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MS samples preparation by acetone precipitation

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We use this protocol and it's working

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Disclaimer

Abstract

In a proteomics pipeline a correct sample preparation is a crucial step as it deeply influence the subsequent data acquisition (usually performed by liquid chromatography couple to mass spectrometry LC-MS) as well as data analysis. This procedure can be conducted using different techniques, from in-gel digestion to filter based sample preparation. The present document illustrate how to perform in-solution sample preparation. Regardless of which procedure is chosen, all sample preparation techniques share the same basic principles and follows similar step-wise procedure (Muntel et al., 2019; Ombrato et al., 2019; Sacramento et al., 2019).

Guidelines

General information and tips:

- For preparing samples to analyze with LC-MS we will need from min 1 µg/µl to max 4 µg/µl (digest check)
- Each cell line/cell type has a different amount of proteins after lysis. There are different ways to estimate proteins amount from a sample:

- If the cell line/tissue is known, protein amount can be estimated based on cell number
- If the cell line/tissue or sample characteristics are unknown, perform a protein quantification assay (like e.g. BCA, or run a Coomassie gel with a known MEF QC sample as comparison)
- If these two alternatives are not practicable (e.g. there's not enough material), estimate the protein amount from cell pellet dimension (use MEF as comparison)

- Always include an additional MEF pellet as a process control (checking pellet dimensions and therefore protein amount, MS runs etc...)

Materials

Reagents

- 1 x PBS (137 mM NaCl, 2,7 mM KCl, 8 mM NA2 HPO4 und 2 mM KH 2PO4, pH 7,4, RNAse free, 25x protease inhibitor and 10x phosphatases inhibitor both diluted to 1 x)
- Protease inhibitor (cOmplete ULTRA Tablets, #05892791001, Roche): 1 tablet in 2 mL milliQ water for a 25x solution, to be aliquoted. The final solution of Protease inhibitor in PBS should be 1x
- Phosphatases inhibitor (PhosSTOP, #4906837001, Roche): 1 tablet in 1 mL milliQ water for a 10x solution, to be aliquoted. The final solution of Protease inhibitor in PBS should be 1x
- 10x Lysis Buffer (1000 mM HEPES, 500 mM DTT, 20%SDS)
  fc 1× 100 mM HEPES, 50 mM DTT, 2% SDS
  NB: always calculate the right volumes of the following reagents depending on the final concentration of SDS that needed in the buffer: 1 M HEPES pH 8 (#H3375, Sigma)(stored at -20° C), 1 M DTT (#6908.3, Roth) prepared freshly; NB: For 10x buffer, SDS has to be used for diluting DTT in order to achieve the right final concentration!) 20% SDS (#75746, Sigma)
  NB: If K^+ inside, precipitation, resuspend in H_2O. NB: SDS is viscous, pipette with caution.
  NB: 1-2% SDS for 8x Acetone precipitation, for mild conditions or low concentration of proteins
  2-4% SDS for 4x Acetone precipitation, for harsh conditions or high amounts of proteins
- 200 mM IAA (#I1149, Sigma), (add 1 ml of MilliQ H_2O to a readymade aliquot stored in DARK! After preparation keep it at room temperature in the dark)
  NB: If acetone precipitation is not performed immediately, long incubation with IAA might lead to the formation of artifacts. If precipitation cannot be done immediately, consider using alternatives like CAA
- Digestion buffer
  1 M Guanidine HCl (#0035.1, Roth)
  100 mM HEPES (#H3375, Sigma)
  NB: Alternatively to Guanidine, Urea 3 M could be used as digestion buffer.
- MilliQ-Water
- 100% Acetone (#0001037801BS, Biosolve)
- 80% Acetone (stored in the -20° C)
- Lysyl Endopeptidase (LysC) (#125-05061, Wako) 0.1 µg/µl starting from a stock solution 0.5µg/µl
  NB: Stock powder is stored in the first -20° C freezer on the right hand side of the central aisle of lab. To prepare it dissolve 20 µg in 40 µl of either water or trypsin buffer to obtain a 0.5µg/µl stock solution, then aliquot it and dilute with digestion buffer or water to 0.1 µg/µl. Always work in ice to prevent degradation of the enzyme and avoid freeze/thaw cycles
  NB: The concentration (µg/µl) of LysC depends on the amount of protein to digest!
- Sequencing Grade Modified Trypsin (#V5111, Promega) 0.1 µg/µl (starting from a stock solution 0.5µg/µl
  NB: To prepare a 0.5 µg/µl stock trypsin follow the same procedure as for LysC
  NB: Trypsin has always to be dissolved in its own buffer, otherwise it will autodigest!
- 10% Trifluoroacetic acid, ULC/MS-CC/SFC (#0020234131BS, Biosolve)
- Acetonitrile, ULC/MS-CC/SFC (#0001204101BS, Biosolve)
- Formic acid 99%, ULC/MS-CC/SFC (#0006914143BS, Biosolve)
- Methanol 100% (#34860-2.5L-R, CHROMASOLV)

- Oasis Buffer A: 0.05% Formic Acid (FA)(v/v) in MilliQ water
- Oasis Buffer B: 80% Acetonitrile, 0.05% FA (v/v), in MilliQ water
- MS Buffer A (5% (v/v) acetonitrile, 0.1% (v/v) TFA in water)
- iRT kit peptides (#Ki-3002-1, Biognosys)
- Milli-Q water system (Merck, Advantage A10)

