Sphingonet Workshop

Lipidomics

September 9th and 10th 2014

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Programme

Tuesday, September 9th

8:30 Introduction: lipid extraction protocol (Thomas Hannich)

9:00 Total lipid extraction

11:30 Sample collection and preparation (Auxiliadora Aguilera-Romero)

12:30 Lunch (Sandwiches)

14:00 Methylamine treatment

14:30 Lipidomics and metabolipidomics (Howard Riezman)

15:15 Break

15:30 Drying of methylamine solvent (Assistants)

15:30 Total lipid fraction: desalting via butanol extraction

17:30 Social activity

Wednesday, September 10th

8:30 Sphingolipid fraction: desalting via butanol extraction

10:30 Break

11:00 Data handling (Olivier Schaad)

12:00 Lunch

13:00 Data processing (Thomas Hannich)

14:00 Descriptive statistics (Aline Santos)

15:00 Mass spectrometer tour

Protocol for the Workshop
Lipidomics on cell cycle phase and pheromone signaling

Introduction

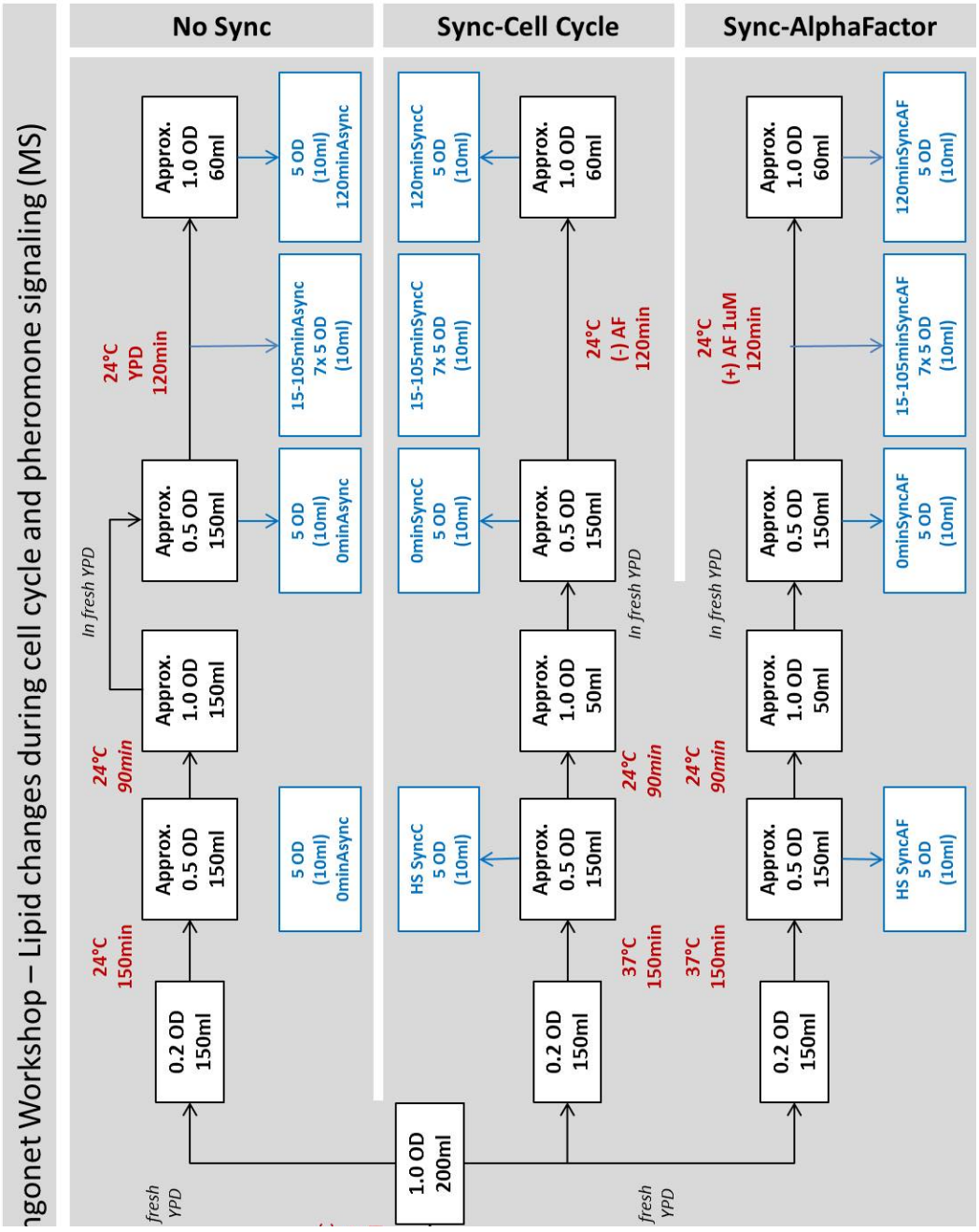
Cell growth description

Cells are first grown at 24°C to exponential phase. The culture is then split into three flasks, one is incubated at 24°C for the non-synchronized culture and the two others are incubated at 37°C for 150 minutes to induce cell-cycle arrest at G1 (synchronized cultures). The synchronized cultures are then switched to 24°C for 90 minutes to allow the cells to recover and reach the maximum response to α-Factor. α-Factor is then added to one of the synchronized cultures, the cells will arrest in G1 after one full cell cycle while the one from the control continue to divide. All the cultures are incubated for 120 minutes at 24°C and an aliquot of cells is taken every 15 minutes.

