COVID-19-MSC Metabolomics and Lipidomics Protocol

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This protocol covers the process from blood collection to dried sample for storage, and includes indicative protocols for subsequent LC-MS/MS analysis. Sample preparation yields sufficient samples for > 5 analytical replicates.

Overall protocol flow:

1. Blood collection, transport, processing.
2. Quenching to inactivate any active virus and generate aliquots.
3. Sample processing and drying.
4. LC-MS/MS analysis

Protocol 1: Blood collection, transportation and centrifugation

Step 1 timing: ~1-1.5h phlebotomy, < 6 h including sample transport.

This sample collection protocol can also be used for sample collection other analysis (e.g. immunological analysis).

The protocol is a recommended protocol. Use locally approved and implemented protocols if necessary.

***Materials and equipment for step 1**:*

- Vacutainer for plasma, non-gel containing, e,g Red cap (plain) BD Biosciences.
- Locally used phlebotomy equipment
- Centrifuge, refrigerated box
- PPE: lab coat or gown, gloves, goggles where there is risk of splash/aerosol generation.

Notes:

This work should be conducted in a BSL-2 hood using protocols approved for SARS-CoV2 as sample inactivation has not taken place.

Samples should only be stored in a properly designated freezer.

Method for protocol 1:

1. Collect venous blood for metabolomics/lipidomics analysis in 3 mL plasma vacutainers, e.g..red cap BD Biosciences. **Do not** use SST (gel containing) tubes.
2. Fill tube if possible.
3. Invert the tube and back gently 8-10 times immediately after blood collection.
4. Wipe the blood tube down with alcohol or Distel wipe.
5. Record time and date at which sample taken (on the blood form and tube).
6. Record ISARIC patient ID (on the tube)
7. Double bag and put the tube in a refrigerated cool box for transport to the processing laboratory.
8. Ensure transport box is labelled with COVID-19.
9. Wipe down box and any surfaces used with distel/alcohol wipes.
10. On arrival, wipe tubes down with distel/alcohol wipe, check samples are undamaged and appropriately labelled and match the number and type of samples expected.

11. If time between sample collection and processing is < 30 minutes, allow tubes to stand at room temperature until 30 minutes from collection has passed to allow full clotting of blood.
12. If time between sample collection and processing is greater than 6 hours, continue to process but clearly label sample record sheet with time delay.
13. Centrifuge the tubes at 1,600 g for 10 mins at 4°C. This must use either buckets with lids or a centrifuge with an inner lid. If centrifuge not within a BSL-2 hood then leave centrifuge for 10 mins before opening for any aerosol to settle.
14. Remove tubes and wipe down tubes and buckets and centrifuge drum.
15. Decant serum into labelled storage tube and store at -80 °C until a batch is ready for processing.
16. If there are signs of excessive lipidemia (cloudiness) or hemolysis (pink colour to solution), or other sample abnormalities, note this on the sample sheet.

Risk mitigation: Treat all samples as infectious until quenching is complete and use a biosafety cabinet (at least BSL-2) for the entire process. All surfaces and equipment must be wiped down with Distel wipe or EtOH after use. Use PPE recommended for handling infectious biofluids.

If a spill occurs on transport perform a local risk assessment before proceeding.

If tube breaks in centrifuge, put lid back on, leave for 30 mins to settle. If in bucket, transfer bucket to BSL-2 hood. Remove samples in BSL-2 hood and clean. Discard broken tube. Autoclave the bucket.

Protocol 2: Preparing tubes for aliquoting.

Step 2 timing: depends on sample numbers.

Materials and equipment for protocol 2:

- 2 mL capped centrifuge tubes, high grade plastic with low extractable/leachable content (e.g. Eppendorf brand).
- 2 mL screw cap glass vials with PTFE faced septa, preferably pre-washed (e.g. Supelco catalogue number 27339).
- Solvent and -80 °C freezer resistant labelling method (preferably machine printed, e.g. LabTAG XyliTUFF).

Note: a solvent and -80 °C freezer resistant labelling method must be used for labelling tubes and vials.

