From QC to quantitation: Utility of QReSS metabolites in FBS measurements

Authors

Andrew Percy[1], Amanda Souza[2], Ioanna Ntai[2], Bashar Amer[2]; [1]Cambridge Isotope Laboratories, Inc., Tewksbury, MA USA; [2]Thermo Fisher Scientific, San Jose, CA USA

Keywords

Quality control metabolomics, QC kits, untargeted metabolomics, quantitative metabolomics, QReSS standards, system suitability

Highlights

- Fetal bovine serum, FBS, is a common supplemental growth medium in cell culture applications
- QCs are imperative to maximizing the quality of metabolite profiling and quantification data
- QReSS™ metabolite mix is useful in assessing performance efficiencies as well as in obtaining qualitative/quantitative information on FBS compounds
- Exceptional stability observed in the HILIC-MS analysis, which supported the data extracted from the targeted and untargeted MS analysis

Introduction

Metabolomics is a rapidly growing field of research that has unique and proven advantages in systems biology and biomarker discovery. Great advances have been made over the past decade on the feasibility of mass spectrometry (MS)-based metabolomics. Despite the advancements, there remains a pervasive need for quality control (QC) as metabolomic experiments can experience unwanted variations that threaten the quality of the acquired data.[1,2] The use of reliable QC measures is therefore critical to assess and prevent unwanted preanalytical and instrumental variation.[3] Any system errors or outliers identified through QC testing must therefore be corrected to ensure consistent and meaningful metabolomics data.

An area of metabolomics garnering great interest, particularly in discovery efforts, is the profiling and quantitation of cell cultures. In this *in vitro* application, fetal bovine serum (FBS) is commonly used as a supplemental growth medium. FBS contains several nutritional and macromolecular components, including a variety of small molecules (e.g., amino acids, carbohydrates, lipids, and hormones) that are essential for cell growth and proliferation.

FBS from different commercial sources, or handled under different processing techniques (e.g., dialyzed, heat-inactivated, unprocessed), may result in inconsistent cell culture phenotypes and growth rates. Incorporating reliable QCs in the evaluation of FBS, when applied in metabolomics to spent and control media, can help increase the overall confidence of interpretations when this supplemental material possesses variation.

Here, the QReSS™ standard mixes from Cambridge Isotope Labs (CIL cat no. MSK-QReSS1 and MSK-QReSS2) were used in the QC of metabolites from variably sourced FBS samples. The QReSS standards are beneficial for QC assessments because they comprise a chemically diverse set of 18 stable isotope-labeled metabolites that span a broad molecular weight range, possess varied ionization propensities, and cover a distribution in class and retention time. Highlighted in addition to the QC results are the absolute quantitation of known compounds from targeted MS experiments and novel findings from an untargeted workflow.

Experimental

Materials

All reagents were of the highest available grade and solvents were LC-MS grade. Manufacture recommendations were adhered to in the storage of these chemicals and reagents. QCs and calibrants were constructed with the QReSS kit mixes (stable isotope-labeled and/or unlabeled; CIL cat no. MSK-QReSS-KIT and MSK-QReSS-US-KIT, respectively). The composition details of the labeled QReSS standard mixes are outlined in Table 1. All QReSS mixes were stored at ambient temperature (protected from light and moisture) in their neat form and at 4 °C upon solubilization. FBS samples (n = 20) were obtained from four commercial vendors supplied across five countries (USA, Canada, Mexico, Panama, and Australia). These were treated by the vendor under different conditions (dialyzed, heat-inactivated, and unprocessed) and were stored according to supplier recommendations.

Table 1. Composition details for the stable isotope-labeled mixes in the QReSS kit. Isotopic enrichments are all ≥98%, while chemical purities are >98%, unless otherwise specified (see* in compound description columns). Note: The concentrations reflect the stock solutions obtained from 1 mL solvent additions.

