Supporting Information S1

Cross-laboratory standardization of pre-clinical lipidomics using differential mobility spectrometry and multiple reaction monitoring

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Study Design

For a detailed overview please refer to Figure S1 below. In the second phase of the study, the laboratories were first asked to perform a thorough cleaning of the mass spectrometry system, including the QTRAP 5500 front end and the SelexION device (the full cleaning protocol can be found below). After cleaning, the laboratories were asked to run a SelexION tuning check and a standard Lipidyzer system suitability test (SST) (details about the SST can be found below). Next, the instrument was benchmarked to ensure that the system partaking in the study was performing according to the vendor's specifications (the full benchmarking protocol can be found below). Each laboratory analyzed five replicates of Lipidyzer control plasma standard (Sigma #P9523) with both extraction protocols and five technical replicates of the pooled samples. The results were shared with the central laboratory to confirm that all partners were following the study protocol with equal success.

The third phase involved a full reproducibility study executed over four days within a single week. Each day, five replicate extractions of SRM 1950 plasma were analyzed with both extraction protocols (BD and MTBE). In addition, five technical replicates of pooled samples were run daily to separate the variance introduced by sample preparation and instrumentation. Before sample acquisition, a daily cleaning of the SelexION device and SST were carried out. The remaining plasma samples in the NIST candidate RM 8231 (DB, HTG and AA) were analyzed in five replicates on a single day using the MTBE extraction protocol. Additionally, 59 EDTA plasma samples from individuals with familial hypercholesterolemia were analyzed using the MTBE extraction protocol over two batches in three participating laboratories. Once the central laboratory received all the data files, the final data analysis phase was started using R (version 3.62) and Microsoft Excel.

Samples

The Metabolomics Quality Assurance and Quality Control Program at NIST provided both SRM 1950-Metabolites in Frozen Human Plasma and candidate RM 8231 Frozen Human Plasma Suite for Metabolomics (HTG, DB, and AA plasma). All NIST plasma samples were collected after informed

consent under approved Institutional Review Board (IRB) protocols reviewed by the NIST Human Subjects Protection Office. The preparation of SRM 1950 has been previously detailed[1]. Briefly, Bioreclamation, Inc. (Hicksville, NY, USA) collected whole blood from 100 donors (1:1 male-to-female ratio) after a 72 h abstention from medication and an overnight fast. Donors were between 40 and 50 years of age. The anticoagulant used was lithium heparin. The collected blood was centrifuged at 8000 × g for 25 min at 4 °C to obtain plasma. All donor plasma was blended and then aliquoted into individual vials to produce the SRM. The candidate RM 8231 diabetic plasma material was produced by Solomon Park Research Laboratories, Inc. (Kirkland, WA, USA). Whole blood was collected from 5 male donors and 6 female donors after an overnight fast. Donors were between 34 and 68 years of age. Each donor met the specified ranges for glucose (>126 mg dL−1) and triglycerides (<150 mg dL−1). Solomon Park Research Laboratories, Inc. also produced the candidate RM 8231 HTG plasma material. Whole blood was collected from 11 male donors after an overnight fast. Donors were between 31 and 72 years of age. Each donor met the NIST-specified ranges for glucose (<100 mg dL−1) and triglycerides (>300 mg dL−1). Both the diabetic and high triglyceride plasma were obtained by centrifuging the respective collected blood at 251 rad s−1 for 10 min, followed by additional centrifugation at 397 rad s−1 . The anticoagulant used was lithium heparin. Plasma from each donor was thawed once and then blended to make the respective donor pools before aliquoting into individual vials. The candidate RM 8231 AA plasma was acquired from BioreclamationIVT (Westbury, NY, USA). Whole blood was collected from 16 donors (8 males and 8 females) after an overnight fast. Donors were between 20 and 25 years of age. K2 ethylenediaminetetraacetic acid (EDTA) was used as the anticoagulant. The 16 units of blood were subsequently thawed and pooled by Solomon Park Research Laboratories, in accordance with procedures used with the diabetic and high triglyceride plasma.

The 59 plasma samples from statin-treated individuals with familial hypercholesterolemia (FH) and cardiovascular disease were provided by the Erasmus MC, Rotterdam, The Netherlands. This cohort has been described in detail elsewhere[2]. In brief, a mutation in the LDL receptor gene was identified in all individuals. A total of 19 individuals had a history of myocardial infarction, 20 patients were asymptomatic but had severe coronary stenoses, and 20 asymptomatic and with low atherosclerotic burden. This is an example of a cohort in which the residual cardiovascular risk can be studied to identify novel targets for additional treatments.

