in LC/MS grade water (**Table 1**).

2.2. **Preparation of internal standard stock solution**

2.2.1. Combine all compounds except for nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide phosphate (NADP), Flavin adenine dinucleotide (FAD), acetyl

coenzyme A (ACA), and glycerol 3-phosphate (Gly3P), with the appropriate volumes to create a 2 mM stock solution of all compounds.

2.3. Combine NAD, NADP, FAD, ACA, and Gly3P with the appropriate volumes to create a 2 mM stock solution.

3. Preparation of sample (Figure 1)

3.1. Quench and precipitate the proteins in a cell-free protein synthesis reaction by adding an equal volume of ice-cold 100% ethanol to the reaction. Centrifuge the sample at 12,000 x *g* for 15 min at 4 °C. Transfer the supernatant to a new centrifuge tube.

NOTE: Samples can be stored at -80 °C at this point and analyzed at a later time

4. Labeling reaction

4.1. **Labeling sample with ^{12}C-aniline solution**

4.1.1. Transfer 6 µL of sample into a new centrifuge tube and bring the volume to 50 µL with water**.**

NOTE: Volume sample size may depend on the specific CFPS reaction.

4.1.2. Add 5 µL of 200 mg/mL EDC solution.

4.1.3. Add 5 µL of ^{12}C-aniline solution.

NOTE: The aniline solution separates into two phases. Mix well before adding to the reaction.

4.1.4. Vortex the reaction with gentle shaking for 2 h at room temperature.

4.1.5. After 2 h, remove the tubes from the shaker and add 1.5 µL of trimethylamine (TEA) to the reaction in a fume hood.

NOTE: Triethylamine raises the pH of the solution which stops the aniline tagging reaction and stabilizes the compounds.

CAUTION: Triethylamine is toxic and causes irritation of the eyes and respiratory tract.

4.1.6. Centrifuge at 13,500 x *g* for 3 min.

4.2. **Labeling internal standards with ^{13}C-aniline solution**

4.2.1. Dilute internal stock solution to 80 µM with a final volume of 50 µL.

NOTE: Concentration of internal standards can be adjusted to levels close to the experimental sample.

4.2.2. Add 5 µL of 200 mg/mL EDC solution.

4.2.3. Add 5 µL of ^{13}C-aniline solution.

4.2.4. Vortex the reaction with gentle shaking for 2 h at room temperature.

4.2.5. After 2 h, remove the tubes from the shaker and add 1.5 µL of TEA to the reaction in a fume hood.

4.2.6. Centrifuge at 13,500 x *g* for 3 min.

4.3. **Combining tagged internal standard and tagged sample**

4.3.1. Mix 25 µL of ^{12}C-aniline labeled sample with 25 µL of ^{13}C-aniline labeled standard.

4.3.2. Transfer to an auto-sampler vial and analyze by the LC/MS procedure.

4.4. **Creating a standard curve for untagged metabolites**

4.4.1. Dilute stock solution of untagged metabolites (NAD, NADP, FAD, ACA, and Gly3P) to final concentrations of 320 µM, 80 µM, 20 µM and 5 µM with a volume of 50 µL.

4.4.2. Add 5 µL of 200 mg/mL EDC solution.

4.4.3. Add 5 µL of ^{12}C-aniline solution.

4.4.4. Vortex the reaction with gentle shaking for 2 h at room temperature.

4.4.5. After 2 h, remove the tubes from the shaker and add 1.5 µL of TEA to the reaction in a fume hood.

4.4.6. Centrifuge at 13,500 x *g* for 3 min.

4.4.7. Transfer supernatant to an auto-sampler vial and analyze by the LC/MS procedure.

NOTE: The untagged metabolites follow the same procedure as the sample to replicate the sample matrix in order to maintain similar ionization efficiency.

5. Setup of LC/MS procedure

5.1. **Preparation of solvents**

5.1.1. Prepare 5 mM tri-butylamine (TBA) aqueous solution adjusted to pH 4.75 with acetic acid.

NOTE: TBA in the mobile phase helps the analytes achieve good resolution and separation[14].

5.1.2. Prepare 5 mM TBA in acetonitrile (ACN).

5.1.3. Prepare wash solvent with 5% water and 95% ACN.

5.1.4. Prepare purge solvent with 95% water and 5% ACN.

5.2. Setup of MS conditions

5.2.1. Set the mass spectrometer to negative ion mode with a probe temperature of 520 °C, negative capillary voltage of -0.8 kV, positive capillary voltage of 0.8 kV, and set the software to acquire data at 5 points/s.

5.2.2. Set selected ion recordings (SIR) for each metabolite with specified cone voltages and mass over charge (m/z) values. See **Table 1**.