Abbrev. description	Abbrev./alt. name	Chemical formula	Metabolic class	Conc. (µg/mL)
Vial 1				
L-Alanine ($^{13}C_3$/^{15}N)	Ala	$^{13}C_3H_7{}^{15}NO_2$	Amino acid	100
1,4-Butanediamine·HCl ($^{13}C_4$)	Putrescine	$^{13}C_4H_{12}N_2$·2HCl	Other (polyamine)	10
Creatinine (D_3)	Crn	$C_4H_4D_3N_3O$	Amino acid	100
Ethanolamine·HCl (D_4)	ETA	$C_2H_3D_4NO$·HCl	Other (1,2-aminoalcohol)	10
Guanosine ($^{15}N_5$)*	Guo	$C_{10}H_{13}{}^{15}N_5O_5$	Nucleoside	2
Hypoxanthine ($^{13}C_5$)	HPX	$^{13}C_5H_4N_4O$	Nucleobase (purine)	10
L-Leucine ($^{13}C_6$)	Leu	$^{13}C_6H^{13}NO_2$	Amino acid	5
L-Phenylalanine ($^{13}C_6$)	Phe	$^{13}C_6C_3H_{11}NO_2$	Amino acid	100
Thymine ($^{15}N_2$)	T	$C_5H_6{}^{15}N_2O_2$	Nucleobase (pyrimidine)	20
L-Tryptophan (^{13}C11)	Trp	$^{13}C_{11}H_{12}N_2O_2$	Amino acid	100
L-Tyrosine ($^{13}C_6$)	Tyr	$^{13}C_6C_3H_{11}NO_3$	Amino acid	100
Vitamin B3 ($^{13}C_6$)	Nicotinamide	$^{13}C_6H_6N_2O$	Vitamin	5
Vial 2				
Citric acid ($^{13}C_3$)	CA	$^{13}C_3C_3H_8O_7$	Organic acid	10
Fumaric acid ($^{13}C_4$)	FA	$^{13}C_4H_4O_4$	Organic acid	100
Indole-3-acetic acid ($^{13}C_6$)	IAA	$^{13}C_6C_4H_9NO_2$	Hormone	5
α-Ketoglutaric acid, disodium salt ($^{13}C_4$)*	α-KG	$^{13}C_4CH_4Na_2O_5$	Organic acid	100
Sodium palmitate ($^{13}C_{16}$)	PA (or 16:0)	$^{13}C_{16}H_{31}O_2Na$	Fatty acid	10
Sodium pyruvate ($^{13}C_3$)	Pyr	$^{13}C_3H_3O_3Na$	Organic acid	100

Sample preparation

The QReSS metabolite mixes were solubilized in 1 mL of 50% methanol before aliquot mixing and further dilution. The FBS and pooled FBS samples were extracted for polar metabolites using a cold 75:25 acetonitrile:methanol solution (at 9:1 solvent:sample) containing the standard QReSS mix. Each sample was extracted sequentially (3×). After centrifugation, the supernatant was stored at 4 °C until HILIC-MS processing. Thirteen-point standard curves were constructed using a consistent concentration of the combined labeled QReSS mix (approximates the experimental sample metabolite concentrations) and a variable concentration of the unlabeled QReSS mix (from level A at low ng/mL to level H at high µg/mL e.g., 5 ng/mL to 50 µg/mL for creatinine). The calibrants were stored at 4 °C in the UPLC autosampler prior to MS acquisition.

Chromatography

Metabolite separations were performed by HILIC on an Thermo Scientific™ Accucore™ HPLC column (150 × 2.1 mm i.d., 2.6 µm particles, 80 Å pores). The column and autosampler were maintained at 30 and 4 °C, respectively. Following 5 µL injections, separations occurred over a 32 min gradient at an analytical flow rate (see Table 2 for details). The mobile phase compositions were as follows:

- **Eluent A:** 0.1% formic acid and 10 mM ammonium formate in water
- **Eluent B:** 0.1% formic acid in acetonitrile

Table 2. HILIC gradient method.