Method for protocol 2:

1. Label 3 x 2 mL microcentrifuge tubes per sample for extraction lipidomic/metabolomic analysis, including unique sample identifier and aliquot number 1 - 3.
2. Label 2 x 2 mL microcentrifuge tubes per aliquot sample (6 per patient sample) for storage for metabolomic analysis, including a unique sample identifier and designation for metabolite analysis.
3. Label 2 x 2 mL glass vials per aliquot sample (6 per patient sample) for storage for lipidomic analysis, including a unique sample identifier and designation for lipid analysis.
4. Label 1 x 2 mL microcentrifuge tubes per sample for Biocrates analysis.
5. For each batch of lipidomics/metabolomics samples label 2 x 2 mL tubes identical to those being used for the samples as “PBS standard”.
6. There may be serum left over after processing, and this should be stored at -80 °C for any future analysis. Additional tubes may need to be prepared for this if the serum cannot be stored in the original containers (e.g. if they are snap cap).

Protocol 3: Lipid and Metabolite extraction

This is adapted from widely used Folch extraction method and is based on the mPLEX method (Nakayasu et al, mSystems 1(3):e00043-16,doi: 10.1128/mSystems.00043-16) which has been shown to inactivate SARS-CoV virus.

Protocol 3 timing: sample and drying method dependent. Processing and drying in a vacuum centrifuge of 10 samples take approximately 1 hour. Increasing numbers extend this, but not linearly.

Materials and equipment for protocol 3:

All chemicals should be of the highest grade available, and solvents must be HPLC grade or better (Preferably LCMS grade). ***Do not*** *use autoclaved plastics for any part of the procedure. Solvents, and samples once methanol has been added, should not come into contact with autoclaved plastics. Use glass or high quality plastics (e.g. Eppendorf brand).* ***Do not*** *use glassware that has been detergent washed. Detergents can severely compromise lipidomics experiments.*

- 100 % Methanol, mass spectrometry grade (e.g. Fisher Scientific Optima LC/MS grade, Honeywell CHROMASOLV LC-MS)
- Water, mass spectrometry grade (e.g. Fisher Scientific Optima LC/MS grade, Honeywell CHROMASOLV LC-MS).
- HPLC or better grade chloroform, ethanol stabilized (e.g. Merck HPLC Plus)
- Glass reservoir for solvents for dispensing, not detergent washed. This glassware should be dedicated for this purpose, and pre-soaked in the solvent.
- Avanti SPLASH® Lipidomix catalogue no: cat no: 330707 (or equivalent lipid standards).
- Pipette, 100 uL for serum aliquots.
- Filtertips for serum aliquoting.
- Phosphate buffered saline
- Positive displacement pipette (e.g. Gilson Microman E M250E) or glass gastight syringe with > 200 µL capacity for handling chloroform/methanol mix (a normal pipette can be used but great care and some speed is needed to avoid loss of solution.
- High quality (low extractable) positive displacement tips for Microman E if using this (e.g. Gilson CP250).
- Parafilm (or equivalent) strips.
- High quality (low extractable) tips for methanol.
- Chemistry PPE (gloves, lab coat, safety glasses)
- Benchtop microcentrifuge for 2 mL tubes capable of 10,000+ g.
- Fume hood

Centrifugation steps should be conducted at room temperature in a fume hood.

Notes:

This work should be conducted in a BSL-2 hood using protocols approved for SARS-CoV2 up to step 8 as sample inactivation has not taken place.

Exposure to chloroform and methanol vapour should be minimised at all times. Use a mechanically extracted, externally vented fume hood wherever possible.

Chloroform and chloroform methanol solutions are volatile and low viscosity, so use of a normal pipette to handle these is not recommended.

Note:
Steps 4 to 10 must be conducted in a BSL2 hood under COVID19 sample handling conditions.
The virus is inactivated at step 10 and samples can be removed from Cat2(+) containment to a fume hood.
The proteomics samples should be processed at the same time to minimise freeze/thaw cycles (see proteomics sample protocol).