5.3. **Initializing LC/MS according to manufacturer's instructions**

5.3.1. Prime solvent lines in the solvent manager for 3 min.

5.3.2. Prime wash solvent (5% water, 95% ACN) and purge solvent (95% water, 5% ACN) for 15 s for 5 cycles.

5.3.3. Set the sample manager to 10 °C.

5.3.4. Install a C18 (1.7um, 2.1mm x 150mm) column and initialize column with 100% ACN at 0.3 mL/min for 10 min.

5.3.5. Condition the column at 95% water and 5% ACN at 0.3 mL/min for 10 min prior to introducing solvents with buffers.

5.3.6. Condition the column at 95% solvent A (5mM TBA aqueous, pH 4.75) and 5% solvent B (5 mM TBA in ACN) at 0.3 mL/min for 10 min.

5.3.7. Set up a gradient protocol with the elution starting at 95% solvent A and 5% solvent B, raised to 70% solvent B in 10 min, raised to 100% solvent B in 2 min and held at 100% solvent B for 3 min. Return to initial conditions (95% solvent A, 5% solvent B) over 1 min and hold for 9 min to re-equilibrate the column.

5.3.8. Condition the column with the gradient protocol 3 times prior to any injections onto the column.

5.4. **Injecting sample and standards**

5.4.1. Inject 5 µL of the sample into the column and acquire the appropriate m/z ion intensities for the ^{12}C-aniline tagged sample.

5.4.2. Inject 5 µL of the same sample again, but this time acquire the m/z ion intensities for the ^{13}C-aniline tagged standards.

NOTE: Our LC/MS system is unable to acquire both ^{12}C and ^{13}C m/z intensities at the specified SIR time windows, since it is too much data to acquire in the specified time window. Therefore, we inject the same sample twice.

5.4.3. Inject untagged metabolite standards from lowest concentration to highest and record the appropriate m/z ion intensities.

6. Quantification

6.1. **Creating Export method**

6.1.1. In data acquisition software, select **File > New Method > Export Method**.

6.1.2. Specify a Filename, such as **AnilineTagging_Date**.

6.1.3. Check the **Export ASCII** File and choose a directory to export the text file to.

6.1.4. In **Report Type**, select **Summary by All**.

6.1.5. In **Delimiters**, for **Column** select a **,**. For **Row**, select **[cr][if]**.

6.1.6. In **Table**, select **Export** and then **Edit Table** to include **SampleName**, **Area**, **Height**, **Amount** and **Units**.

6.1.7. Save export method.

6.2. **Quantifying metabolites with internal standards using data acquisition software**

6.2.1. Under the **Sample Sets** tab, right click the corresponding **LC/MS** run and select **View as > Channels**.

6.2.2. Select all SIR channels for the ^{13}C-aniline internal standards of one injection, right click and select **Review**.

6.2.3. If the **LC Processing Method Layout** window does not automatically appear, go to **View > Processing Method Layout**.

6.2.4. In **Processing Method Layout**, go to the **Integration** tab and set **ApexTrack** as the algorithm.

6.2.5. Go to the **Smoothing** tab and set the type to **Mean** and the smoothing level to **13**.

NOTE: Any smoothing level can be selected, as long as it is consistent across all samples.

6.2.6. In the **MS Channel** tab, disable **MS 3D Processing**.

6.2.7. In the SIR channel window, integrate each peak, one channel at a time. Once a peak is integrated, go to **Options > Fill from Result** and the details of the peak will be filled in the **Components** tab. Change the peak name to the corresponding compound name.

6.2.8. Once all the SIR channels have been evaluated, save the processing method and close window.

6.2.9. Select all SIR channels of the ^{13}C-aniline and ^{12}C-aniline tagged sample, right click and select **Process**.

6.2.10. Check the **Process** box, select **Use specified processing method**, and choose the processing method that is just saved. Also check the **Export** box, select Use specified export method and choose the saved export method created earlier. Click **OK.**

6.2.11. Open the exported text file with Excel and calculate the concentration of the unknown compound using:

$$C_{x,i} = \frac{A_{x,i}}{A_{std,i}} C_{std,i} D$$

where $C_{x,i}$ is the concentration of the unknown sample for metabolite i, $A_{x,i}$ is the integrated area of the unknown metabolite i, $A_{std,i}$ is the integrated area of the internal standard of metabolite i, $C_{std,i}$ is the concentration of the internal standard of metabolite i, and D is the dilution factor.

6.3. **Quantifying untagged metabolites with standard curve**

6.3.1. Under the **Sample Sets** tab, right click the corresponding LC/MS run and select **View as > Channels**.

6.3.2. Select all SIR channels for the untagged standards of one injection, right click and select **Review**.

6.3.3. If the **LC Processing Method Layout** window does not automatically appear, go to **View > Processing Method Layout**.

6.3.4. In Processing Method Layout, go to the **Integration** tab and set **ApexTrack** as the algorithm.

6.3.5. Go to the **Smoothing** tab and set the type to **Mean** and the smoothing level to **13**.

NOTE: Any smoothing level can be selected, as long as it is consistent across all samples.

6.3.6. In the **MS Channel** tab, disable **MS 3D Processing**.

6.3.7. In the SIR channel window, integrate each peak, one channel at a time. Once a peak is integrated, go to **Options > Fill from Result** and the details of the peak will be filled in the **Components** tab. Change the peak name to the corresponding compound name.