Time (min)	Flow rate (mL/min)	% Eluent B
0.0	0.40	95
0.5	0.40	95
10.5	0.25	40
17.5	0.25	40
18.0	0.40	95
32.0	0.40	95

Mass spectrometry

The Thermo Scientific™ Accucore™ HILIC column (contained within a Thermo Scientific™ Vanquish™ Horizon UHPLC system) was coupled to a Thermo Scientific™ Orbitrap Exploris™ 120 mass spectrometer via a Thermo Scientific™ OptaMax™ NG electrospray ion (ESI) source. The source was operated under positive and negative polarity switching. Data were acquired in the full scan MS mode and in the data-dependent MS^2 mode. MS^2 acquisitions involved top four precursor selections and dynamic exclusions. External and internal mass calibration of both ESI modes were achieved with the Thermo Fisher™ Pierce™ FlexMix™ calibration solution and Easy-IC™ source respectively. The MS^1 and MS^2 parameter settings are outlined in Tables 3 and 4, respectively, with ultra-high-purity nitrogen serving as the carrier gas throughout.

Table 3. ESI source settings for the Orbitrap Exploris 120 mass spectrometer. AU refers to arbitrary units.

Parameter	Value
Sheath gas	35 AU
Auxiliary gas	10 AU
Sweep gas	1 AU
Spray voltage	3.2 kV (ESI+)
	2.7 kV (ESI-)
Capillary tube temperature	275 °C
Probe	250 °C

Table 4. MS^1 and MS^2 settings for the Orbitrap Exploris 120 mass spectrometer.

MS^1 parameters	Value
Mass resolution	60,000 at *m/z* 200
Scan range	60–800 *m/z*
RF lens	70%
Normalized AGC target	100%
Maximum injection mode	Custom
Maximum injection time	200 ms
Data type	Profile
MS^2 parameters	**Value**
Mass resolution	60,000 at *m/z* 200
Isolation window	2 *m/z*
Dissociation type	Higher energy collisional dissociation
Collision energy mode	Fixed
HCD collision energy	30%
Collision energy type	Normalized
Scan type	Data-dependent acquisition—Thermo Scientific™ AcquireX™ intelligent MS/MS data acquisition workflow

Data Processing and Analysis

Data were acquired using Thermo Scientific™ Xcalibur™ software. The run order involved 13 calibrants, five pooled FBS (i.e., pooled QC), and 20 randomized FBS samples (see Figure 1 for run sequence).

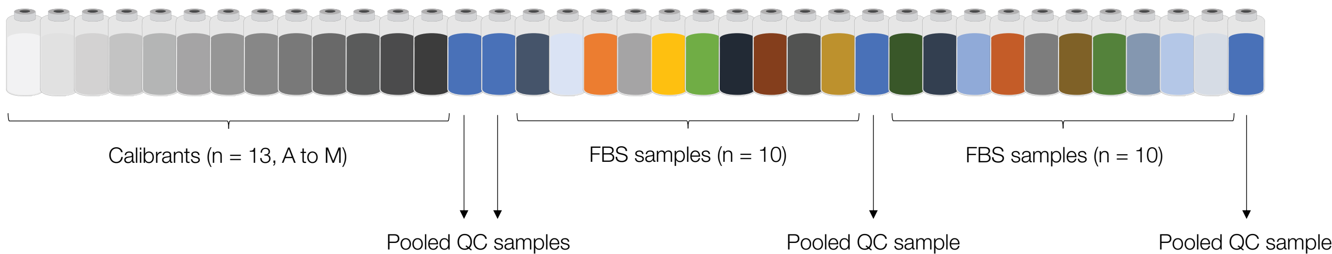

Figure 1. Order of one complete batch sequence. Note: The first series was fronted by a pooled QC and the FBS samples were randomized between batch replicates.

Batch sequence runs were processed in triplicate with interspersed solvent blanks. Thermo Scientific™ TraceFinder™ software (v5.1) was used for the targeted data analysis using a 3 ppm mass tolerance filter, while the untargeted data were analyzed in Thermo Scientific™ Compound Discoverer™ software (v3.2). In both cases, peak selections and integrations were validated manually by visual inspection of the target metabolites before the interpretation of the QC and qualitative/quantitative results. For metabolite quantification, 13-point standard curves were prepared with a 1/× weighting. The dynamic range reflected the difference between the qualified concentration levels.