Strain
cdc15-2 (RH210-3c)

Keywords
TL : phospholipid (total) lipid
SL : sphingolipid lipid

Flowchart of the experiment



Cell collection

Previously done by the assistants to have the cells ready to extract.

YPUAD medium (1L)

20g glucose
10g Bacto Peptone
20g Bacto Yeast extract
1.95g MES hydrate
40mg of each L-Tryptophan, Uracil and Adenine

Day 1 : Grow a 3 ml YPUAD preculture at 24°C until saturation.

Day 2 : Dilute culture to 0.005 OD/ml in 200 ml YPUAD.
Grow cells o/n at 24°C in a water bath shaker until OD 1.0

Day 3 :

1. Measure OD, dilute to 0.2 OD/ml in 450ml fresh YPUAD and split into 3 flasks : 150 ml NonSync (Non synchronized), 150 ml Sync_C (Synchronized Control) and 150 ml Sync_AF (Synchronized α-Factor)
2. Incubate at 24°C (NonSync) or 37°C (Sync) for 150min in water bath shakers.
3. Measure OD and collect a 5 OD aliquot (HS), see below "9. Aliquot processing".
4. Transfer the cultures to a 24°C water bath shaker for 90min.
5. Measure OD and dilute to 0.5 OD/ml in 150ml YPUAD.
6. Add 1 μM α-Factor to Sync_AF culture (*150ul from stock solution 1mg/ml)*.
7. Collect a 5 OD aliquot.
8. Grow for 120 minutes in a water bath shaker at 24°C and collect a 5 OD aliquot at the following time points : 15, 30, 45, 60, 75, 90, 105 and 120 minutes.
9. Aliquot processing :
   - Prepare a 15 ml Falcon tube with 526 μl 100% TCA.
   - Take 10 ml of the culture and transfer to the Falcon tube (5% TCA final).
   - Incubate for at least 15 minutes on ice.
   - Centrifuge for 5 minutes at 800g (2000rpm on Eppendorf 5810).
   - Wash the cell pellet with 5 ml ice cold 5% TCA.
   - Centrifuge for 5 minutes at 800g (2000rpm on Eppendorf 5810).
   - Resuspend the cell pellet in 2 ml ice cold water and transfer the appropriate amount to have 5 OD into a 13mm glass tubes.
   - Centrifuge for 5 minutes at 800g (2000rpm on Eppendorf 5810).
   - Remove the supernatant and freeze the tube at -80°C.

Pyridine extraction for phospholipid and sphingolipid analysis

Pyridine Extraction solvent

45 ml ethanol

45 ml water

15 ml diethyl ether

3 ml pyridine

54 μl ammonium hydroxide (4.2 N) (stable for 1-2 weeks)

Protocol

1. Prepare the standard mix: Resuspend the dried standards in 250 μl Chloroform:Methanol (1:1) MS grade and sonicate for 5 minutes.
2. Take the tubes containing the cell pellets out of the -80°C.
3. Add 100 μl glass beads and 20 μl of internal standard mix.
4. Add 0.75 ml extraction solvent.
5. Vortex for 6 minutes on a multivortexer.
6. Incubate for 20 minutes at 60°C.
7. Centrifuge for 5 minutes at 800g (2000rpm on Eppendorf 5810).
8. Transfer the supernatant to a clean 13 mm diameter tube.
9. Add 0.75 ml extraction solvent to the beads.
10. Vortex for 6 minutes on a multivortexer.
11. Incubate for 20 minutes at 60°C.
12. Centrifuge for 5 minutes at 800g (2000rpm on Eppendorf 5810).
13. Combine the second supernatant with the first one.
14. Divide into 2 aliquots of equal amounts, one for the TL analysis and one for the SL analysis.
15. Dry under a flow of N_2 or in the Centrivap (start at room temperature, then increase gradually the temperature every 15 minutes : 30, 35, 40, 50, 60 °C).
16. Do the methylamine treatment on SL and store the TL in the cold room.