6.3.8. Once all the SIR channels have been evaluated, save the processing method and close window.

6.3.9. Under the **Sample Sets** tab, right click on the sample set and select **Alter Sample**.

6.3.10. Select **Amount** in the new window.

6.3.11. Select copy from **Process** method and choose the process method that was just saved.

6.3.12. Enter the concentration of each metabolite for each vial and enter the unit as <μM for each component (or the corresponding unit) and select **OK**.

6.3.13. Select the sample set again, right click, **View as > Channels**.

6.3.14. Select all SIR channels of the untagged metabolites for the standards, right click and select **Process**.

6.3.15. Check the **Process** box and choose **Use specified processing method**. Select the appropriate processing method and click **OK**.

6.3.16. Select SIR channels for all untagged metabolites for the samples, right click and select **Process**.

6.3.17. Check the **Process** box, select **Use specified processing** method, and choose the processing method that was just saved. Also check the **Export** box, select **Use specified export method** and choose the saved export method created earlier. Click **OK**.

6.3.18. Quantify the untagged metabolites with the standard curve and export the results to a text file to the directory specified.

REPRESENTATIVE RESULTS:

As a proof-of-concept, we used the protocol to quantify metabolites in an *E. coli* based CFPS system expressing green fluorescent protein (GFP). The CFPS reaction (14 μL) was quenched and deproteinized with ethanol. The CFPS sample was then tagged with ^{12}C-aniline, while standards were tagged with ^{13}C-aniline. The tagged sample and standards were then combined and injected into the LC/MS (**Figure 1**). The protocol detected and quantified 40 metabolites involved in central carbon and energy metabolism using internal standards, while a standard curve for 5 of the metabolites that were not tagged with aniline was also developed (**Figure 2**). The diverse metabolites involved in these pathways were a class of phosphorylated sugars, phosphocarboxylic acids, carboxylic acids, nucleotides, and cofactors. The derivatization with aniline introduced a hydrophobic moiety into hydrophilic molecules which facilitated more effective separation using reversed-phase chromatography[12]. In addition, the method enabled the separation of structural isomer pairs such as glucose 6-phosphate and fructose 6-phosphate in a single LC/MS run. Each compound's mass over charge (m/z) ratio and retention time were identified prior to the experiment by injecting 1 mM of one compound at a time and comparing the mass spectrum to the blank (**Table 1**).

The limit of detection and range of linearity for all compounds was estimated by producing a standard curve that ranged from 0.10 μM to 400 μM (**Table 2**). The average correlation coefficient (R^2) for all compounds was 0.988 and most compounds had a linear range of 3-orders of magnitude. Three compounds had notable saturation effects, especially alpha-ketoglutarate which had a linear range from 0.1 μM to 25 μM. Isocitrate and citrate also had saturation effects above 100 μM.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic of workflow for aniline tagging. The cell-free protein synthesis reaction is deproteinized and tagged with ^{12}C-aniline, while a standard stock mixture is tagged with ^{13}C-aniline. Both mixtures are then mixed at a 1:1 volumetric ratio and analyzed by LC/MS.

Figure 2: Overlapped selected ion chromatograms for 40 metabolites. Mass chromatogram from a single LC/MS run of a 40 μM standard mixture of 40 metabolites. Peaks were identified by their retention time and m/z values for each compound. Complete compound names and their abbreviations are listed in **Table 1**.

Table 1: Identification and labeling results of metabolites. Each compound's corresponding peak number, retention time, m/z value for unlabeled, ^{12}C and ^{13}C labeled, and MS species. MS Species, A stands for Aniline tag.

Table 2: Metabolite quantification in a representative CFPS sample. The concentration of each metabolite and the standard deviation. Limit of detection, range of linearity and correlation coefficient identified from standard curves.

DISCUSSION:

Cell-free systems have no cell wall, thus there is direct access to metabolites and the biosynthetic machinery without the need for complex sample preparation. However, very little work has been done to develop thorough and robust protocols to quantitatively interrogate cell-free reaction systems. In this study, we developed a fast, robust method to quantify metabolites in cell-free reaction mixtures and potentially in whole-cell extracts. Individual quantification of metabolites in complex mixtures, such as those found in cell-free reactions, or whole-cell extracts, is challenging for several reasons. Central amongst these reasons is chemical diversity. The array of functional groups simultaneously present in these mixtures (e.g., carboxylic acids, amines, phosphates, hydroxyls, etc.) greatly increases the analytical complexity. To circumvent this, we used an aniline derivatization method in combination with ^{13}C internal standards to introduce hydrophobic components to the metabolite mixtures. Using this method, we robustly detected and quantified 40 metabolites in a cell-free reaction in a single LC/MS run. The protocol tagged 35 of the 40 compounds in this study, while the remaining 5 compounds were quantified with a standard curve method. Earlier work suggested the reaction conditions formed an intramolecular salt between the amine and phosphate group that inhibited derivatization[12]. Reaction conditions were not identified for simultaneous derivatization of all 40 compounds; however, the current alternative is quantification with a standard curve method. While we demonstrated this technique in a cell-free reaction mixture, it could also likely be applied to whole-cell extracts, thus, potentially allowing the absolute quantification of intracellular metabolites concentrations. The latter application has relevance to a variety of important questions in biotechnology and human health.