Calculation of consensus values and associated uncertainties

The consensus value was calculated as the median of the site means without removing outliers. The associated standard uncertainty of consensus value was calculated as $u = \sqrt{(\pi/2m)} \times 1.483 \times MAD$, where u, m and MAD denote standard uncertainty, number of sites, and median absolute deviation of the sites means, respectively. Further, the usefulness of consensus values was evaluated by calculating the sample coefficient of dispersion (COD) expressed as the percentage ratio of standard uncertainty to MEDM for each lipid, which is analogous to the sample coefficient of variation[3]. For a consensus value of a lipid to be considered valid, COD > 20% was set as an exclusion criterion i.e., COD value less than or equal to 20% were deemed acceptable. The final consensus concentration values were compared with the concentrations published previously by the LIPID MAPS consortium and Bowden et al[4, 5].

Experimental Section

The Lipidyzer Platform analysis

The Lipidyzer platform consists of a SCIEX QTRAP 5500 mass spectrometer equipped with a SelexION differential ion mobility (DMS) interface and Nexera X2 UHPLC-system operated with Analyst software. Each sample was analyzed using multiple reaction monitoring (MRM) in two consecutive flow injection analysis (FIA) runs with positive and negative polarity switching (MRM, internal standard concentrations, assignment and MS-DMS settings can be found in Table S1 in Supplementary Materials S2). In the first run, PC, LPC, PE, LPE, and SM were separated with the SelexION DMS cell using field asymmetric ion mobility mass spectrometry after ionization in the Turbo V source of the mass spectrometer. 1-propanol was added to the curtain gas as chemical modifier to improve DMS separation[6]. In the second run, FFA, TAG, DAG, CER, DCER, LCER, HCER, and CE were measured with the DMS-cell switched off. 50 µL of the reconstituted samples were injected using a Shimadzu SIL 30AC autosampler into the running solvent (10 mM ammonium acetate in dichloromethane: methanol (50:50, vol/vol)) pumped at an isocratic flow rate of 7 µL/min by a Shimadzu Nexera LC30 system. After six minutes, the flow rate was ramped to 30 µL/min for two minutes to allow for washing.

Lipidomics Workflow Manager (LWM) software, written explicitly for the Lipidyzer platform, was used for automated data acquisition, processing, and reporting. The software accurately quantifies lipid species using 54 deuterated IS, developed with Avanti Polar Lipids, that cover 10 major lipid classes present in plasma/serum[7]. The IS assignment and their respective concentrations are described in Supplementary Table S1 and elsewhere[8]. Most lipid classes have multiple IS, with verified fatty acid composition, to minimize the influence of fatty acid chain length variation and degree of unsaturation for accurate quantification. The software calculates a particular lipid species concentration as an average intensity of the analyte MRM, divided by the average intensity of the most structurally similar IS MRM, multiplied by its concentration in nmol/mL. Finally, the software reports concentration in nmol/g, assuming 1 mL of plasma is equal to 1 g.

System suitability test

An ampoule from the SST standard kit (Sciex #5040407) is opened and three solutions were prepared. 0.01 mL SST standard was diluted with 0.99 mL sample running buffer (see below) to prepare a QC_LOD solution. Additionally, two vials with 0.05 mL SST standard diluted with 0.2 mL sample running were prepared to obtain two QC_RSD solutions. QC_RSD solutions were only used for the comprehensive test performed after complete cleaning of the instrument at the start of the project. QC_LOD solution was used for both the comprehensive and quick SST tests. Quick SST test was performed on each day before running samples. One injection of blank sample (sample running buffer) and QC_LOD, and multiple injections of QC_RSD were conducted (with the DMS ON method only), to measure the limit of detection (LOD) and relative standard deviation (RSD). The software reports a simple PASS or FAIL based on meeting a threshold cutoff in RSD and counts per second (cps) for 20 scans collected. Both, quick and comprehensive SST are carried out following the instructions of the Lipidyzer workflow manager.

Instrument cleaning and benchmarking

Front End Cleaning of QTRAP 5500 System

Cleaning of the QJet Ion Guide

1. Use SCIEX Concentrated Powder Instrument Cleaner (P/N 5045687) for cleaning. Prepare a solution of cleaning solution with ~ 1 packet per 1.5 L in deionized water.

2. Wear clean neoprene or powder free nitrile gloves at all times. Replace gloves after using the cleaning solution and before the rinse steps to ensure no soap is left behind.
3. Vent the QTRAP 5500 system and remove the QJet Ion Guide and place it on a clean, stable surface.
4. Remove the O-ring from the rear of the QJet. Remove the two hex screws holding on the IQ0 lens. Then remove the c-clip to release the IQ0 lens (using small needle nose pliers or forceps).
5. Soak the QJet and IQ0 lens and soak or rinse well with isopropanol.
6. Rinse well with water.
7. Immerse the cleaning brush in the cleaning solution then insert the brush into the center of the QJet ion guide to clean the inside of the rods. Rinse with clean flowing water. Repeat at least 3 times.
8. Soak a cleaning swab in cleaning solution and use to scrub both sides of the IQ0 lens. Rinse with water. Repeat at least 3 times with a new cleaning swab each time.
9. Change gloves.
10. Pour MilliQ water over and through the QJet ion guide for one minute, to ensure all soap has been washed away.
11. Pour MilliQ water over and through the IQ0 lens guide for one minute, to ensure all soap has been washed away.
12. Use clean dry air (CDA) or nitrogen to dry the QJet ion guide. Be sure to remove all droplets.
13. Reassemble the QJet.