Results and discussion

Quality control is an integral aspect of experimental design. Here, the QReSS metabolite mixes were used to qualify a series of qualitative and quantitative metabolomic measurements pertaining to the FBS sample analyses. The following sections discuss the results and their broader impact, beginning with the system QCs.

Performance QCs

For QC tracking purposes, the master labeled QReSS mix was added to all sample types (process blanks excluded) at a consistent concentration. Samples containing labeled internal standards encompassed calibrants and FBS samples (both pooled and individual), with processing by HILIC-MS/MS in analytical triplicate. Owing to the point of QReSS addition, the experiments enabled instrument performance metrics to be evaluated over the entirety of the batch sequences. Figure 2 illustrates an example compilation of QC data.

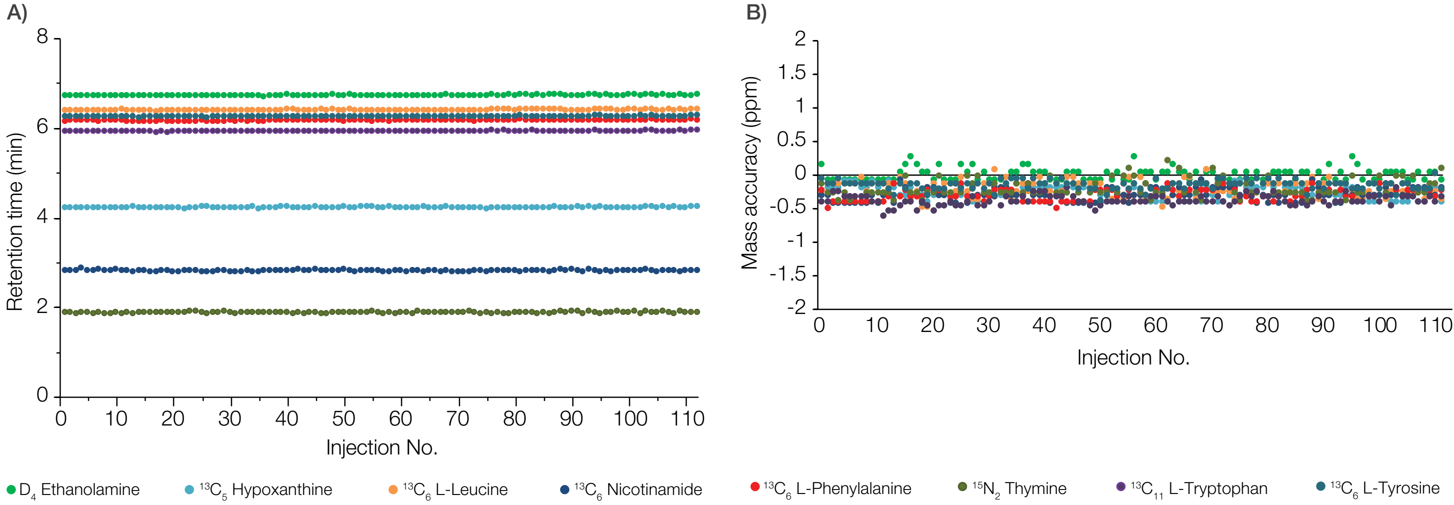

Figure 2. QC results for a subset of isotope-labeled QReSS metabolites measured across the calibrants, QCs, and FBS samples. Acquisitions were by HILIC-MS (ESI+). Variability of retention times are shown in (A) and mass accuracy in (B).

Overall, the results demonstrated excellent maintenance of metrics within and between batch polarities/replicates. The retention times, for example, were observed to have a <1% CV across the 112 total injections, while mass accuracy was consistently sub-ppm. Low variability in the labeled standards were additionally evidenced by the signal responses (<10% CV, on average). Slightly higher signal variability, however, was observed in the dialyzed FBS sample measurements in comparison to the heat-inactivated and unprocessed samples. This reduction is posited to be attributed to the dialyzation process. That notwithstanding, the high precision in metabolite measurement across the injection series provides confidence in the reliability of the instrument acquisitions and the labeled standards from which the qualitative/quantitative findings are derived.