The method presented here was based on a previous technique (GSIST) that was applied to whole-cell extracts of the yeast *S. cerevisiae*[12,13]. In this study, we expanded the number of compounds which could be detected and quantified, including all 12 nucleotides (xMP, xDP, xTP, where x is A, C, G and U). Addition of these compounds could have important biological implications. For example, these nucleotides are heavily involved in transcription and translation processes, which is one of the central processes of interest in CFPS applications, and more generally the compounds are important in a variety of physiological functions. In addition, we were able to detect acetic acid which is an important metabolite when examining overflow metabolism. However, we did not include it in the study because there was a significant reduction of signal in multiple compounds, especially nicotinamide adenine dinucleotide reduced (NADH) and nicotinamide adenine dinucleotide phosphate reduced (NADPH) when acetic acid was added to the standard mixture. Acetic acid had a high limit of detection of 612 µM, thus at these high levels it had a negative effect on the other metabolites' signals. Despite this, acetic acid can still be detected and quantified in samples by creating a standard curve with just acetic acid in the vial. Acetic acid had a m/z value of 134.0, retention time of 5.78 min, and a linear range from 612 µM to 5000 µM (R^2 = 0.986) when tagged with ^{12}C-aniline. The remaining metabolites did not alter each other's ion signal and represent a comprehensive mixture to characterize CFPS metabolism. The protocol presented here is limited to metabolites involved in central carbon and energy metabolism. Thus, the current method is unable to measure metabolite abundance from other pathways that may be of importance, such as fatty acid and amino acid metabolism.

Taken together, we developed a fast, robust protocol for the characterization and absolute quantification of 40 compounds involved in glycolysis, the pentose phosphate pathway, the tricarboxylic acid cycle, energy metabolism, and cofactor regeneration in CFPS reactions. The method relied on internal standards tagged with ^{13}C-aniline, while the sample was tagged with ^{12}C-aniline. The internal standards and sample compounds co-eluted and eliminated ion-suppression effects which enabled accurate quantification of individual metabolites in complex metabolite mixtures. We identified a total of 40 compounds (41, if including acetic acid) that can be detected and quantified in a cell-free reaction mixture; however, the list of metabolites could be further expanded and adjusted towards the particular biochemical process of interest. Thus, the method provides a robust and accurate approach to characterize cell-free metabolism, which is potentially critical to improving the yield, productivity and energy efficiency of cell-free processes.

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DISCLOSURES:

The authors have nothing to disclose.