Methylamine treatment for sphingolipid analysis (basic hydrolysis of ester bonds to remove glycerolipids)

Modified after Clarke and Dawson (1981), Bioch.J. 195; 301-306 and Cheng et al, Molecular and Cellular Biology, Vol. 21, N° 18, Sept.2001, p. 6198-6209

Monomethylamine reagent

4ml methanol

3ml H_2O

1ml n-butanol

5ml Monomethylamine

Protocol

1. Add 0.5 ml freshly made Monomethylamine reagent to the dried aliquot for the SL analysis and vortex well.
2. Sonicate for 5 minutes and vortex well.
3. Incubate for 1 hour at 53°C with the cap tightly closed.
4. Dry the lipids with the Centrivap (start for 30 minutes at 50°C and then increase to 60°C).
5. Store at -80°C.

n-butanol extraction to desalt samples for MS analysis

n-butanol saturated with H_2O

- In a glass bottle, prepare a solution n-butanol/H_2O (2:1; v/v) (LC-MS grade).
- Shake well and let phases separate.
- Take the upper phase to do the extraction.

Protocol

1. Resuspend the dried lipid extracts in 300 µl n-butanol saturated with H_2O.
2. Vortex well and sonicate for 5 minutes.
3. Add 150 µl H_2O (LC-MS grade), vortex well and centrifuge for 10 min at 3200g (4000 rpm on Eppendorf 5810).
4. Transfer the upper phase (BP1) in a 2ml amber glass tube. This can be done with a plastic yellow tip.
5. Add 300 µl n-butanol saturated with H_2O, vortex well and centrifuge for 10 min at 3200g (4000 rpm on a Eppendorf 5810).
6. Combine upper phase (BP2) with BP1.
7. Add 300 µl n-butanol saturated with H_2O, vortex well and centrifuge for 10 min at 3200g (4000 rpm on Eppendorf 5810).
8. Combine upper phase (BP3) with BP1+BP2.
9. Dry the combined butanol phases with the Centrivap (start for 30 minutes at 50°C and then increase at 60°C).
10. Flush the samples with N_2.
11. Store at -80°C.

Lipidomic analysis : Yeast, Mammals and Worms

Yeast sample collection and extraction

Collection of yeast samples

YPUAD medium (1L)

20g glucose
10g Bacto Peptone
20 g Bacto Yeast extract
1.95 g MES hydrate
40mg of each L-Tryptophan, Uracil and Adenine

Protocol

- Day 1: If needed, take the strain out of the –80°C and plate on appropriate plates.
- Day 3: Evening, inoculate a 3ml preculture from one colony and grow in YPUAD.
- Day 4: Evening, inoculate a 50 ml culture (YPUAD) with 1.3 µl of the preculture.
- Day 5: Grow cells until OD_{600nm} = 1.1-1.5 and collect 2 x 25 OD_{600nm}
  - In a 50 ml FALCON tube, pipette 2.5 ml 100% TCA.
  - Fill with the culture to 50 ml (5% TCA final).
  - Incubate for at least 10 minutes on ice.
  - Centrifuge for 5 minutes at 800g (2000rpm on Eppendorf 5810).
  - Wash the cell pellet with 10 ml 5% TCA.
  - Centrifuge for 5 minutes at 800g (2000rpm on Eppendorf 5810).
  - Resuspend the cell pellet in 5 ml water and transfer the appropriate amount to have 25 OD into two 10 ml glass tubes (one for the pyridine extraction and one for the chloroform/methanol extraction).
  - Centrifuge for 5 minutes at 800g (2000rpm on Eppendorf 5810).
  - Remove the supernatant and freeze the tubes at -80°C.

Important : The analysis should be done on at least two biological replicates, three ideally. Biological replicates means separate cultures started from different colonies.

Yeast pyridine extraction for phospholipid and sphingolipid analysis

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Extraction solvent

45 ml ethanol
45 ml water
15 ml diethyl ether
3 ml pyridine
54 μl ammonium hydroxide (4.2 N) (stable for 1-2 weeks)

Protocol

- Take the tubes containing the cell pellets out of the -80°C.
- Add 500 μl glass beads and 25 μl of internal standard mix.
- Add 1.5 ml extraction solvent.
- Vortex for 6 minutes on a multivortexer.
- Incubate for 20 minutes at 60°C.
- Centrifuge for 5 minutes at 800g (2000rpm on Eppendorf 5810).
- Transfer the supernatant to a clean 13 mm diameter tube (13mm diameter tubes are the only ones that can fit in the Centrivap).
- Add 1.5 ml extraction solvent to the beads.
- Vortex for 6 minutes on a multivortexer.
- Incubate for 20 minutes at 60°C.
- Centrifuge for 5 minutes at 800g (2000rpm on Eppendorf 5810).
- Combine the second supernatant with the first one.
- Divide into 2 aliquots of equal amounts, one for the phospholipid (total) lipid analysis and one for the sphingolipid analysis.
- Dry under a flow of N_2 or in the Centrivap (start at room temperature, then increase gradually the temperature every 15 minutes : 30, 35, 40, 50, 60 °C). The time for drying depends on the number on samples, it takes 5-6h for 24 samples (= 48 tubes).
- Flush the samples dried in the Centrivap with N_2 and store at -80°C.
- Do an n-butanol extraction (protocol p.17) on the aliquot for the total lipid analysis and a methylamine treatment (protocol p.16) on the aliquot for the sphingolipid analysis.