Cleaning of the Orifice Plate

1. Soak cleaning swab with cleaning solution.
2. Wipe both back and front of orifice plate thoroughly, rinse then dispose of the swab.
3. Repeat 3 times.
4. Change gloves.
5. Pour MilliQ water over both sides of orifice plate for one minute, to ensure all soap has been washed away.
6. Use clean dry air (CDA) or nitrogen to dry. Be sure to remove all droplets.

Cleaning of the SelexION Cell

1. Remove the stainless-steel plates from the ceramic plate and place them on a clean, stable surface.
2. Sonicate with isopropanol or 50:50 methanol: dichloromethane. Then rinse with water.
3. Soak cleaning swab with cleaning solution.
4. Scrub the flat surfaces of the cell thoroughly for one minute with the soaked swabs.
5. Repeat at least 3 times, or until the surfaces look clean.
6. Change gloves.
7. Pour MQ water over the plates for one minute. Make sure all soap has been rinsed away.
8. Sonicate in beaker for 15 minutes in high purity water (or deionized water that is high purity).
9. Use clean dry air (CDA) or nitrogen to dry the plates. Be sure to remove all droplets. Reinstall the plates.

Cleaning of Curtain Plate

1. Dampen a lint-free wipe with HPLC water and wipe down the front of the curtain plate.
2. Using 50:50 methanol: dichloromethane, dampen a lint-free wipe and wipe down the front of the curtain plate.

3. Repeat steps 1 and 2 until curtain plate is clean.

SelexION Tuning Check

After installing the DMS device, check the performance using the SelexION Tuning test for Lipidyzer. Follow the vendor protocols and ensure that it is passing the vendor's specifications. Save the results and ensure good separation is observed between the lipid classes.

Daily Quick Cleaning of SelexION Device

During the study, a quick clean of the DMS cell is performed at the end of each batch.

1. Remove the DMS cell.
2. Place over 500 mL beaker with the plates facing down.
3. Under a fume hood, pour 150 mL of 50:50 dichloromethane: methanol down the center of the cell.
4. Swirl around to remove excess and use clean dry air (CDA) or nitrogen to dry.
5. Be sure the cell is completely dry before reinstalling.

Sample Preparation Protocols

Chemicals and Consumables

Chemicals & Solutions	**P/N number (examples)**	**Company (example)**
Methyl tert-butyl ether **HPLC grade** (MTBE)	34875-1L	Honeywell
Methanol **LC-MS grade** (MeOH)	34966-2.5L	Sigma Aldrich
Water **LC-MS grade** (H_2O)	14263-1L	Sigma Aldrich
Lipidyzer running buffer	*See SCIEX Lipidyzer manual*	*n/a*
Lipidyzer internal standards	*See SCIEX Lipidyzer manual*	*n/a*
Lipidyzer Plasma Standard	*SCIEX PN 4386703*	
NIST reference Plasma	*SRM 1950* *Candidate reference materials 8231*	*NIST*

Consumables	**P/N Number**	**Company**
Certified Screw cap vials, clear 100/ PK	5182-0714	Agilent
Certified Blue screw caps 100/ PK	5182-0717	Agilent
Vial inserts, 250 µL glass with polymer feet	5181-1270	Agilent

2.0 mL Safe-Lock tubes, pcr clean.	0030 123.344	Eppendorf AG
Dichloromethane, HPLC, >99.8%, amylene as stabilizer	34856	Sigma Aldrich
Disposable Borosilicate Glass Tubes with Plain End (16 x 100 mm)	14-961-29	Fisher
Lipidyzer Platform Internal Standards Kit	P/N 5040156	SCIEX

Preparation of Internal Standard Kit

1. Take internal standards kit for Lipidyzer Platform out of freezer and allow them to warm up at bench top (~30 minutes).
2. Prepare thirteen 2mL threaded vials with PTFE lined caps. Alternatively, the proven standard tools of a lab can be used
3. Vortex each ampule in internal standard kit and shake liquid down to bottom of ampule (spin briefly if necessary).
4. Open ampule and transfer entire volume to 2 mL vial with new glass Pasteur pipette; discarding each pipette after use. Alternatively, the proven standard tools of a lab can be used. Cap vial immediately
5. Transfer the ampule label with the standard info and lot number on it to corresponding vial.