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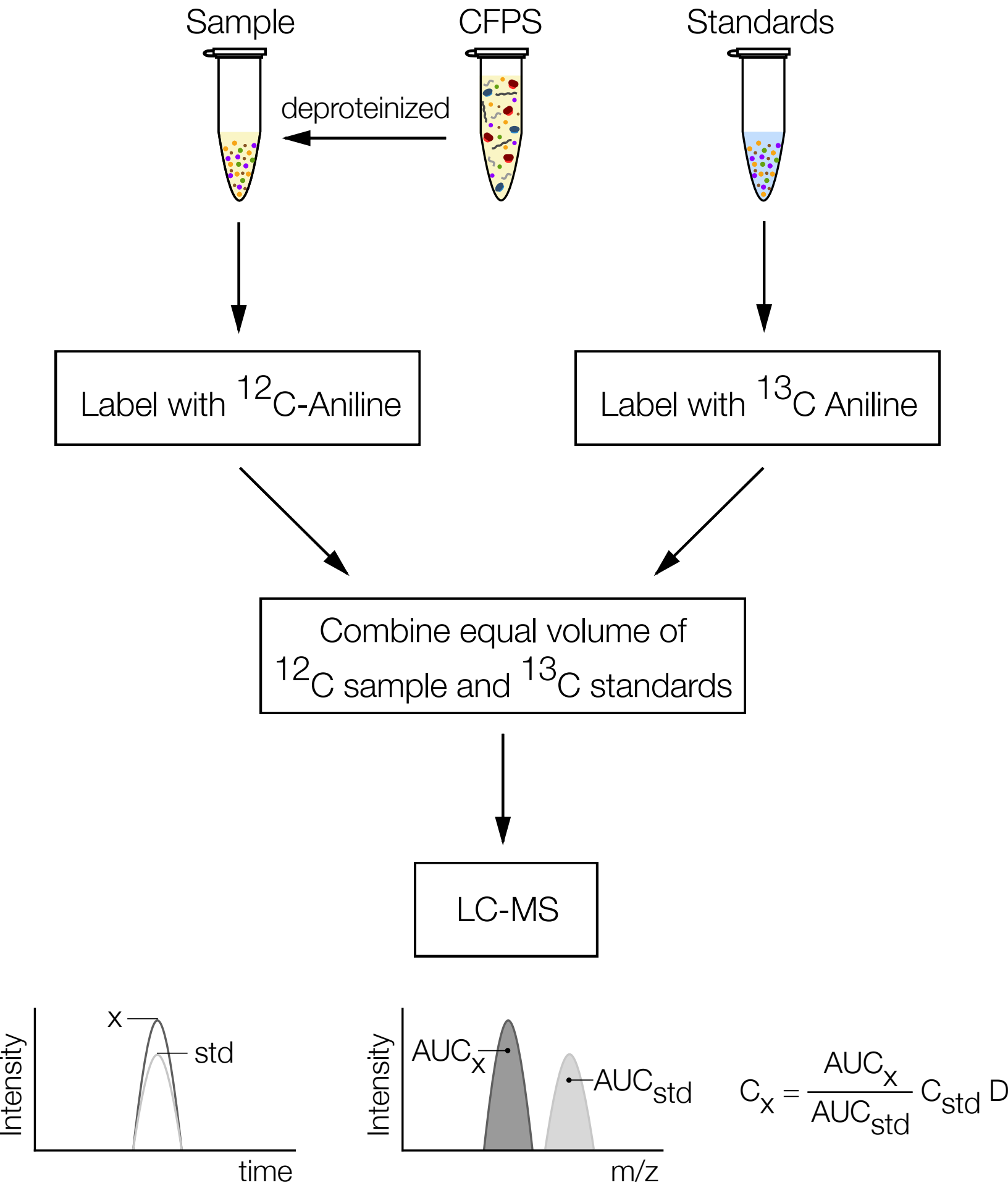
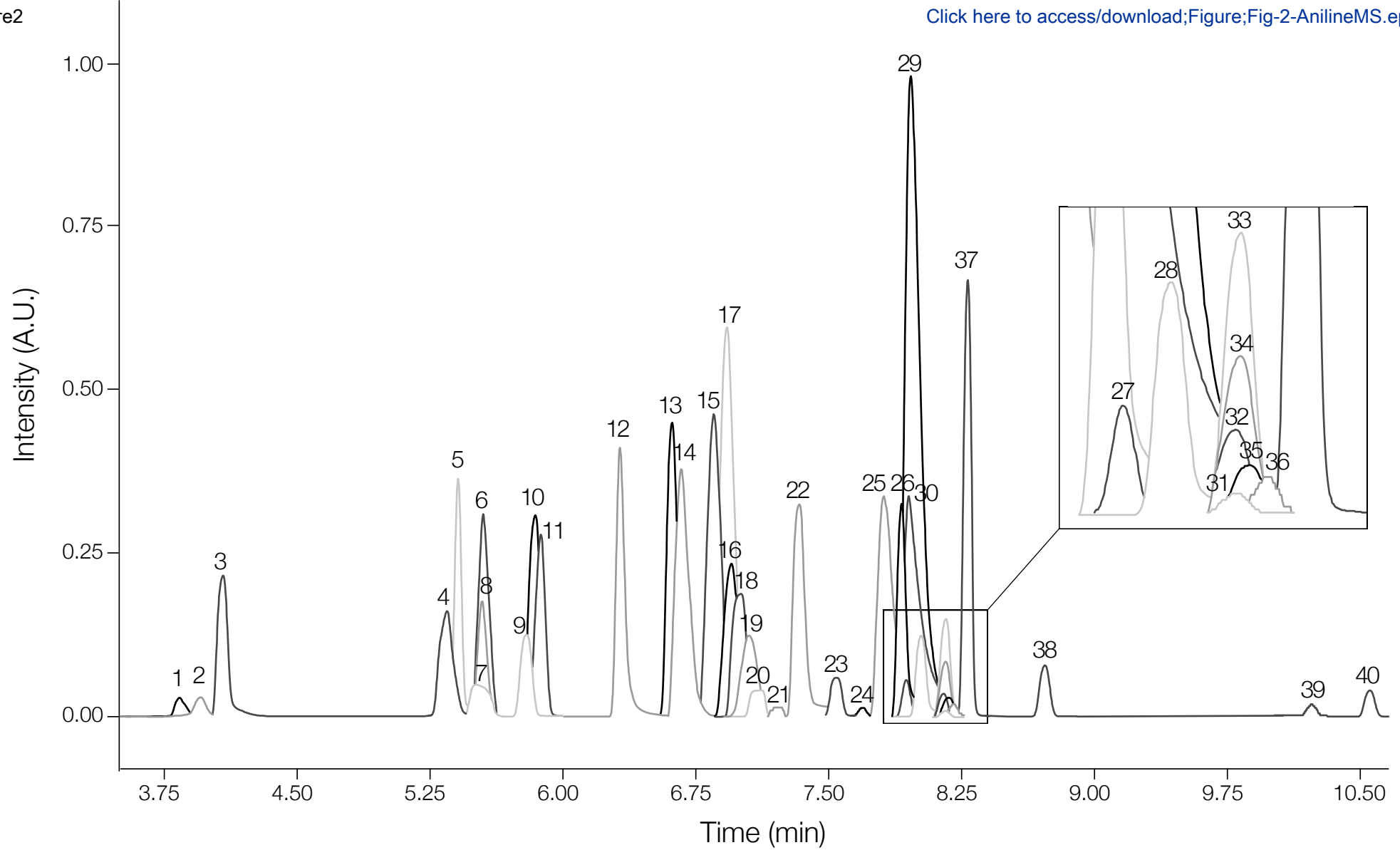