Yeast CHLOROFORM :METHANOL extraction for total lipids GC-MS analysis

Modified after Folch, J., Lees, M. and Stanley, G.H.S. (1957), *J. Biol. Chem*., **226**, 497-509

Solvents and solutions

Methanol, Chloroform
Chloroform/Methanol (1:2, v/v)
0.034% $MgCl_2$
2M KCl/Methanol (4:1, v/v)
Artificial Upper Phase : Chloroform/Methanol/Water (3:48:47, v/v/v)

Protocol

- Add 500 μl glass beads and the internal standard (Cholesterol).
- Resuspend in 600 μl H_2O.
- Add 1500 μl methanol.
- Vortex for 1 minute on the multivortexer.
- Add 750μl chloroform.
- Vortex for 6 minutes on the multivortexer.
- Transfer the supernatant to new tubes.
- Add 600 μl chloroform:methanol (1:2) to the beads.
- Vortex and combine the second supernatant with the first one.
- Add 400 μl 0.034% $MgCl_2$ to the supernatants.
- Vortex for 1 minute on the multivortexer.
- Centrifuge for 5 minutes at 800g (2000rpm).
- Take off the aqueous upper phase without removing the protein layer.
- Add 400 μl 2M KCl/methanol (4:1) to the lower phase.
- Vortex for 1 minute on the multivortexer.
- Centrifuge for 5 minutes at 800g (2000rpm).
- Take off the aqueous upper phase without removing the protein layer.
- Add 400 μl Artificial Upper Phase to the lower phase.
- Vortex well and centrifuge for 5 minutes at 800g (2000rpm).
- Take off the aqueous upper phase without removing the protein layer.
- Transfer the organic phase in a new tube.
- Add 400 μl Artificial Upper Phase to the organic phase.
- Vortex well and centrifuge for 10 minutes at 3200g (4000rpm).
- Take off the aqueous upper phase without removing the protein layer.
- Transfer the organic phase in 2 amber glass vials (one for direct analysis and one for the backup).
- Dry under a flow of N_2 or in the Centrivap.
- Flush samples with N_2 if dried in the Centrivap and store at –80°C.

Mammalian sample collection and extraction

Collection of mammalian samples

From cell culture

- Plate cells so that they have about the same confluency at the time of cell harvest. **This is very important. Confluency greatly influences lipid concentration!**
- You need one confluent 6 or 10cm dish per lipid extraction.
- Cell collection can be done under non-sterile conditions on the bench.
- Take off medium and wash cells carefully with ice-cold PBS.
- Place cells on ice; tilt the dish so that remaining rest of PBS can be collected. Never let the cells dry out! Always close the lid.
- Add 500µl of fresh ice cold PBS per plate. Scrape the cells off the dish using a Costar cell lifter. Work on ice, try to be fast, collect cells on one side of the dish.
- Transfer 500µl cell suspension to a 1.5 ml eppendorf tube.
- Take an aliquot aside for BCA protein assay (typically 50µl)
- Spin down cells at 2500rpm, 5min, 4°C.
- Take off the PBS using a pipet (do not aspirate). The cell pellets can be frozen at -20°C or directly used for BCA assay and lipid extraction.
- For BCA assay : resuspend pellet in 55µl of lysis buffer (M-PER, Thermo Scientific).
- For lipid extraction: continue with MTBE extraction protocol.

From tissue

Best way is to pulverize the tissue in liquid nitrogen. If this is not possible, this is very important to have at least pieces of the same size.

Mammalian methyl-*ter*-buthyl ether (MTBE) extraction for total lipids GC/LC analysis

Modified after Matyash (2008) J.LipidRes. 49; 1137-1146

Solvents

MTBE

Artificial upper phase : MTBE/methanol/water (10:3:1.5, v/v)

Protocol

- From cell culture samples :
  - Start with a confluent 6 or 10cm dish.
  - Resuspend cell pellet in 100 µl H_2O.
  - Transfer cell suspension into a 2ml eppendorf tube.
  - Add 360 µl methanol
  - Add the internal standard mix.
  - Vortex well
  - Add 1.2 ml MTBE.
  - Vortex for 10 minutes at 4°C (coldroom).
- From mammalian tissue samples:
  - Cool down the Cryolysis machine to 3°C.
  - Start with 20-30mg, keep the samples on ice until the breaking.
  - Resuspend tissue material in 100 µl H_2O.
  - Transfer cell suspension into a 2ml eppendorf tube for the Cryolysis system.
  - Add 50 µl Zirconium beads.
  - Add 360 µl methanol
  - Add the internal standard mix.
  - Break the tissue with 3 bursts of 45’ at 6200 rpm with 45’ interruptions.
  - Add 1.2 ml MTBE.

