

Jun 17, 2025

Version 1

Metabolites, Lipids, Protein extraction protocol (MPLEx) V.1

DOI

<https://dx.doi.org/10.17504/protocols.io.36wgqd15ovk5/v1>

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Protocol Citation: Heather Olson, Geremy Clair, Ernesto S Nakayasu, WEI-JUN QIAN 2025. Metabolites, Lipids, Protein extraction protocol (MPLEx). protocols.io <https://dx.doi.org/10.17504/protocols.io.36wgqd15ovk5/v1>

**Manuscript citation:**

Ernesto S. Nakayasu, Carrie D. Nicora, Amy C. Sims, Kristin E. Burnum-Johnson, Young-Mo Kim, Jennifer E. Kyle, Melissa M. Matzke, Anil K. Shukla, Rosalie K. Chu, Athena A. Schepmoes, Jon M. Jacobs, Ralph S. Baric, Bobbie-Jo Webb-Robertson, Richard D. Smith, Thomas O. Metz. "MPLEx: a Robust and Universal Protocol for Single-Sample Integrative Proteomic, Metabolomic, and Lipidomic Analyses". mSystems. 2016 May 10;1(3):e00043-16. doi: 10.1128/mSystems.00043-16

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Protocol status: Working

We use this protocol and it's working

Created: February 14, 2025

Last Modified: June 17, 2025

Protocol Integer ID: 120442

Keywords: Metabolites, Lipids, Proteins, Extraction, Sample preparation, LC-MS, termed mplex, mplex, protein extraction protocol, solvent extraction method, metabolite, including biofluid, lipid, proteins from the same sample, solvent, protein

Funders Acknowledgements:

HubMAP Kidney (NIH U54)

Grant ID: U54-DK127823

HubMAP Lung (NIH U54)

Grant ID: U54-HL165443

LungMAP2 PNNL (NIH U01)

Grant ID: U01-HL148860

Abstract

This protocol describes a solvent-solvent extraction method to obtain Metabolites, Lipids and Proteins from the same sample. This method, termed MPLEx, can be used on a variety of samples including biofluids (e.g., bronchoalveolar lavage fluid, plasma, blood, urine), mammalian tissues, bacteria, viruses, soil, etc.

Guidelines

This extraction method is mainly used for two reasons: 1) to obtain metabolite, lipid and protein components from the same sample or 2) to remove contaminants from the protein portion of a sample. It is best to keep the samples on ice at all times during processing. Keeping the samples cold not only assists with the separation of the various layers, it also slows any metabolic processes that can occur in the sample upon warming to room temperature.

Materials

Chemical supplies

1. Methanol (HPLC grade)
2. Chloroform (Ethanol stabilized)
3. Ultrapure water (e.g. Milli-Q or Nanopure)

Non-Chemical Supplies

1. Vortexer mixer
2. Centrifuge
3. Ice bucket
4. Vacuum concentrator
5. Pellet Pestle homogenizer or other tissue homogenizer
6. 0.2um PTFE 96 filter well plate
7. Breathable tube membrane
8. Microcentrifuge tube made with virgin PE
9. Pasteur pipette and bulb or gentle suction device
10. Automatic Liquid Sampler vials (e.g. Waters Corp Clear Glass 12 × 32 mm Screw Neck Total Recovery Vial)
11. Caps for ALS vials (e.g. 12 mm screw cap with Red PTFE / White Silicone Rubber / Red PTFE Septa)
12. 96-well positive pressure system connected to a N2 source

Safety warnings

-  If the samples to be prepared are from human origin or may contain infectious organism, the procedure has to be performed in conditions meeting the bio-safety laboratory (BSL) requirements adapted to the type of samples.

Due to the utilization of solvent, it is essential to perform most of the steps in a ventilated hood. If working with samples that are RG2 or higher, a ducted B2 BSC must be utilized for this protocol.

Before start

If performing LC-MS Metabolite or Lipid analysis of samples using this extraction method, it is suggested to add 3-4 process blank samples utilizing the same tubes, solvents, homogenizers, etc. Ensure blanks follow the same procedure as the samples you are preparing to observe background MS peaks coming from the process.