Preparation of Internal Standard Mixture

1. Within Lipidyzer software workflow, input or select standard mix with appropriate standard lot number and number of samples and it will suggest IS volumes assuming 100 µL of plasma will be processed. For this study, only 25 µL of plasma will be used, so divide actual sample number by 4 in the Lipidyzer Workflow Manager software.
   Note: The LWM software will add 5 extra samples (20 samples worth of 1:4 standard) to any sample number under 10. As such, it is worthwhile to simply calculate the 'per sample' volumes needed for each standard on your own and create a cocktail for your sample number + 20%.
2. Lay out the 13 internal standards, appropriate Hamilton syringes, glass tube or vial for standard mix and wash beakers containing 50:50 methanol: chloroform or DCM in a fume hood. Alternatively, the proven standard tools of a lab can be used
3. Transfer appropriate volumes of standard to standard mix tube, washing syringe 6-7 times between each transfer. Minimize time internal standard stocks are open to reduce evaporation.
4. Dry down standard mix in evaporator
5. Resuspend in appropriate solvent (methanol/DCM for B&D extraction, methanol/MTBE for MTBE extraction). Use final volume prescribed in LWM software workflow.
6. Aliquot 25 µL (rather than 100 µL) per sample for 1:4 standard.

Preparation of Lipidyzer Plasma Standard

1. Resuspend the dried plasma standard in 5 mL of high grade water.
2. Vortex well to resuspend.
3. Aliquot into 10× 500 µL and freeze for the study.

Bligh-Dyer (BD) Extraction Protocol

1. Add the following to a 16 x 100 mm (or 16 x 125 mm) glass culture tube
    a. 25 µL plasma sample (or standard plasma)
    b. 975 µL H_2O
    c. 900 µL of CH_2Cl_2 (DCM)
    d. 2 mL of MeOH
2. Vortex lightly after all are added
3. Add 25 µL of the Internal Standards (I.S. as prepared above), vortex lightly again and allow to sit in the monophase for 30 min at room temperature (RT)
4. After 30 min add 900 µL of CH_2Cl_2 followed by 1 mL of Water and quickly vortex. This will break the monophase and you will have three layers. The **lower** layer is the DCM and contains the lipids.
5. Centrifuge for 10 min at 2000 g @15 °C or higher
6. Remove the DCM layer using a glass Pasteur pipette and transfer to a clean glass test tube (16 x 100 mm). For optimum extraction add 1.8 mL of CH_2Cl_2 to the aqueous layer, vortex and extract a second time. Add the CH_2Cl_2 layer to the first layer and dry using a nitrogen drying system (i.e. TurboVap).
7. When dried, add 250 µL of running buffer (for preparation of the running buffer, see protocol provided by the Lipidyzer) and transfer to an Agilent sample vial with a glass insert (alternative vials with proven performance can also be used). If necessary the samples can be centrifuged before analysis.
8. Analyze using the Lipidyzer methods.

MTBE Extraction Protocol

Of note: based on Matyash et al.[9] with small modifications taking the experiences made by all participating laboratories into account.

1. Add the following to a 2 mL Eppendorf PCR clean plastic tube
    a. 25 µL plasma sample (or standard plasma)
    b. 75 µL LC-MS grade water
    c. 25 µL IS Lipidyzer mix in MTBE (as prepared in section above).
    d. 575 µL MTBE
    e. 160 µL MeOH
2. Vortex all and shake in the monophase at room temperature for 30 minutes
3. Add 200 µL water to the tube, this will break the monophase and you will have three layers. The pellet are the proteins and other insoluble matter, the lower layer is the aqueous phase and the **upper** is the MTBE and contains the lipids.
4. Centrifuge 3 minutes at 12.000 xg (or higher), at RT.
5. Take the upper layer and transfer to an Agilent glass vial using Pasteur pipette. Alternatively, the proven standard tools of a lab can be used.
6. Take the PCR tube and repeat the extraction:
    a. Add 300 µL MTBE
    b. Add 100 µL MeOH
    c. Add 100 µL H_2O
    d. Shake 5 min at room temperature
    e. Centrifuge 3 minutes at 12.000 xg (or higher), at room temperature.
7. Take the upper layer and combine with the previous extract using a Pasteur glass pipette, this makes approximately 800 µL in total. Alternatively, the proven standard tools of a lab can be used.

8. Evaporate the upper layer under a gentle stream of nitrogen (e.g Turbovap).
9. When dried, add 250 µL of Lipidyzer running buffer (for preparation of the running buffer, see protocol provided by the Lipidyzer).
10. Check if an extra spin down step is necessary, samples should be very clear and clean before injecting them.
11. Transfer to an Agilent glass vial with insert, then analyze on Lipidyzer.

Preparation of pooled samples (technical replicates)

1. Remove 80 µL from each extraction (BD and MTBE) to create a pooled sample of 800 µL each day. Then, aliquot 140 µL from this pool into 5 separate vials for replicate injections.