Figure2



1. Gly3P	9. LAC	17. UDP	25. CTP	33. MAL
2. NAD	10. AMP	18. FAD	26. GTP	34. GAP
3. GLC	11. UMP	19. F16P	27. OAA	35. ACA
4. S7P	12. NADP	20. 6PG	28. aKG	36. NADPH
5. F6P	13. 3PG	21. NADH	29. UTP	37. PEP
6. GMP	14. CDP	22. G6P	30. ATP	38. SUCC
7. RL5P	15. GDP	23. R5P	31. FUM	39. ICIT
8. CMP	16. ADP	24. E4P	32. PYR	40. CIT

Table1

Click here to access/download;Table;Table1-MS.xlsx

Peak Number	Metabolite	Abbreviation	KEGG ID	Retention Tim	12C m/z	13C m/z
1	Glycerol 3-ph	Gly3P	C00093	3.85		
2	Nicotinamide	NAD	C00003	3.96		
3	Glucose	GLC	C00031	4.06	289.9	296
4	Sedoheptulos	S7P	C05382	5.41	364	370
5	Fructose 6-pl	F6P	C00085	5.48	334	340
6	Guanosine m	GMP	C00144	5.57	437.05	443
7	Ribulose 5-pl	RL5P	C00199	5.58	304	310
8	Cytidine mon	CMP	C00055	5.59	397.09	403
9	Lactate	LAC	C00186	5.77	164.05	170
10	Adenosine m	AMP	C00020	5.85	421.1	427.1
11	Uridine monc	UMP	C00105	5.88	398.07	404
12	Nicotinamide	NADP	C00006	6.39		
13	3-Phosphogly	3PG	C00197	6.63	242	248.06
14	Cytidine diph	CDP	C00112	6.72	477	483
15	Guanosine di	GDP	C00035	6.87	517	523
16	Adenosine di	ADP	C00008	6.94	501	507
17	Uridine diphc	UDP	C00015	6.97	478	484
18	Flavin adenin	FAD	C00016	7.03		
19	Fructose 1,6-	F16P	C05378	7.1	395.95	402.1
20	Gluconate 6-p	6PG	C00345	7.11	425.1	437
21	Nicotinamide	NADH	C00004	7.23	633.13	639.08
22	Glucose 6-ph	G6P	C00668	7.32	409.1	421.1
23	Ribose 5-pho	R5P	C00117	7.54	379.1	391.1
24	Erythrose 4-p	E4P	C00279	7.71	348.9	361
25	Cytidine triph	CTP	C00075	7.84	557	563
26	Guanosine tri	GTP	C00044	7.93	597	603
27	Oxalacetate	OAA	C00036	7.94	281	293
28	Alpha-ketoglu	aKG	C00026	7.95	295	307.1
29	Uridine triphc	UTP	C00075	7.97	558	564
30	Adenosine tri	ATP	C00002	8.03	581	587
31	Fumarate	FUM	C00122	8.09	265	277.1
32	Pyruvate	PYR	C00022	8.09	162	168
33	Malate	MAL	C00149	8.09	283.06	295.15
34	D-glyceraldeh	GAP	C00118	8.09	319	331.1
35	Acetyl-coenzy	ACA	C00024	8.16		
36	Nicotinamide	NADPH	C00005	8.23	694.92	700.82
37	Phosphoenol	PEP	C00074	8.28	317	329.1
38	Succinate	SUCC	C00042	8.64	267.07	279.1
39	Isocitrate	ICIT	C00311	10.13	398	416
40	Citrate	CIT	C00158	10.46	416.1	434.06

nonlabel m/z	CV	MS Species
153	10	M – H2O – H
698	10	M + Cl – H
	15	M + A + Cl - H
	10	M + A – H
	10	M + A – H
	10	M + A – H
	10	M + A – H
	10	M + A – H
	10	M + A – H
	10	M + A – H
	10	M + A – H
724	10	M - H2O – H
	15	M + A – H2O – H
	10	M + A – H
	10	M + A – H
	10	M + A – H
	10	M + A – H
784.15	15	M – H
	10	M + A – H2O – H
	10	M + 2A – H
	10	M + A + H2O – nicotinamide – H
	10	M + 2A – H
	15	M + 2A – H
	10	M + 2A – H
	5	M + A – H
	5	M + A – H
	25	M + 2A – H
	15	M + 2A – H
	10	M + A – H
	15	M + A – H
	10	M + 2A – H
	25	M + A – H
	10	M + 2A – H
	5	M + 2A – H
790	10	M – H2O – H
	10	M + A – nicotinamide – H
	20	M + 2A – H
	15	M + 2A – H
	10	M + 3A – H2O – H
	20	M + 3A – H