Method for protocol 3:

1. In a fume hood make a sufficient quantity of 2:1 chloroform:methanol solution in a glass screw cap container (e.g. Duran bottle) allowing for at least 500 µL per sample to be extracted, and pre-chill and keep at -20 °C. Avoid contact of solution with cap as much as possible. This is the solvent mix for step 2. (note: Chloroform is volatile and low viscosity, so use of a normal pipette is not recommended).
2. **As short a time as possible before extraction**, in a fume hood add 500 µL (5 x the serum sample volume) of the solvent mix from above and 10 µL Avanti SPLASH® Lipidomix® internal lipid standard to each of the lipidomics/metabolomics sample extraction tubes from protocol 2 step 1, and the PBS standard tubes from protocol 2 step 5. Keep at -20 °C prior to plasma addition.
3. **As short a time as possible before extraction**, add 200 µL (2 x the serum sample volume) of ehanol to each of the Biocrates analysis tubes from protocol 2 step 4. Keep at -20 °C prior to plasma addition.

4. Thaw serum samples on ice.
5. Add 100 µL of each serum sample to each of the 3 tubes for extraction for metabolomics/lipidomics analysis.
6. Add 100 µL of each serum sample to each of the tubes for Biocrates analysis.
7. Add 100 µL of PBS to the PBS standard tubes.
8. Vortex for 30 seconds (an additional 10 seconds sonication can assist extraction).
9. Stand sample on ice for 5 minutes.
10. Vortex for 30 seconds (an additional 10 seconds sonication can assist extraction).

11. Store samples for Biocrate analysis at -80°C or transfer to analysis lab.
12. Centrifuge the metabolomic/lipidomic samples at > 10,000 g for 10 minutes.

13. Carefully remove the majority of the upper phase and divide equally between the 2 fresh 2 mL centrifuge tubes from protocol 2, step 2. There should be around 200 µL of upper phase. This is the aqueous MeOH phase for ***metabolomics***.
    Note: The sample can be further aliquoted at this stage if required. 20 µL aliquots should contain enough metabolite for analysis.
14. Carefully remove the majority of the lower phase and divide equally between 2 x 2 mL glass vials prepared in protocol 2 step 3. There should be around 300 µL of lower phase. This is the chloroform phase for ***lipidomics***.
    Note: The sample can be further aliquoted at this stage if required. 50 µL aliquots should contain enough lipid for analysis.
15. Add 200 µL of ice cold methanol to the protein disc left behind, vortex for 20 seconds and create a pellet by centrifugation at 10,000 g for 5 minutes.
16. Decant the methanol solution and allow the pellet to dry in fume hood for 15 minutes. Store frozen at -80°C. This will be used for alternative ***proteomic*** analysis (Aston/Manchester).
17. Evaporate solvents from metabolomic and lipidomic sample tubes. Drying under a nitrogen stream at 35°C is a good method for chloroform samples (lipidomics) and works for methanol samples (metabolomics) but can be quite slow. Vacuum centrifugation can be used as an alternative for methanol samples. Air drying at 35°C can be used if other methods not available, but is not recommended.
18. Store samples at -80°C.
19. Transfer aliquots in batches to partner analysis labs as dried samples on dry ice.

Notes:

Risk mitigation:
Virus will be inactivated at step 11 in this protocol.

Methanol is toxic and exposure should be minimised. Volumes used are small and likely exposure is low risk.

Chloroform is highly toxic, a potent embryotoxin, and is listed as a potential human carcinogen Exposure should be minimised, and where possible samples containing chloroform should be handled in a mechanically extracted, externally vented hood using appropriate PPE. This is essential for the drying step.

Transfer to analysis labs

Samples should be shipped in batches, in cryoboxes with inserts with a manifest and map of the sample contents.

Samples must be transferred on dry ice. Samples should be transferred in a container with enough dry ice to maintain the samples frozen for 24h longer than the expected delivery time.

Step 4: LC-MS/MS methods.

General Notes:

All chemicals should be of LCMS grade or better. Do not use autoclaved plastics for any part of the procedure. Do not use detergent washed glassware for any part of the procedure. Solvents, and samples once methanol has been added, should not come into contact with autoclaved plastics. Use glass wherever possible for lipidomics samples.