Translation of Lipidyzer annotations to LIPID MAPS shorthand annotation

Lipidyzer abbreviation (examples)	LIPID MAPS shorthand annotation according to[10, 11]
CE(14:0)	CE 14:0
CER(18:0)	Cer d18:1/18:0
DAG(12:0/16:0)	DG 12:0_16:0
DCER(16:0)	Cer d18:0/16:0
FFA(12:0)	FA 12:0
HCER(16:0)	HexCer d18:1/16:0
LCER(16:0)	LacCer d18:1/16:0
LPC(16:0)	LPC 16:0
LPE(16:0)	LPE 16:0
PC(16:0/18:0)	PC 16:0_18:0
PE(16:0/18:0)	PE 16:0_18:0
PE(O-16:0/18:0)	PE O-16:0/18:0
PE(P-16:0/18:0)	PE P-16:0/18:0
SM(14:0)	SM d18:1/14:0
TAG36:0-FA12:0	TG 36:0-FA 12:0

Table S-1A: Translation of annotations to LIPID MAPS shorthand annotation

Supplementary Figures

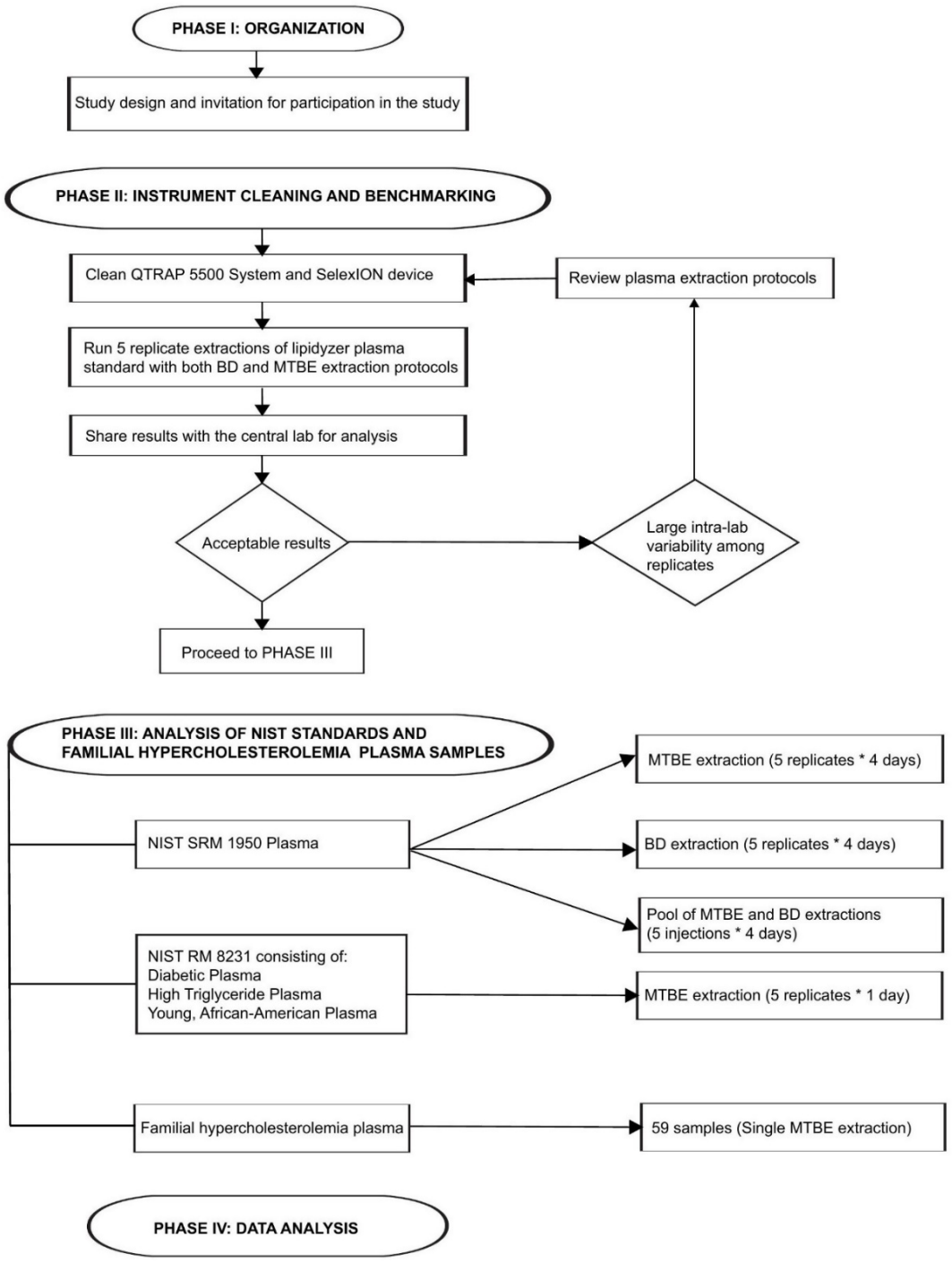


Figure S-1 Complete overview of study design and workflow of the inter-laboratory comparison study