Table2

Peak No.	Metabolite	Abbreviation	KEGG ID	Concentration	SD (n = 3)
1	Glycerol 3-ph	Gly3P	C00093	0.377	0.034
2	Nicotinamide	NAD	C00003	0.052	0.010
3	Glucose	GLC	C00031	0.002	0.000
4	Sedoheptulos	S7P	C05382	0.007	0.000
5	Fructose 6-ph	F6P	C00085	0.029	0.004
6	Guanosine m	GMP	C00144	0.007	0.001
7	Ribulose 5-ph	RL5P	C00199	0.035	0.002
8	Cytidine mon	CMP	C00055	0.045	0.001
9	Lactate	LAC	C00186	2.134	0.048
10	Adenosine m	AMP	C00020	0.020	0.002
11	Uridine monc	UMP	C00105	0.021	0.000
12	Nicotinamide	NADP	C00006	0.014	0.002
13	3-Phosphogly	3PG	C00197	6.125	0.239
14	Cytidine diph	CDP	C00112	0.202	0.029
15	Guanosine di	GDP	C00035	0.146	0.027
16	Adenosine di	ADP	C00008	0.797	0.161
17	Uridine diphc	UDP	C00015	0.212	0.036
18	Flavin adenin	FAD	C00016	0.008	0.001
19	Fructose 1,6-	F16P	C05378	3.643	0.105
20	Gluconate 6-p	6PG	C00345	0.017	0.001
21	Nicotinamide	NADH	C00004	0.063	0.028
22	Glucose 6-ph	G6P	C00668	0.046	0.002
23	Ribose 5-pho	R5P	C00117	0.055	0.005
24	Erythrose 4-p	E4P	C00279	0.038	0.007
25	Cytidine triph	CTP	C00075	0.896	0.078
26	Guanosine tri	GTP	C00044	0.870	0.109
27	Oxalacetate	OAA	C00036	0.023	0.008
28	Alpha-ketoglu	aKG	C00026	0.391	0.020
29	Uridine triph	UTP	C00075	0.845	0.092
30	Adenosine tri	ATP	C00002	1.557	0.188
31	Fumarate	FUM	C00122	0.576	0.100
32	Pyruvate	PYR	C00022	5.813	0.804
33	Malate	MAL	C00149	2.548	0.269
34	D-glyceralde	GAP	C00118	2.194	0.367
35	Acetyl-coenz	ACA	C00024	0.196	0.044
36	Nicotinamide	NADPH	C00005	0.006	0.010
37	Phosphoenol	PEP	C00074	3.442	0.345
38	Succinate	SUCC	C00042	5.683	0.573
39	Isocitrate	ICIT	C00311	0.003	0.006
40	Citrate	CIT	C00158	0.002	0.001

Limit of Detection (µM)	Limit of Linea	R^2
0.1	400	0.995
0.39	400	0.993
0.1	400	0.997
0.16	400	0.988
0.1	400	0.986
0.39	100	0.992
0.39	400	0.996
0.1	100	0.992
0.1	400	0.988
0.1	100	0.992
0.1	100	0.997
0.34	400	0.950
0.1	100	0.996
0.39	400	0.997
1.5625	400	0.984
0.39	400	0.995
0.39	400	0.991
0.1	400	0.958
0.39	400	0.989
0.39	400	0.989
0.39	100	0.972
0.1	400	0.984
0.39	100	0.999
0.39	400	0.979
6.25	100	0.998
6.25	100	0.993
0.56	400	0.997
0.1	25	0.979
1.5625	400	0.998
1.5625	400	0.991
1.5625	100	0.999
0.39	400	0.993
0.1	400	0.991
0.1	100	0.974
0.1	100	0.991
0.14	100	0.990
0.1	100	0.962
0.1	320	0.999
0.39	100	0.998
0.1	100	0.981