- The following steps are for both starting material:
    - Incubate on a shaker for 1 hour at room temperature.
    - Add 200 μl H_2O to the mixture to induce phase separation and vortex.
    - Incubate for 10 minutes at room temperature.
    - Centrifuge for 10 minutes at 1000g.
    - Transfer the upper phase in a new 13mm glass tube.
    - Add 400μl of the artificial upper phase to the lower phase and vortex.
    - Incubate for 10 minutes at room temperature.
    - Centrifuge for 10 minutes at 1000g.
    - Combine the second upper phase with the first one.
    - Divide extracts into three parts, (one for phospholipid analysis (TL) and one for sphingolipid analysis (SL) and one for sterols analysis (S)).
    - Dry samples either under a stream of nitrogen or in a Centrivap at 50°C.
    - Do a methylamine treatment on the sphingolipid part (protocol p.16).

Worm sample collection and extraction

Collection of worm samples

Synchronization

- Collect gravid adults and their eggs from five 10 cm plates
- Add 1.5 mL bleaching solution (4% NaOH in commercial bleach) to worms in 3.5 mL water, incubate until mothers start breaking open (ca. 4-5 min)
- 3x wash with ddH_2O
- Resuspend in 1.5 mL M9 buffer
- Incubate at 20-22°C with shaking for 24 hours to let eggs hatch.

Growth

- Dilute the starved larvae in 10 mL
- Take 10 µL and count the larvae
- Spin at 600 g for 1 min and resuspend in 1 mL
- Plate 8000 larvae per 15 cm NGM plate seeded with 1 mL of 10x concentrated OP50 bacteria
- Grow for 3 days at 20°C until they are young adults and just start laying eggs. They will have some eggs inside, but no larvae on the plate.

Collection

- Wash the worms but not the eggs gently off the plate with ddH_2O
- Put them in a 15 mL Falcon tube
- Spin at 600 g for 1 min and wash bacteria away with ddH_2O
- Resuspend in 1 mL and transfer to a Cryolysis tube
- Spin at 1000 g for 2 min and wash with 1mL MS-H_2O (I put the eppendorf tubes through a falcon tube cap and spin like this on a swing rotor table top to get a nice and dense pellet)
- Spin at 1000 g for 2 min and take off the maximum of supernatant
- Freeze in liquid nitrogen
- Keep at -80°C.

Worm CHLOROFORM :METHANOL extraction for total lipids GC/LC-MS analysis

Modified after Bligh, EG et Dyer, WJ (1959) *Canadian Journal of Biochemistry and Physiology*, **37(8)**: 911-917

Solvents

Methanol, Chloroform, LC-MS Water

Chloroform/Methanol (1:2, v/v)

Protocol

- Cool down the Cryolysis machine to 3°C.
- Add 100 μL 1.4 mm zirconium oxide beads and 800 μL MS-H_2O.
- Break worms with 3 bursts of 45' at 6200 rpm with 45' interruptions; keep at 3-4°C
- Elute lysate into a glass tube with lipid standards (use wide rim glass tubes where the cryolysis tubes fit in, make wholes into lid and bottom of the tubes with a hot needle, and centrifuge them in the table top centrifuge at 600g).
- Elute again with 200 μL MS-H_2O.
- Add 3.6 mL chloroform:methanol (1:2) and vortex well (at this point there should only be one phase; in case of phase separation, some methanol can be added).
- Spin at 800g for 5min and transfer the supernatant to a new glass tube.
- Induce phase separation by adding 0.5 mL of LC-MS-H_2O and 0.5 mL of chloroform.
- Vortex well.
- Centrifuge at 800g for 5min.
- Transfer the lower organic phase and split into two 13mm glass tubes (total lipids and sphingolipids).
- Treat the sphingolipid fraction with methylamine (protocol p. 16).

Worm Sterol Preparation

Hannich et al. (2009) *Dev Cell* **16(6)**:833-43

- Do a Bligh and Dyer extraction from approximately 40,000 young adults adding 8nmoles of ergosterol standard
- Saponify total lipid extracts in 1 mL 3M KOH:methanol (1:9; v:v) at 80°C for 1 hour
- Extract unsaponified lipids three times with 0.5 mL hexane
- Remove fatty acids using a QAE-Sephadex A-50 column (Pharmacia Fine Chemicals AB, Uppsala, Sweden)
- Analyze remaining lipids by GC/MS

Methylamine treatment for sphingolipid analysis on MS (basic hydrolysis of ester bonds to remove glycerolipids)

Modified after Clarke and Dawson (1981), Bioch.J. 195; 301-306 and Cheng et al, Molecular and Cellular Biology, Vol. 21, N° 18, Sept.2001, p. 6198-6209

Monomethylamine reagent

4ml methanol

3ml H_2O

1ml n-butanol

5ml methylamine

Protocol

- Add 0.5 ml freshly made Monomethylamine reagent to the dried aliquot for the sphingolipid analysis and vortex well.
- Sonicate for 5 minutes and vortex well.
- Incubate for 1 hour at 53°C with the cap tightly closed.
- Dry the lipids under N_2 or with the Centrivap (start for 30 minutes at 50°C and then increase to 60°C). It takes 1-2h to dry.
- Do an n-butanol extraction on the dried lipids.
- Flush the samples dried in the Centrivap with N_2 and store at -80°C if the n-butanol extraction (protocol p. 17 or 18) is not done the same day.

n-butanol extraction to desalt samples for MS analysis

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n-butanol saturated with H_2O

- In a glass bottle, prepare a solution n-butanol/H_2O (2:1 v/v) (LC-MS grade).
- Shake well and let it decant.
- Take the upper phase to do the extraction.