Targeted quantitation and metabolite profiling

Forward response curves were prepared using the unlabeled and labeled QReSS mixes (comprises 18 metabolites) in buffer. This type of standard curve is generated from a dilution series of unlabeled (i.e., light) concentrations with constant labeled (i.e., heavy) across a set of calibrant levels.[4,5] The absolute concentrations of the 18 target metabolites in the FBS samples were then determined by applying their experimental response ratios (i.e., light vs. spiked-in heavy) to the metabolite-specific standard curves (relative response vs. light concentration plots). This strategy can be readily adopted here as it has been demonstrated previously to be robust in precisely quantifying endogenous compounds in varying sample matrices. An example collection of quantitative results for a target metabolite is illustrated in Figure 3.

Overall, the curves demonstrated excellent linearity (R^2 >0.999), with a dynamic range approximating four orders of magnitude. The metabolite XICs in the control and experimental sample analyses were Gaussian and interference-free (see Figure 3B example).

That, taken together with the stable performance metrics, provided confidence in the metabolite concentrations derived from the FBS samples. The quantitative results for an example metabolite in the experimental FBS samples is shown in Figure 3C, while Figure 4 plots a collection of metabolite quantitations in the pooled FBS.

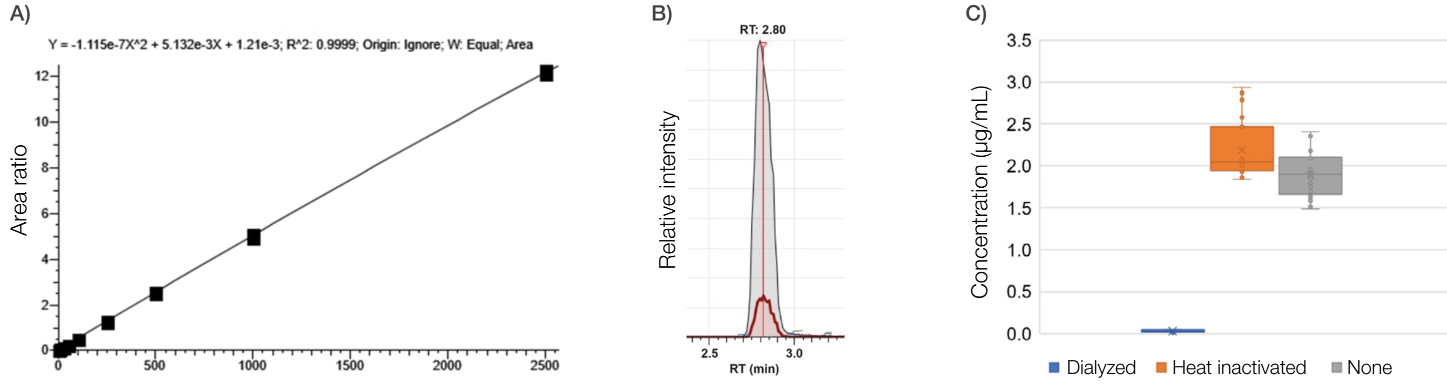

Figure 3. Vitamin B_3 results in the control and experimental FBS samples. Standard control curve in (A), XIC metabolite overlay of an unprocessed FBS sample in (B), and determined metabolite concentrations in the FBS samples in (C).

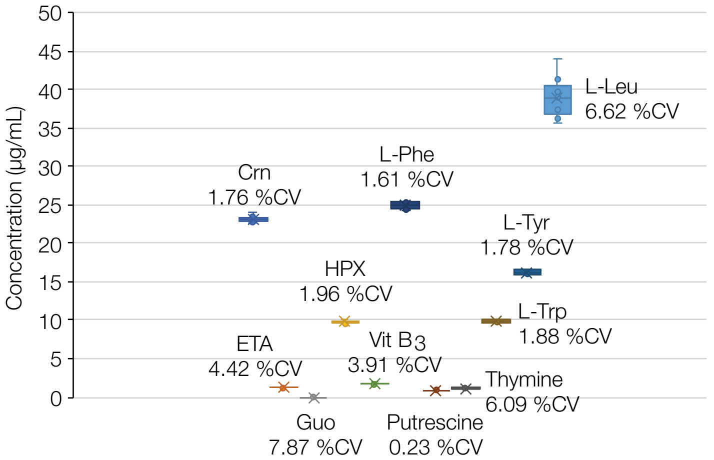

Figure 4. Example collection of metabolite concentrations determined in the pooled QCs. Targets were measured by HILIC-MS (ESI+).