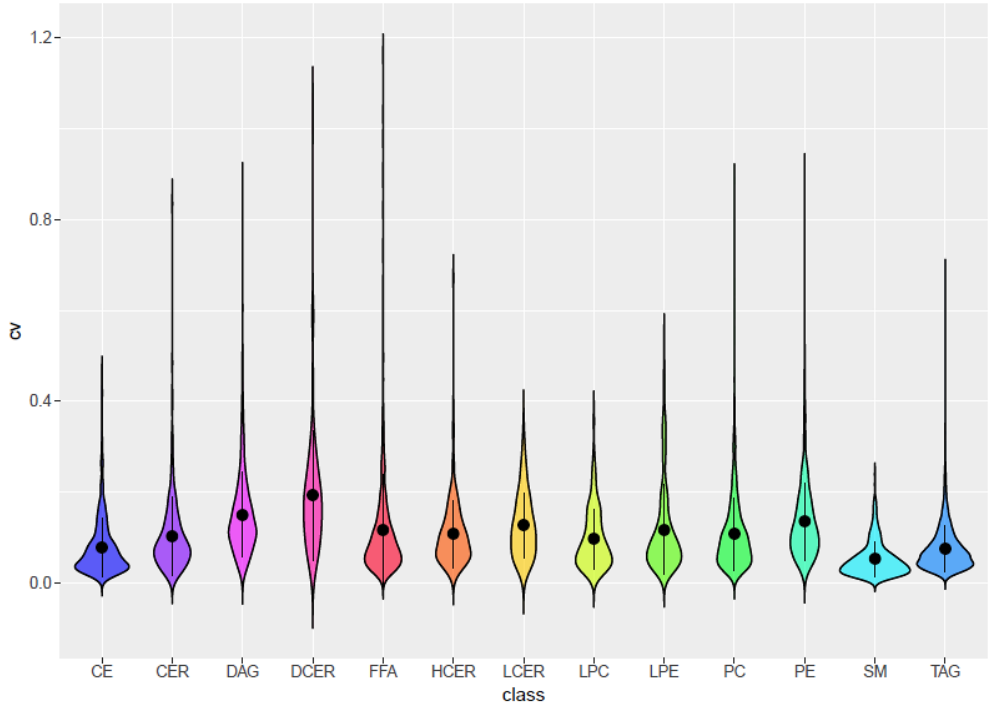


Figure S-2 CV distribution along 13 lipid classes measured with MTBE extraction method

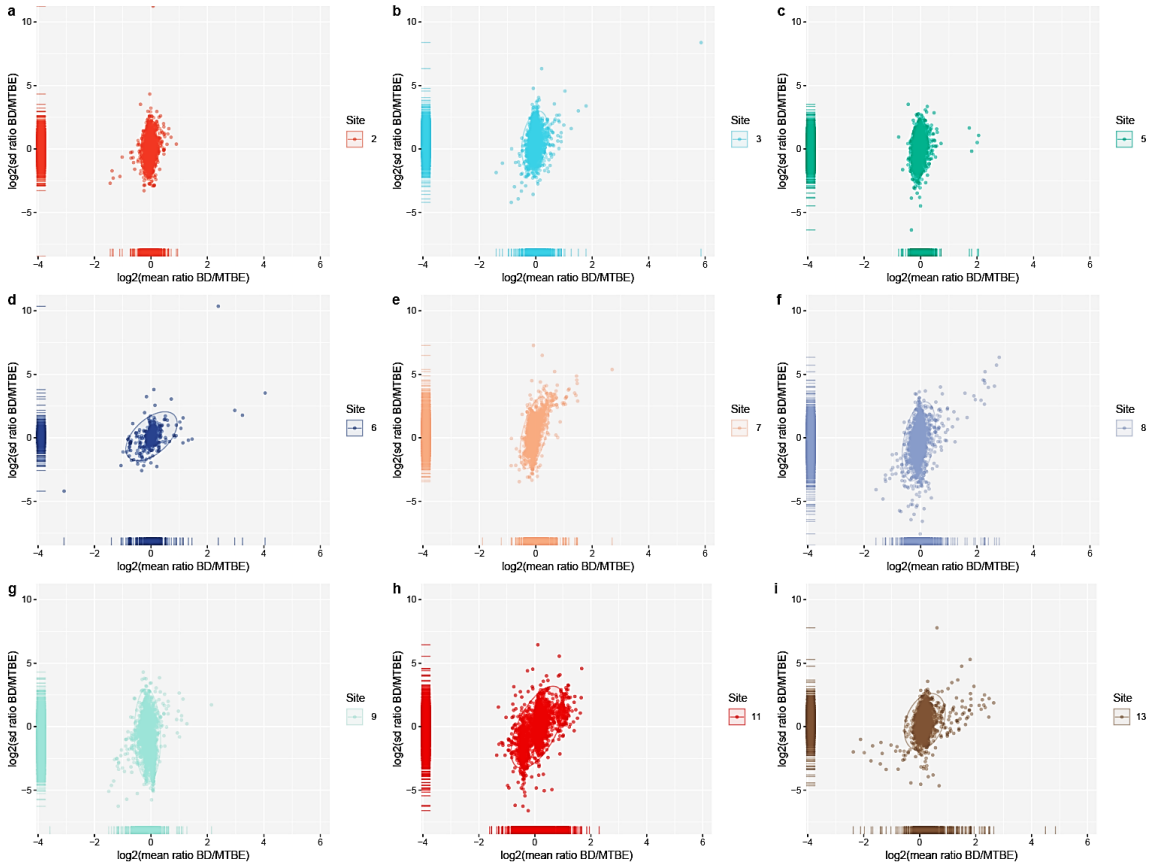


Figure S-3 Scatter plot to summarize the performance of each laboratory between the methods. x-axis represents ratio of mean concentration of BD method to MTBE method in base-2 logarithmic scale and y-axis represents ratio of standard deviation of two methods in base-2 logarithmic scale.