Protocol

- Resuspend the dried lipid extracts in 300 µl n-butanol saturated with H_2O.
- Vortex well and sonicate for 5 minutes.
- Add 150 µl H_2O (LC-MS grade), vortex well and centrifuge for 10 min at 3200g (4000 rpm on a Eppendorf 5810).
- Transfer the upper phase (Butanol Phase (BP) 1) in a 2ml amber glass tube. This can be done with a plastic yellow tip.
- Add 300 µl n-butanol saturated with H_2O, vortex well and centrifuge for 10 min at 3200g.
- Combine upper phase (BP 2) with BP 1.
- Add 300 µl n-butanol saturated with H_2O, vortex well and centrifuge for 10 min at 3200g.
- Combine upper phase (BP 3) with BP 1+BP 2.
- Optional: divide in two aliquots: one for the direct analysis and one for a backup.
- Dry the combined butanol phases under N2 or with the Centrivap (start for 30 minutes at 50°C and then increase at 60°C). It takes 2-3h.
- Flush the samples dried in the Centrivap with N_2.
- Store at -80°C.

Alternative n-butanol extraction to desalt samples for MS analysis

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n-butanol saturated with H_2O

- In a glass bottle, prepare in advance a solution 2/3 n-butanol, 1/3 H_2O (LC-MS grade).
- Shake well and let the phases separate.
- Take the upper phase to do the extraction.

Protocol

- Resuspend lipids in in 300 μl n-butanol saturated with H_2O.
- Sonicate for 3min.
- Prepare original eppendorf tubes and add 150μl H_2O to each.
- Transfer the resuspended lipids into the new tubes.
- Vortex well and centrifuge for 5 min. at 13000rpm.
- Transfer the upper phase (**Butanol Phase 1**) into a MS glass vial (final vial).
- Repeat extraction. Add 300 μl n-butanol saturated with H_2O to the original glass tube to rinse remaining lipids from the wall of the tube.
- Transfer the resuspended lipids into the tube containing the remaining aqueous phase (150μl H_2O).
- Vortex well and centrifuge for 5 min. at 13000rpm.
- Transfer the upper phase (**Butanol Phase 2**) from the final glass vial.
- Add 300ul n-butanol saturated with H_2O to the aqueous phase (wash of the H_2O phase).
- Vortex well and centrifuge at 13000rpm for 5min.
- Transfer the upper phase (**Butanol phase 3**) into the final glass vial.
- Dry the combined butanol phases under N_2 or with the centrivap at 50°C for 30min, then at 60°C (3-4h).
- Flush the dried samples with N_2 and the store the samples at -80°C.

Phospholipids and Sphingolipids : Sample preparation, running on TSQ and analysis through the pipeline

Sample preparation

- Resuspend samples in 250 µl chloroform / methanol (CHROMASOLV LC-MS grade, 1:1, v/v) and sonicate for 5 minutes.
- Always work with Hamilton syringes.
- Dilutions of the samples are made directly in an Eppendorf twin.tec PCR 96 well plate to a final volume of 100 µl. Take up the solvent first and then fill up to 100 µl with your sample. For example, for PC, PE take up 99 µl of solvent then 1 µl of sample and pipette into the appropriate well.
- Cover your wells with parafilm to avoid evaporation of your sample during pipetting.
- After you are done, seal the plate with Easy Pierce 20µm Foil (Thermo Scientific).
- Samples should be run immediately. Alternatively, they can be stored overnight at 4°C. Stored samples need to be sonicated before use.

Positive mode solvent
Chloroform/Methanol/Water (2:7:1, v/v) + 5mM Ammonium Acetate
Negative mode solvent
Chloroform/Methanol (1:2, v/v) + 5mM Ammonium Acetate

Always prepare fresh solvent and a fresh solution of 1M Ammonium Acetate (MS grade) in water (MS grade).