Name of Material/Equipment	Company	Catalog Number	Comments/Description
12C Aniline	Sigma-Aldrich	242284	Aniline 12C
13C labeled aniline	Sigma-Aldrich	485797	Aniline 13C6
3-Phosphoglyceric acid	Sigma-Aldrich	P8877	3PG
Acetic Acid	FisherScientific	AC222140010	ACE
Acetonitrile, LCMS	JT BAKER	9829-03	ACN
Acetyl-coenzyme A	Sigma-Aldrich	A2056	ACA
Acquity UPLC BEH C18 1.7 µM, 2.1 x 1!	Waters	186002353	Column
Adenosine diphosphate	Sigma-Aldrich	A2754	ADP
Adenosine monophosphate	Sigma-Aldrich	A1752	AMP
Adenosine triphosphate	Sigma-Aldrich	A2383	ATP
Alpha-ketoglutarate	Sigma-Aldrich	K1128	aKG
Citrate	Sigma-Aldrich	251275	CIT
Cytidine diphosphate	Sigma-Aldrich	C9755	CDP
Cytidine monophosphate	Sigma-Aldrich	C1006	CMP
Cytidine triphosphate	Sigma-Aldrich	C9274	CTP
D-glyceraldehyde 3-phosphate	Sigma-Aldrich	39705	GAP
Erythrose 4-phosphate	Sigma-Aldrich	E0377	E4P
Ethanol	Sigma-Aldrich	EX0276	EtOH
Fisher Scientific accuSpin Micro 17 Cer	FisherScientific		Centrifuge
Flavin adenine dinucleotide	Sigma-Aldrich	F6625	FAD
Fructose 1,6-bisphosphate	Sigma-Aldrich	F6803	F16P
Fructose 6-phosphate	Sigma-Aldrich	F3627	F6P
Fumarate	Sigma-Aldrich	F8509	FUM
Gluconate 6-phosphate	Sigma-Aldrich	P7877	6PG
Glucose	Sigma-Aldrich	G8270	GLC
Glucose 6-phosphate	Sigma-Aldrich	G7879	G6P
Glycerol 3-phosphate	Sigma-Aldrich	G7886	Gly3P
Guanosine diphosphate	Sigma-Aldrich	G7127	GDP
Guanosine monophosphate	Sigma-Aldrich	G8377	GMP
Guanosine triphosphate	Sigma-Aldrich	G8877	GTP
Hydrochloric acid	Sigma-Aldrich	258148	HCl
Isocitrate	Sigma-Aldrich	I1252	ICIT
Lactate	Sigma-Aldrich	L1750	LAC

Malate	Sigma-Aldrich	02288	MAL
myTXTL - Sigma 70 Master Mix Kit	ArborBiosciences	507024	Cell-free protein synthesis
N-(3-dimethylaminopropyl)-N′-ethylca	Sigma-Aldrich	03449	EDC
Nicotinamide adenine dinucleotide	Sigma-Aldrich	43410	NAD
Nicotinamide adenine dinucleotide ph	Sigma-Aldrich	N5755	NADP
Nicotinamide adenine dinucleotide ph	Sigma-Aldrich	481973	NADPH
Nicotinamide adenine dinucleotide rec	Sigma-Aldrich	N8129	NADH
Oxalacetate	Sigma-Aldrich	O4126	OAA
Phosphoenolpyruvate	Sigma-Aldrich	P0564	PEP
Pyruvate	Sigma-Aldrich	P5280	PYR
Ribose 5-phosphate	Sigma-Aldrich	R7750	R5P
Ribulose 5-phosphate	CarboSynth	MR45852	RL5P
Sedoheptulose 7-phosphate	CarboSynth	MS07457	S7P
Succinate	Sigma-Aldrich	S3674	SUCC
Tributylamine	Sigma-Aldrich	90780	TBA
Triethylamine	FisherScientific	O4884	TEA
ultrapure water	FisherScientific	10977-015	water
Uridine diphosphate	Sigma-Aldrich	U4125	UDP
Uridine monophosphate	Sigma-Aldrich	U6375	UMP
Uridine triphosphate	Sigma-Aldrich	U6625	UTP
VWR Heavy Duty Vortex	VWR		Vortex
Water, LCMS	JT BAKER	9831-03	WATER
Waters Acquity H UPLC Class Quaterna	Waters		LCMS
Waters Acquity H UPLC Class Sample M	Waters		LCMS
Waters Acquity Qda detector	Waters		LCMS
Waters Empower 3	Waters		Software
Waters LCMS Total Recovery Vial	Waters	186000384c	LCMS Vial

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Author(s):	Michael Vilkhovoy, David Dai, Sandra Vadhin, Abhinav Adhikari, Jeffrey D. Varner

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Date:	5/28/2019

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JoVE60329

Title: Absolute quantification of cell-free protein synthesis metabolism by reversed-phase liquid chromatography-mass spectrometry
Authors: Michael Vilkhovoy, David Dai, Sandra Vadhin, Abhinav Adhikari, and Jeffrey D. Varner
Manuscript ID: JoVE60329
Journal: Journal of Visualized Experiments

Dear Dr. Bing Wu,

We thank the reviewers and JoVE for providing feedback on our manuscript entitled: Absolute quantification of cell-free protein synthesis metabolism by reversed-phase liquid chromatography-mass spectrometry. We have read the comments and have addressed them below.

Editorial Comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

> We thank the editor for taking the time to go through the manuscript. We have thoroughly proofread the manuscript.

2. The Summary is over the 50 word limit.

> We updated the summary to 50 words:
>
> > In this study, we present a robust protocol to quantify 40 compounds involved in central carbon and energy metabolism in cell-free protein synthesis reactions. The cell-free synthesis mixture is derivatized with aniline for effective separation using reversed-phase liquid chromatography and then quantified by mass spectrometry using isotopically labelled internal standards.

3. Please define all abbreviations before use.

> We have gone through the manuscript and defined all abbreviations.

4. Step 6.3.12: Please write this step in the imperative tense.

> We removed step 6.3.12, since it described what would happen following step 6.3.11. It wasn't an actual step in the protocol and therefore it was removed.