If the method is being used to clean a protein sample, discard the metabolite and lipid layers to waste instead of transferring to vials/tubes for analysis.

Ensure samples are extracted in tubes that are made with virgin polymer (not recycled plastics). Retailers that provide this type of tube is Eppendorf, Olympus (15- or 50-mL centrifuge tubes) or Sorenson (microcentrifuge tubes). If tubes made with recycled plastics are used, polymer leaching will occur with exposure to chloroform.

Solvent-Solvent extraction of liquid samples

- 1 This protocol works best on samples where the volume is 200 μ L or less. For samples that have a higher volume (but potentially low protein concentration such as Bronchoalveolar Lavage Fluid (BALF)) it is best to lyophilize to dryness and reconstitute with water. Using larger initial volumes will result in the metabolite/lipid extraction volumes being much larger and the protein "puck" in the interphase will be thinned out in a larger tube and more problematic to deal with. The protocol can be scaled up or down, as long as the ratio of Chloroform:Methanol:Water is 8:4:3.
- 2 For samples that are less than 200 μ L, bring them up to 200 μ L with water in a 1.5-mL microcentrifuge tube. Do not use any type of buffer as this will show up in the metabolite layer and could mask metabolic changes.
- 3 Add 800 μ L of cold (stored at -20°C) 2:1 Chloroform:Methanol to the 200 μ L of sample. This will create the 8:4:3 Chloroform:Methanol:Water ratio needed for the extraction. If working with multiple samples, vortex sample briefly following solvent addition before placing sample tube in ice.
- 4 Following solvent addition to all samples, vortex samples at highest speed for 1 minute followed by 5 minutes of rest on ice.
- 5 Vortex samples again for 1 minute then centrifuge at 10,000 $\times$ g for 10 minutes at 4°C . Samples will be separated into three distinct layers following this step.
- 6 Using a Pasteur pipette, transfer the top (polar, metabolite) layer into a new tube or vial for metabolite analysis (see metabolite preparation section for which container is appropriate).
- 7 Using a new Pasteur pipette, gently push past the protein interphase layer to the lower (non-polar, lipid) layer and transfer to a Waters Corp Clear Glass 12 $\times$ 32 mm Screw Neck Total Recovery Vial.
- 8 Wash the protein interlayer with 1 mL of cold (-20°C) Methanol and then centrifuge again at 10,000 $\times$ g for 10 minutes at 4°C .
- 9 Following centrifugation, the protein should be in a pellet at the bottom of the microcentrifuge tube. Gently pour the methanol off of the pellet and place the tubes upside down inside a fume hood for 5 minutes to dry. Ensure pellet doesn't dry for longer than 5 minutes or reconstitution can be an issue.

Solvent-Solvent extraction of tissues or pellets



- 10 Select appropriate size of tube for tissue mass. For example, a small biopsy sample (100–200 mg) will fit in a 1.5-mL microcentrifuge tube. Much larger than that, you should put the sample into a 5-mL microcentrifuge tube for processing. Additionally, for metabolite/lipid normalization, it is a good practice to obtain the weight of each tissue before processing.
- 11 For samples in a 1.5-mL microcentrifuge tube, add 470 μ L of ice-cold (-20°C) 4:3 Methanol:Water.
- 12 Homogenize tissue/cell pellet with pellet pestle homogenizer (similar to ones used for DNA homogenization)
- 13 Once all samples have been homogenized, 530 μ L of ice-cold (-20°C) chloroform is added (this will result in the 8:4:3 CHCl_3 :MeOH:H₂O ratio mentioned above) followed by brief vortexing. Once chloroform has been added to all samples, vortex all samples at highest speed for 1 minute
- 14 Place all samples on ice for 5 minutes following vortexing step.
- 15 Vortex all samples again for 1 minute, followed by centrifugation at 10,000 $\times$ g for 10 minutes at 4°C . Samples will be separated into 3 distinct phases following this step.
- 16 Follow steps 9–12 of the "Solvent-solvent extraction of liquid samples" to obtain the metabolite, lipid and protein phases from these samples.