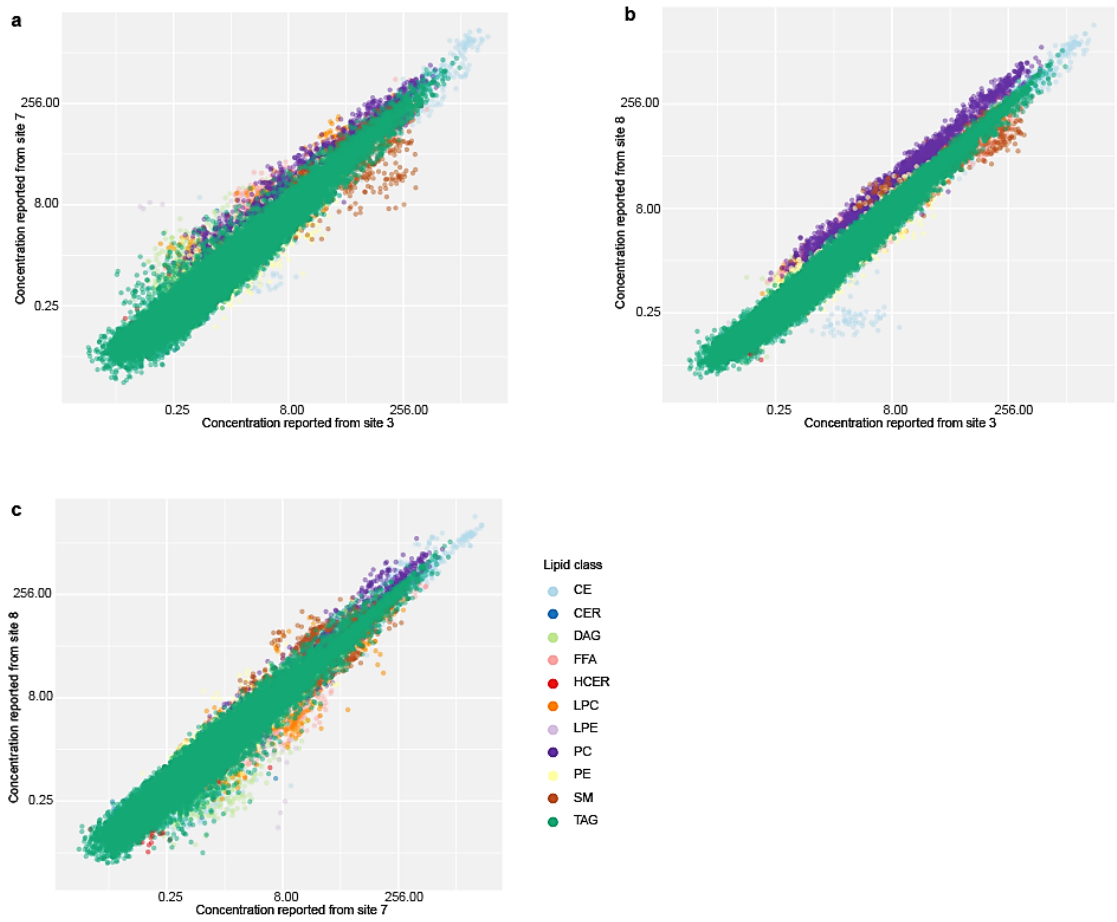


Figure S-4 Correlation of reported concentration of lipids including TAG lipids in 59 plasma samples related to familial hypercholesterolemia between three sites.