Lipid Class	Dilutions by Organism		
PC,PE	1/100	1/10	1/10
PI,PS,CL	1/100	1/4	1/10
SL (positive mode)	1/10	1/10	1/4
SL (negative mode)	1/10	1/4	1/4

Sample Running on the Mass Spectrometer (TSQ Vantage)

- Prepare a .csv sample file for the sequence on the TSQ.
- Naming samples should follow this pattern:

  FreeTextOnSample_MrmMethod_BiologicalReplicate_TechnicalReplicate
- There are 4 different MRM methods:

  - p1 (positive mode, **T**L)
  - p2 (positive mode, **S**L)
  - n1 (negative mode, **T**L)
  - n2 (negative mode, **S**L)

- Biological and technical replicates should be named in numerical or alphabetical order. **Example:** Con_p1_1_1 or Con_p1_1_A means: Control (Con)_ positive mode TL_biological replicate 1_technical replicate 1.
- Be careful with the naming of the folder and of the files since it is very important if you want to send the experiment through the calculation pipeline.
- At the end of the sequence have a quick look at the runs in Excalibur and check that everything is fine for all the samples.
- If there is one run with no signal rerun it immediately (same solvent, same time for the machine) and replace it in the raw file's folder. Zip the folder containing only the experimental raw files and load on http://lipidx.vital-it.ch/base/exps.

Analysis on the LipidX pipeline

- Go to the http://lipidx.vital-it.ch/base/pages/1 website and log in with your user name.
- Go to "Experiments" (menu up the page) and click on New experiment (bottom of the page).
- Fill up the page (Experiment type: MRM analysis) and click on Create button.
- After a certain time the "Experiments" page appears, but the new experiment may not be listed, because it takes some time to convert the .raw files in .txt files.
- When the new experiment is listed, click on the link Show (right of the line). This will open a new page with a listing the experiment files and on top of it a line with different links.
- First, click on Standard concentrations and check that the amount of input for each standard is correct.
- Second, click on Signals. A page with a color code for the intensities appears. If the signal is missing for one of the cycle (very light pink, white), there is a possibility to eliminate it by clicking in the small box up the column. Click then on "Update cycle selection" (bottom right of the page). The data is now ready to be sent to the calculation part of the pipeline.
- Click on "Tools" (menu up the page), then on MRMquant (menu on the right of the page) and on New MRMquant job (top or bottom of the page).
- Fill up the page, be careful with the selection of the calibration curve and click on create.
- Refresh the page until the message "Success" appears in the column status.
- The MRMquant job can then be seen by clicking on Show the (right of the line).
- To download a MRMquant job, select it by clicking in the box on the left and click on "Download".
- The downloaded folder contains for each mode p1, n1, p2 and n2, the following . csv files:
  - _concentrations_by_cycle
  - _log10_median-signal
  - _median_concentration
  - _median_concentration_by_biorepl
  - _st_dev_concentration
  - _st_dev_log10_signals

Sterols: Sample preparation, running on the VARIAN 320MS and analysis

Sample preparation

- Resuspend samples in 250 μl chloroform/methanol (CHROMASOLV LC-MS grade, 1:1) and sonicate for 5 min.
- Dilute in the same solvent (C/M, 1:1).
    - Yeast : dilution 1:5
    - Mammalian : dilution 1:2
    - Worms : very low amount of sterols, big prep needed
    - Flush the samples briefly with N_2 to prevent oxidation of sterols.

Sample Running on the Mass Spectrometer (320MS)

- Fill the Sample List directly on the machine.
- For each sample load 5 μl.
- Run the method : 080604 FS sterols

Analysis

- For each sterol, extract the specific ion.
  Ergosterol (m/z = 396)
  Cholesterol (m/z=386)
  Ergosterol esters (m/z = 378)
  Cholesterol esters (m/z = 368)
- Integrate the area under the peak.
- Transfer these values into Excel and calculate the quantities using the standard curves for cholesterol and ergosterol.

Internal standards for LC-GC / MS

Important

- Keep the standard solutions in amber glass vials.
- Always pipette the standards with Hamilton glass syringes.
- To avoid evaporation and variations, it is strongly recommended to prepare a set of standard mixes for several experiments and keep them dry at -80°C.
- Prepare this set of standard mixes for different numbers of samples (ex.: for 6, 12, and 24 samples) to avoid waste of standards.
- Always prepare at least one more standard mix than the number of samples, e.g. a standard mix for 25 samples will be sufficient for 24 samples. You will have losses on the tube wall and due to evaporation.
- Always use CHROMASOLV, LC-MS grade if possible, solvents.

Standards used for lipidomic

Standard	Organism	Provider	Product Nr
PC31:1 Phosphatidylcholine (PC)17:0-14:1	Y	Avanti	LM-1004
DLPC Phosphatidylcholine (PC)12:0-12:0	M-W	Avanti	850335
PE31:1 Phosphatidylethanolamine (PE)17:0-14:1	Y-M	Avanti	LM-1104
DLPE Phosphatidylethanlamine (PE)12:0-12:0	W	Avanti	850702
PI31:1 Phosphatidylinositol (PI)17:0-14:1	Y-M-W	Avanti	LM-1504
PS31:1 Phosphatidylserine (PS)17:0-14:1	Y-M	Avanti	LM-1304
DLPS Phosphatidylserine (PS)12:0-12:0	W	Avanti	840038
CL56:0 Cardiolipin (CL)14:0-14:0-14:0-14:0	Y-M-W	Avanti	710332
C17Cer Ceramide (Cer)d18:1-17:0	Y-M-W	Avanti	860517
C8GC Glucosylceramide (GlcCer)d18:1-8:0	Y-M-W	Avanti	860540
C12SM Sphingomyelin (SM) d18:1-12:0	Y-M-W	Avanti	860583
C16 deoxyDHCer	W	Avanti	860462
Cholesterol	Y	Sigma	C-8667
Ergosterol	M-W	Fluka	45480