Metabolite Sample Processing for GC-MS

- 17 Transfer metabolite layer from above process into a 2-mL glass autosampler vial.
- 18 Apply a breathable tube membrane to the top of the vial and dry sample completely in a centrifugal concentrator (e.g. Labconco Centrivap, Thermo SpeedVac).
- 19 Once dry, remove membrane and apply a non-slit autosampler vial cap with Red PTFE / White Silicone Rubber / Red PTFE Septa. Store sample at -20°C until needed for derivatization and analysis by GC-MS

Metabolite Sample Processing for LC-MS

- 20 Transfer metabolite layer from above process into a 1.5-mL microcentrifuge tube similar to the ones used for the MPLEx extraction process



- 21 Apply a breathable tube membrane to the top of the vial and dry sample completely in a centrifugal concentrator (e.g. Labconco Centrivap, Thermo SpeedVac).
- 22 Add 150-200 μ L of 80:20 methanol:water to dried sample in tube. Vortex and sonicate until sample dissolves. There may be residue that does not dissolve after vortexing and sonicating, do not spend more than 5 minutes on resuspension attempts.
- 23 Centrifuge samples at 21,000 $\times$ g for 10 minutes
- 24 While samples are centrifuging, pre-condition a 1-mL, 0.2 μ m PTFE 96 filter well plate (filter plate) with 2 $\times$ 0.5 mL aliquots of methanol followed by 2 $\times$ 0.5 mL ultrapure water using a N2 going through a 96-well positive pressure system. The waste from the conditioning steps should be discarded. Ensure that the PTFE membranes do not dry as this will cause difficulty in filtering the samples. Once the water has been pushed through, it is difficult to see if all wells are empty, so tap plate upside down on a kimwipe to ensure that water is not present in any wells.
- 25 Place the pre-conditioned filter plate on top of a new 1-mL 96-well collection plate.
- 26 Transfer the supernatant from each centrifuged sample into a single well (a template can be utilized to track sample location on the wellplate). If process blanks have been used, add them to the filter plate as well.
- 27 Once all samples (and/or blanks) have been transferred to the filter wellplate, use the 96-well positive pressure system to push the samples through the filter. Do not go higher than 9 psi or you will risk splashing as the samples go into the wellplate below the filter plate.
- 28 Once filtering step is complete, transfer each sample from its well into a Waters Corp Clear Glass 12 $\times$ 32 mm Screw Neck Total Recovery Vial. Apply a non-slit autosampler vial cap with Red PTFE / White Silicone Rubber / Red PTFE Septa to the vial and store sample at -20C until needed for analysis.
- 28.1 Note: our laboratory takes a small portion of each filtered sample (amount depends upon number of samples in the batch) and pools them to create a quality control sample used to help normalize the LC-MS data.

Lipid sample processing for LC-MS

- 29 Once the lipid sample (or process blank) has been placed into the Waters autosampler vial, apply a breathable tube membrane to the top of the vial and dry sample completely in a centrifugal concentrator (e.g. Labconco Centrivap, Thermo SpeedVac).



- 29.1 Note, due to the volatility of the chloroform, this step shouldn't take more than 30-45 minutes (depending on volume, of course). Do not let samples sit dry longer than necessary to avoid oxidation of lipids.
- 30 When sample is dry, remove membrane and add 500 uL of chloroform to vial. Seal vial with a non-slit autosampler vial cap with Red PTFE / White Silicone Rubber / Red PTFE Septa and vortex briefly to dissolve any residue in the bottom of the vial.
- 31 Store samples at -20C until needed for LC-MS Analysis.

Protein Sample processing for LC-MS

- 32 The protein pellets, after being rinsed with methanol and air dried, unless processed immediately, it is best to keep the samples at -80C until preparation.

Note: We recommend sample re-solubilization in a buffer compatible with a protein digestion protocol.

We recommend to use the protocol.io titled : "Sample Preparation (Urea/trypsin digestion) for Proteomics - Mammalian Organisms" after resolubilization in 50 mM NH_4HCO_3 , pH ~8 buffer.



Protocol references

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