Important Notes:

1) **System suitability QA samples (standard mix, e.g. Avanti SPLASH Lipidomix, 10 x dilution of stock) should be run, checked and recorded before using the instrument for samples and at the beginning and at the end of the batch.**
2) **Pooled patient samples (10 µL of sample from each sample in the batch) should be run as QC samples for each batch every 5 samples for correction and normalization purposes.**
3) **After running the system suitability sample, instruments should be prepared by running 2 initial blanks to check for residual interference, and 10 pooled standard samples to condition the instrument, and a blank to check for carry over before the main analysis.**
4) **If any of the initial system suitability samples or blanks fail, the analysis should be stopped and the instrument cleaned and performance tested again.**
5) **Blanks should be run at the end of the batch to check for residual interference.**
6) **Internal or external mass calibration should be intrinsic to the batch protocol.**
7) **Study samples should be blinded and analysed in randomized patient blocks.**
8) **All mobile phases should be made fresh and used within 48h.**
9) **It is worth taking solvent samples from new bottles and running these to check for impurities before use.**
10) **If sufficient pooled standard is available, and machine time allows, run a 5 point dilution series of the pooled sample at the end of the run (1:2, 1:5, 1:10, 1:20, 1:50) to assess linearity of quantification.**

Step 4a: LC-MS/MS method for lipidomics.

General materials and equipment for step 4 (excluding LC-MS equipment and MS consumables):

- Methanol, LCMS grade (e.g. Fisher Scientific Optima LC/MS grade)
- Isopropanol, LCMS grade (e.g. Fisher Scientific Optima LC/MS grade)
- Water, LCMS grade (e.g. Fisher Scientific Optima LC/MS grade)

- Ammonium formate, LCMS grade (e.g. Fisher Scientific Optima LC/MS grade)
- Formic acid, LCMS grade (e.g. Fisher Scientific Optima LC/MS grade)
- 1.7 µm, C18 reversed phase column

Outline protocol for analysis

General notes:

- Batches should include randomised triplicates, pooled samples and QA/QC samples.
- UPLC should be used for the separation, using < 2 µm core-shell standard C18 stationary phase.
- All samples should be run as triplicates, preferably triplicates generated at Step 3 (***Lipid and Metabolite extraction***), action 11.
- Internal standard should be added as soon as possible in the sample preparation protocol. Standards should cover a number of analyte classes at appropriate levels for plasma (e.g. Avanti SPLASH Lipidomix for lipidomics).
- Solvent mixes for separation should be made fresh and used within 48 hours.

Liquid Chromatography

1) Place dried samples on ice.
2) Reconstitute samples in 100 µL isopropanol (IP) with vigorous vortexing for 30 seconds.
3) Aliquot in 20 µL portions to new, labelled sample vials. Keep 1 sample vial for analysis and dry the rest under nitrogen. These can be distributed to other analysis labs for repeat analysis.
4) Only reconstitute the number of samples that can be analysed within 24h and according to the capacity of cooled autosampler trays for your LC system.
5) Prepare a pooled-QC sample by taking 10 µL of each reconstituted sample into an appropriately sized glass vial. The pooled QC sample should be representative of your entire experiment, and should be injected at the start of the batch, end of the batch and between every 5 sample injections. Make the appropriate number of aliquots from the pooled sample.
6) Store prepared samples and pooled QC at -80°C until analysis.

An example LC separation protocol is given below.

Mobile phase A:	50:50 MeOH:H_20 (v/v), 10 mM ammonium formate, 0.1% formic acid
Mobile phase B:	90:9:1 IPA: MeOH:H_20 (v/v/v), 10 mM ammonium formate, 0.1% formic acid.
Column:	ACQUITY UPLC BEH C18 column (length 100 mm, diameter 2.1 mm, particle size 1.7 µm, Waters (cat. no. 176000864, UK)
Colum oven temp:	50 °C

Flow rate: 400 µL/min

1 – 5 µL of sample should be injected, depending on system sensitivity and dynamic range. Standards should cover a number of lipid classes at appropriate levels for plasma (e.g. Avanti SPLASH Lipidomix or similar).

Gradient program:	t	%B
	0	10
	4	30
	5	40
	20	80
	21	99
	24	99
	25	10
	30	10

MS Protocol

Samples should be run using electrospray ionization in both positive and negative ion mode (either switching or duplicate runs). Lockmass or regular (< every 5 samples) inter-run calibration should be used for accurate mass-based analysis (lower resolution targeted analysis for validation will require less rigorous mass correction).