As demonstrated by Figure 4, reproducible quantitation was observed across the replicate measurements of pooled QCs (predominantly <5% CV). This adds further credence to the consistency in the instrument platform performance and the robustness of the labeled standard mixture. In terms of the processing techniques, the dialyzed samples delivered substantially lower endogenous concentrations compared to the heat-inactivated and unprocessed samples (see Figure 3C example), which could impact the results of cell culture applications. This finding was replicated in the targeted ESI- mode and in the untargeted MS/MS data (e.g., putatively annotated urea). While these results indicate general consistency between sourced materials for a given processing method, as evidenced by PCA clustering plots (data not shown), retrospective mining of the profiling data did, however, reveal metabolic differences in FBS samples according to material origin (e.g., putatively annotated uridine).

The untargeted data additionally enabled biochemical pathways, such as choline synthesis, to be evaluated. Detected in the choline biosynthesis pathway was L-serine and choline along with a collection of its metabolic intermediates (e.g., ethanolamine, *N*-monoethanolamine, *N*-dimethylethanolamine). Due to the presence of isotopically labeled ethanolamine in the QReSS mix, ethanolamine could be confidently identified, while the remaining metabolites were putatively assigned based on Thermo Scientific™ mzCloud™ spectral matching. Figure 5 illustrates the variation in the response of these pathway metabolites between groups of FBS sourced materials treated without preprocessing.

Interestingly, the end points of this choline synthesis pathway were found to provide lower variability than its intermediates (i.e., ethanolamine, *N*-monoethanolamine, and *N*-dimethylethanolamine). Stemming from this observation, evaluating metabolite levels in FBS, and other media, may be useful in better understanding cell growth rates and their impacts.