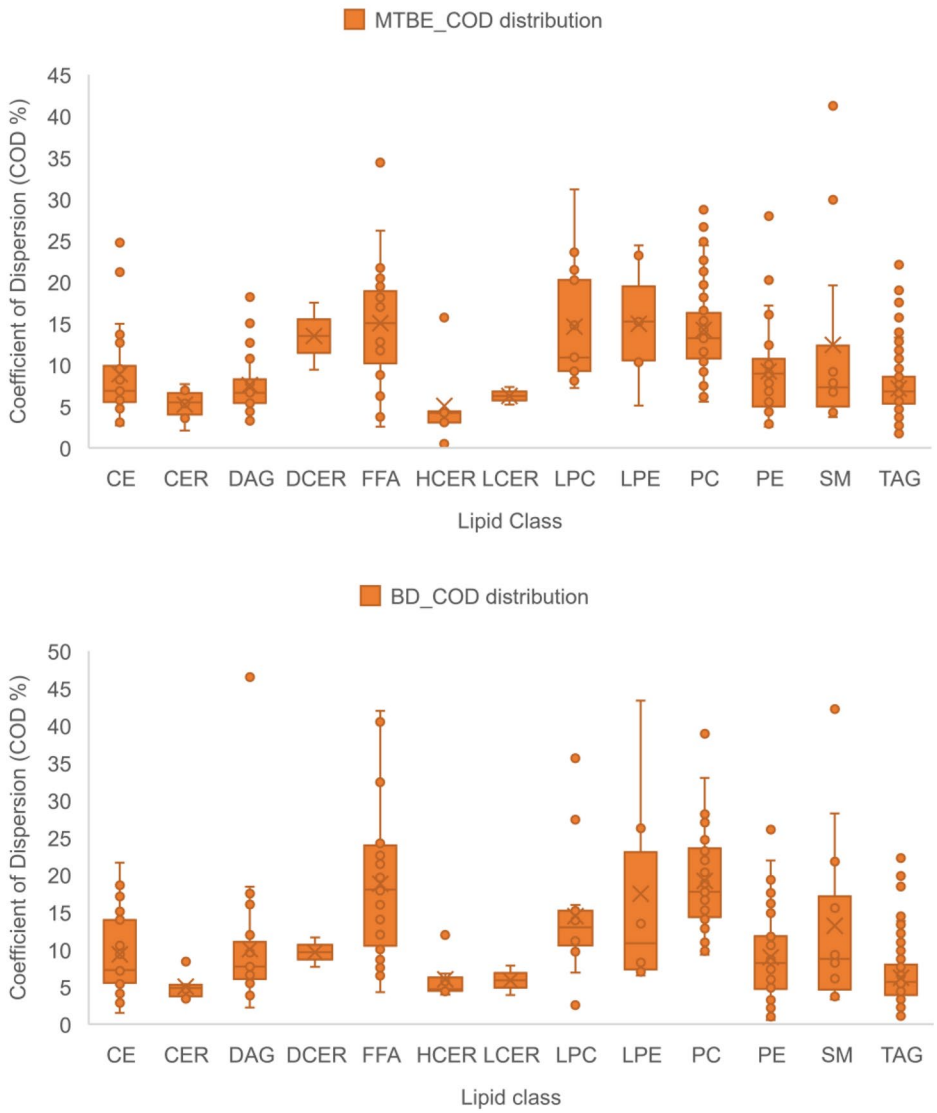
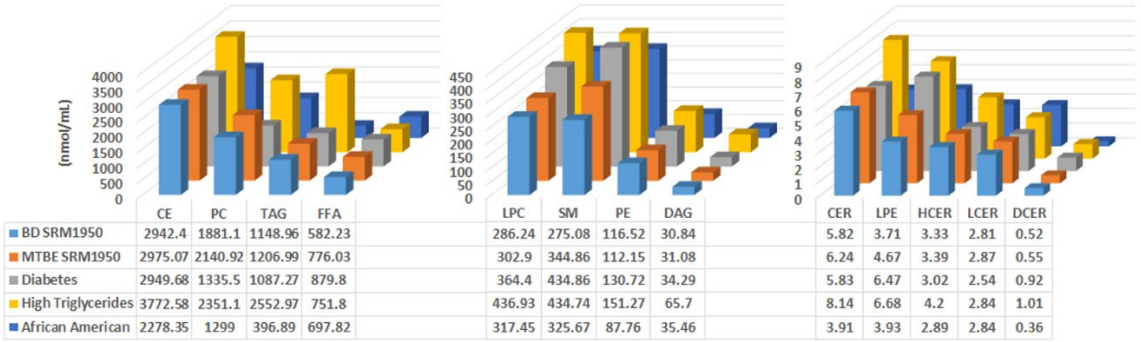


Figure S-5 Comparison of Coefficient of Dispersion (COD) distribution among 13 lipid classes between BD and MTBE lipid extraction methods.



	CE	PC	TAG	FFA	LPC	SM	PE	DAG	CER	LPE	HCER	LCER	DCER
BD SRM1950	2942.4	1881.1	1148.96	582.23	286.24	275.08	116.52	30.84	5.82	3.71	3.33	2.81	0.52
MTBE SRM1950	2975.07	2140.92	1206.99	776.03	302.9	344.86	112.15	31.08	6.24	4.67	3.39	2.87	0.55
Diabetes	2949.68	1335.5	1087.27	879.8	364.4	434.86	130.72	34.29	5.83	6.47	3.02	2.54	0.92
High Triglycerides	3772.58	2351.1	2552.97	751.8	436.93	434.74	151.27	65.7	8.14	6.68	4.2	2.84	1.01
African American	2278.35	1299	396.89	697.82	317.45	325.67	87.76	35.46	3.91	3.93	2.89	2.84	0.36

Figure S-6 Breakdown of plasma lipidome of NIST plasma standards representing different metabolic health states and ethnicities.

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