Samples should be run in a mass range 200- 2,000 Da for survey scans and 100-2,000 Da for MSMS scans. MS resolution should be >30,000. DIA, DDA (top 10) approaches should be used to confirm mass-based identifications, with DIA preferred. Incorporation of additional separation, e.g. ion mobility, will be of value but is not essential and should only be used where the duty cycle does not compromise quantification and depth of analysis. Instrument settings should be optimized using standards. Standards are available from Avanti.

Suggested fragmentation energy: fixed at approximately 35 V, with a -15 V to +15 V slope.

Step 4b: LC-MS/MS method for Metabolomics

Liquid Chromatography

Recommended stationary phase:

Hypersil GOLD UHPLC C18 column (length 100 mm, diameter 2.1 mm, particle size 1.9 µm, Thermo-Fisher Ltd. Hemel Hempsted, UK)

-OR-

ACQUITY UPLC BEH C18 column (length 100 mm, diameter 2.1 mm, particle size 1.7 µm, Waters, cat. no. 176000864, UK)

Mobile phase and wash solutions:

1. Make 1L mobile phase A: 95:5 (*v/v)* water: methanol with 0.1% formic acid.
2. Make 1L mobile phase B: 95:5 (*v/v)* methanol: water with 0.1% formic acid.
3. Prepare a weak needle wash solution by adding 50 mL methanol to 950 ml water and mixing thoroughly.
4. Prepare a strong needle wash solution by adding 800 mL methanol to 200 ml water and mixing it thoroughly.
5. Column operating temperature should be set to 50 °C.
6. Mobile phase gradient elusion profile should be set as below.

Note: standard operating pressures should be checked by allowing mobile phase through the column. The composition of mobile phase during this check should be 50:50, A:B at 0.4 ml per minute. The pressure of LC system should stay below 12,000 psi. If you notice higher pressure, please clean the system with 100% acetonitrile for 30 minutes and re-check for 30 minute with 50:50, A:B at 0.4 ml per minute.

Sample preparation

1. Thaw metabolite pellets at 4 °C. (*note: if samples are not dried to pellet, we recommend using heat block at 35 °C or using vacuum centrifuge to achieve complete dryness of sample first).*
2. Reconstitute pellet in 100 µL of mobile phase A.
3. Only reconstitute samples that can be analysed within 24h and according to the capacity of cooled autosampler trays for your LC system.
4. Prepare a pooled-QC by taking 10 µL of each reconstituted sample into an appropriately sized glass bottle.
5. Pooled QC should be representative of your entire experiment; should be injected at the start of the batch, end of the batch and between every 5 sample injections. Make appropriate number of aliquots from the pooled stock made in step 4.
6. Store prepared samples and pooled QC at -80°C until analysis.
7. LC Gradient conditions are shown in the table.

Mass spectrometry

Mass calibration should be performed for high and low range *m/z* using manufacturer's guidance depending on mass spectrometer used.

Example acquisition settings (Orbitrap QE):

1. Set resolution at > 30,000 in centroid mode.

2. Scan time to be set at 1 µ-scan per 400 ms in the 100–1000 m/z range.
3. Source gases: sheath gas = 40 arbitrary units, aux gas = 0 arbitrary units, sweep gas = 5 arbitrary units.
4. Set ESI source voltage to 3.5 kV and capillary ion transfer tube temperature at 275 °C.

Note: you may have to adjust these settings according to the tune file of your instrument.

LC Gradient Conditions

Time (min)	*Flow rate (ml min^{-1})*	*Mobile phase A (%)*	*Mobile phase B (%)*	*Curve*
				Positive ion mode (ES+)
Initial	0.4	98	2	5
1.0	0.4	98	2	5
16.0	0.4	2	98	5
20.0	0.4	2	98	5
22.0	0.4	100	2	1
				Negative ion mode (ES–)
Initial	0.4	100	2	4
2.0	0.4	100	2	4
17.0	0.4	2	98	4
22.0	0.4	2	98	4
24.0	0.4	100	2	1