Polar Metabolites Extraction (2-Phase) from Blood Plasma or Serum

For serum, the blood is sampled into additive-free blood collection tubes, clot activator tubes, or serum-separating tubes (SSTs) containing a gel separating the serum from blood cells. The clotting step is important and is usually done at RT for 30 min depending on the type of serum tube and the coagulation enhancer added.

For plasma, anti-coagulating substances are used to prevent clotting of the blood samples by binding calcium ions from coagulation proteins. EDTA vacutainers (lavender capped) are recommended as EDTA is not interfering with endogenous metabolites.

Additionally, to avoid pre-analytical variations, the sample pre-processing should be done within 1 h following the sample collection and the samples should be stored on ice (4 °C) during this period. Pre-analytical variation, such as the storage temperature or the pre-centrifugation delay can highly impact the sample quality and should be standardized and monitored.

Sample pre-processing procedures

- For serum, allow the samples to clot for 30 min at RT (in accordance with the collection tube manufacturer's guide), then centrifuge at 2000 x g for 10 min at 4 °C. Transfer supernatant to a new tube.
- For plasma, centrifuge the tubes at 2000 x g for 20 min at 4 °C. Transfer supernatant to a new tube.

The samples can either be used directly for the metabolite extraction or can be stored at -80 °C until further sample processing.

Metabolite extraction

Perform the procedure on ice *unless otherwise stated. Wear a double layer of nitrile gloves with the chloroform steps.*

1. Thaw the plasma/serum samples on ice.
2. Prepare tubes with 50 µl H_2O and 400 µl 100% methanol (ice-cold). Place on ice.
   It is a good idea to take along a 'processing blank'. For this, use 100 µl H_2O and 400 µl 100% methanol.
   - Optional: Include 1 nmol norvaline as an internal standard for sample processing.
     From a 10 mM norvaline stock solution in methanol (stored at -20°C), make a *fresh* 1:500 dilution in water to 20 µM; use 50 ul of this 20 µM solution (containing 1 nmol norvaline) instead of the plain H_2O above. For the blank, use 50 µl 20 µM norvaline and 50 µl H_2O.
3. Gently vortex the thawed plasma/serum samples to mix; add 50 µl to the prepared tubes on ice.
4. Vortex the mixtures in the tubes for 10 sec.
5. Place the samples for at least 20 min at -80°C.
6. Allow the samples to warm up a bit on ice. Vortex for 10 sec.
7. Spin for 10 min at top speed, 4°C.
8. Prepare new Safe-Lock Eppendorf™ tubes [(1)]. Add 300 µl H_2O and 400 µl chloroform to each tube.
9. Transfer the supernatant from the spun samples to the newly prepared tubes (the ratio of MeOH:H_2O:$CHCl_3$ is now 1:1:1).
10. Vortex to homogenize for 1 minute.

11. Place on ice for 10 min to allow the phases to separate.
12. Spin for 10 min at top speed, 4°C.
13. Let the phases equilibrate at RT for 15 min (optional).
14. Remove 600 µl of the top aqueous layer – taking care not to disrupt the interphase – and transfer to a glass vial (or Eppendorf tubes if drying in a regular speed vac).

    Dispose of the remaining chloroform layer as hazardous waste!

    Alternatively: Transfer the bottom chloroform layer to a glass vial – taking care not to take along the interphase layer – for lipidomics analysis, if desired.
15. Dry the aqueous polar metabolites extract in a Genevac EZ2 Elite evaporator (or in a speed vac).
16. Store the samples at -80C until ready for LC-MS analysis.

Supplies and reagents needed for this protocol

(1) Snap-Cap Safe-Lock Eppendorf™ brand tubes (Eppendorf.com, #22363204) are recommended for working with chloroform.

	05-402-25	FisherScientific	Snap-Cap Safe-Lock™ microcentrifuge tubes, 1.5 ml
H2O:	W5-1	FisherScientific	Water; 1L (HPLC grade)
MeOH:	A456-1	FisherScientific	Methanol; 1L (Optima LC/MS grade)
Norvaline:	N7502-25G	Sigma	DL-Norvaline

Used as an internal standard: dissolve 11.7 mg of norvaline powder in 1 ml water to make a 100 mM stock solution. Dilute 1:10 in water (or 50% MeOH) to make a 10 mM working stock solution. Store both stock solutions at -20C.

$CHCl_3$:	423550010	FisherScientific	Reagent ACS 99.8%, ACROS Organics™
glass vials:	13-622-351	FisherScientific	Thermo Scientific™ Chromacol™ GOLD-Grade Inert Glass Vials; Thermo 2SVWGK
caps:	03-452-327	Fisher Scientific	9 mm Screw Caps, SureSTART™ Level 2 (Silicone/PTFE septum); Thermo Scientific 6ASC9STB1
	or		
	03-379-123	FisherScientific	9 mm autosampler vial screw thread caps (PTFE/silicone septum); Thermo Scientific C500054A