Y=Yeast M=Mammalian W=Worms

The lipid standards are synthetic analogs of natural gycerophosphoipids and sphingolipids which can be obtained from Avanti Polar Lipids. For sterol analysis we use cholesterol for yeast samples as standard since the endogenous sterol of yeast cells is ergosterol and ergosterol for mammalian samples and worms as the endogenous one is cholesterol.

Yeast standard mix

For the yeast, a mix of 7 different lipid standards is used for the glycerophospholipid and sphingolipid analysis and cholesterol for the sterol analysis

Glycerophospholipid and sphingolipid standard mix for 25OD **:**

Standard	nmole	µl stock
PC31:1	7.5	7.5
PE31:1	7.5	7.5
PI31:1	6	6
PS31:1	4	4
CL56:0	4	4
C17Cer	1.2	12
C8GC	2	2

Cholesterol standard for 25OD :

Standard	nmole	µl stock
Cholesterol	20	20

Mammalian standard mix

For mammalian lipid analysis, a mix of 9 different lipid standards is used.

One mix is for a confluent 6-10cm dish of mammalian cells (≈ 1 x 10^6 CHO cells) or 20-30mg of tissue.

Standard	nmole	µl stock
DLPC	0.4	0.4
PE31:1	1	1
PI31:1	1	1
PS31:1	3.3	3.3
CL56:0	0.7	0.7
C12SM	2.5	2.5
C17Cer	0.5	0.5
C8GC	0.1	0.1
Ergosterol	8	8

Worm standard mix

For worm lipid analysis, a mix of 9 different lipid standards is used.

One mix is for 8000 worms.

Standard	nmole	µl stock
DLPC	0.4	0.4
DLPE	1	1
PI31:1	1	1
DLPS	3.3	3.3
CL56:0	0.7	0.7
C12SM	2.5	2.5
C17Cer	0.5	0.5
C8GC	0.1	0.1
C16 deoxyDHCer	0.1	0.1

Preparation of the standard mixes

Preparation of standards stock solutions

- Prepare a stock solution of 1.0 μmol/ml of each standard in Chloroform/Methanol (1:1).
- Confirm the concentration of PC, PE, PI, PS and CL by a phosphate assay.

Pipetting and handling

- Pipette the appropriate amount of each standard in a 2 ml amber vial.
- Dry and keep at -80°C.
- Just before use, resuspend the mix in the appropriate amount of Chloroform/methanol (1:1): (number of samples + 1) *20 or 25 µl.
- Vortex and sonicate for 5min.
- Add 20 or 25 µl of the standard mix or sterol standard to each sample at the beginning of the extraction (use Hamilton glass syringes).

Detection on the Mass Spectrometer

LC-MS on TSQ

Detection of Lipids by MS/MS

Lipid class	standard	Polarity	Mode	m/z ion
Phosphatidylcholine $[M+H]^+$	**PC31:1**	+	Product ion	184.07
Phosphatidylethanolamine $[M+H]^+$	**PE31:1**	+	Neutral ion loss	141.02
Phosphatidylinositol $[M-H]^-$	**PI31:1**	-	Product ion	241.01
Phosphatidylserine $[M-H]^-$	**PS31:1**	-	Neutral ion loss	87.03
Cardiolipin $[M-2H]^{2-}$	**CL56:0**	-	Product ion	acyl chain
Ceramides $[M+H]^+$	**C17Cer**	+	Product ion	264.30
MIPC $[M-H]^-$	**C8GC**	-	Product ion	241.01
MIPC $[M-H]^-$	**C8GC**	-	Product ion	421.07
M(IP)2C $[M-H]^-$	**C8GC**	-	Product ion	241.01

LC-MS standard transitions

Standard	Polarity	m/z (Q1)	m/z (Q3)
DLPC	+	622.44	184.07
PC31:1	+	718.54	184.07
DLPE	+	580.40	439.38
PE31:1	+	676.49	535.47
C12SM	+	647.51	184.07
C8GC	+	588.43	264.30
C17Cer	+	552.54	264.30
C16 deoxyDHCer	+	524.54	268.30
PI31:1	-	793.49	241.01
DLPS	-	622.37	535.34
PS31:1	-	718.47	631.43
CL56:0	-	619.42	227.20
C8GC	-	586.43	424.38

GC-MS on Varian

- Full scan
- Mode EDR

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