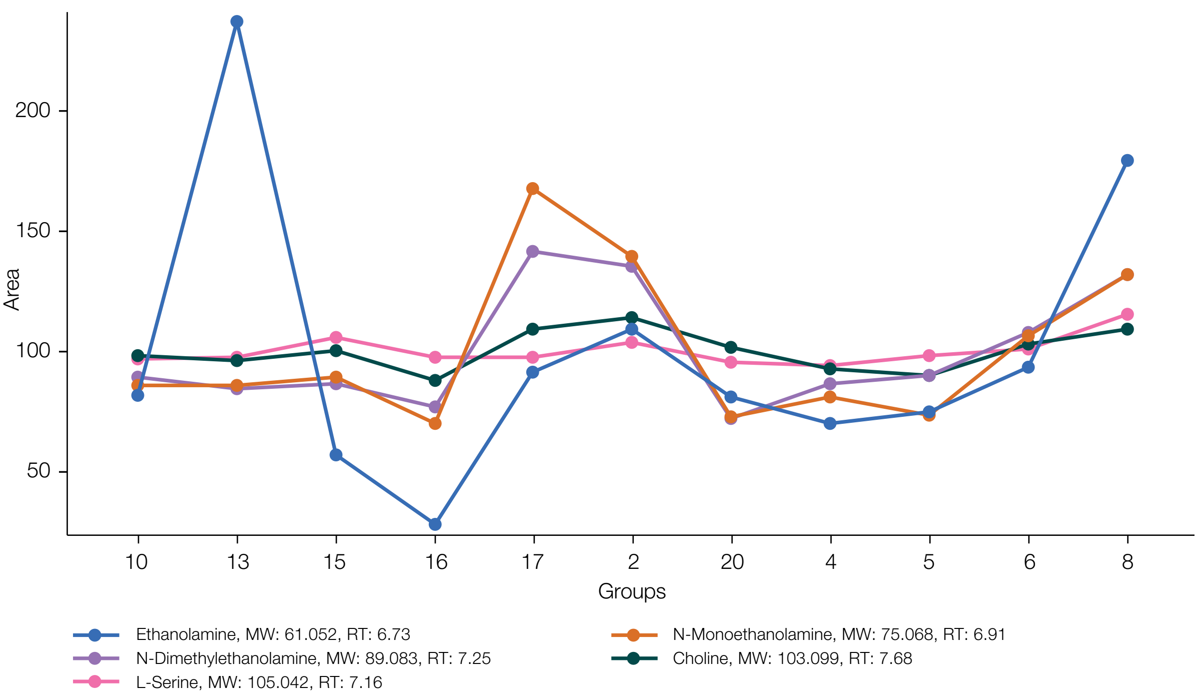

Figure 5. Results of unprocessed FBS samples for metabolites in the choline synthesis pathway.

Conclusions

Reliable QC practices are imperative to maximizing the quality of metabolite profiling and quantification data. In this study, the QReSS metabolite mixtures were applied to FBS samples to assess performance efficiencies and to obtain qualitative/quantitative information on FBS-pertaining metabolites. The standard mixture demonstrated exceptional performance throughout the measurements, while enabling reproducible quantitation of target compounds in the control and experimental samples. The quality of the LC-MS performance was also found to be conserved between targeted and untargeted MS analyses. The endogenous metabolite levels in the FBS were demonstrated to be dependent on the applied processing protocol and country of origin the FBS was sourced from, outcomes of which should be factored when using this growth medium in *in vitro* metabolomic applications.

References

1. Lippa, K.A., Aristizabal-Henao, J.J., Beger, R.D., et al. **2022**. Reference materials for MS-based untargeted metabolomics and lipidomics: a review by the metabolomics quality assurance and quality control consortium (mQACC). *Metabolomics, 18(4)*, 24-53.
2. Dudzik, D.; Barbas-Bernardos, C.; García, A.; et al. **2018**. Quality assurance procedures for mass spectrometry untargeted metabolomics. a review. *J Pharm Biomed Anal, 147*, 149-173.
3. Broadhurst, D.; Goodacre, R.; Reinke, S.N.; et al. **2018**. Guidelines and considerations for the use of system suitability and quality control samples in mass spectrometry assays applied in untargeted clinical metabolomic studies. *Metabolomics, 14(6)*, 72-89.
4. Percy, A.J. Borchers, C. **2021**. Detailed method for performing the ExSTA approach in quantitative bottom-up plasma proteomics. *Methods Mol Biol, 2228*, 353-384.
5. Nakayasu, E.S.; Gritsenko, M.; Piehowski, P.D. **2021**. Tutorial: best practices and considerations for mass-spectrometry-based protein biomarker discovery and validation. *Nat Protoc, 16(8)*, 3737-3760.

Ordering information

Description	Unit size	Cat. No
Metabolomics QReSS Standard 1	1 vial	MSK-QReSS1
Metabolomics QReSS Standard 2	1 vial	MSK-QReSS2
Metabolomics QReSS Kit	1 kit	MSK-QReSS-KIT
Metabolomics QReSS Standard 1 (unlabeled)	1 vial	MSK-QReSS1-US
Metabolomics QReSS Standard 2 (unlabeled)	1 vial	MSK-QReSS2-US
Metabolomics QReSS Kit (unlabeled)	1 kit	MSK-QReSS-US-KIT

Please refer to isotope.com for pricing and product-related application information.

Chemical purity (CP) is 98% or greater, unless otherwise indicated.

Learn more at **thermofisher.com/metabolomics**

For Research Use Only. Not for use in diagnostic procedures. © 2022 Thermo Fisher Scientific Inc. All rights reserved. QReSS is a trademark of Cambridge Isotope Laboratories, Inc. All other trademarks are the property of Thermo Fisher Scientific and its subsidiaries unless otherwise specified. **AN001189-EN 0922M**