Equipment and materials

- Eppendorf 1.5 ml SafeLock tubes (#0030 120.086, Eppendorf)
- Eppendorf 0.5 ml SafeLock tubes (#0030 121.023, Eppendorf)
- 1 mL, 200 µl and 10 µl pipette tips (Sarsted)
- 1 mL, 200 µl and 10 µl LoBind pipette tips (Starlab)
- PCR stripes (#SP-1502-125, Ratiolab)
- Bioruptor plus (#B01020001, Diagenode)
- Centifuge 5804R (#5805000010, Eppendorf) or Eppendorf 5810R (#5811000015, Eppendorf)
- Heatblock (#SBH130D3 or #SBH200D3, Stuart)
- Thermo Mixer C (#5382000015, Eppendorf)
- Vacuum pump
- OASIS Oasis 96-well plate vacuum manifold (# 186001831, Waters)
- Waters Oasis HLB µElution plates 30 µg(for maximum 100 µg proteins) (#186001828BA, Waters)
- SpeedVac (Concentrator Plus/Vacofuge Plus, #5305000100, Eppendorf)
- Glass vials and glass inserts for LC-MS (#88909355 vials, #93909134 inserts, #88849362 caps, VDS optilab)

Safety warnings

- This protocol includes many steps that involve the use of dangerous chemical substances. Please read carefully the chemical safety sheets before starting any experiment!
- This protocol includes steps that involve heating and work under pressure. Please be extremely careful when working with vacuum instruments or around heated surfaces!
- This protocol includes steps that involves centrifugation. Please always balance centrifuges before usage to avoid property damages and incidents!

Before start

Abbreviations

- MEF: Mouse Embryonic Fibroblast
- PBS: Phosphate Buffer Solution
- HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonicacid
- DTT: Dithiothreitol
- SDS: Sodium dodecyl sulphate
- IAA: Iodoacetamide
- ACN: Acetonitrile
- TFA: Trifluoroacetic acid
- FA: Formic Acid
- QC: Quality control
- DC: Digest Check
- DIA: Data Independent Acquisition

DAY 1

1 **Lysis, protein denaturation/alkylation**

1.1 Add 1xPBS to cell pellet. The exact volume in µl has to be calculated to have a good resuspension of the cell pellet (e.g. 200 µl for 2 million cells)

NB: To obtain a better resuspension, vortex and biorupt the cell pellet (5 cycles, 60 sec on, 30 sec off, 4 ° C), or pipet up and down the pellet multiple times

NB: : Add PBS always first. If lysis buffer is added first, the cell will be disrupted and surrounded by sticky DNA → no complete lysis

1.2 Add 2x Lysis buffer.
Calculate the exact volume in µl in order to have a fc 1× 100 mM HEPES (pH 8), 50 mM DTT, 1-4% SDS

NB: the concentration of SDS depends on the conditions of the specific lysis, see introduction and reagents

1.3 Biorupt (10 cycles, 60 sec on, 30 sec off, 20 ° C) ~ 15 min. total time

1.4 Vortex and spin down briefly → a DNA ring will appear and sample getting milky

1.5 Boil 10 min 95°C (turn on the heater at the beginning of the biorupt cycle)

1.6 Biorupt (10 cycles, 60sec on, 30sec off, 20°C). NB: for tissue even 3 biorupting cycles, always check if the DNA is sheered or not

1.7 Vortex and spin down briefly

1.8 Add 200 mM IAA. Calculate the exact volume in µl in order to have a fc of 15 mM

NB: in case no DTT is present in the lysis buffer, add DTT to a fc of 10 mM and incubate 15 min at 45°C BEFORE the IAA step

NB: If precipitation is not performed immediately, long incubation with IAA might lead to the formation of artifacts. If precipitation cannot be done immediately, consider using alternatives like
CAA.

1.9 Incubate 30 min in the dark at RT

1.10 If needed, aliquot the samples in new tubes to get the desired amount of proteins for the following steps. Correctly label the lysate leftovers (name, date and amount of protein in µg/ µl)

NB: This step can be done also before adding IAA
NB: Always lyse all the pellet, it can always store after lysis step
NB: Always label the new sample tubes on top and on side to avoid a complete wash off of ink if acetone drops will fall on it
Tips: To have a better estimation of the protein amount, use some lysate for a protein amount test (e.g. SDS-PAGE gel followed by Coomassie Staining). If there's not enough material, check cell pellet dimension.

2 **Acetone precipitation**

2.1 Add 4 or 8 volumes of ice-cold acetone, depending on lysis conditions

NB: 1-2% SDS for 8x Acetone precipitation, for mild conditions or low concentration of proteins
2-4% SDS for 4x Acetone precipitation, for harsh conditions or high amounts of proteins

2.2 Vortex and spin down

2.3 Let it precipitate at -20°C o/n

NB: samples can be stored in acetone before further sample preparation. Samples can last ca. 1-2 weeks.

DAY 2

3 Centrifuge at 4 °C, 14000rpm, 30 min
NB: 0.5-2 ml Eppendorf Tubes: spin 30 min at 14.000 rpm and 4°C (Eppendorf 5804R ~20800g)

5 ml Eppendorf Tubes: spin 30 min at 4.000 rpm and 4°C (Eppendorf 5810R ~3220g)
Tips: The time for the centrifuge is automatically set. However, samples need to be taken out from the centrifuge immediately after centrifugation. To avoid problems with this, run the centrifuge on infinite settings and remove the samples whenever ready. Longer centrifugation times are not harmful to the samples in this step

4 Prepare LoBinding Tips for precipitation (small tips, better for removing the liquid and to dispense without losing material)

5 Remove acetone.
Tips: To avoid pellet detachment use LoBinding Tips.

6 Wash 2x with appropriate amount of cold 80% acetone (for most of the cases, 300 µl). Vortex the samples each time, then centrifuge:
1. wash: centrifuge 4 °C, 14000 rpm 10 min
2. wash: centrifuge 4 °C, 14000 rpm 2 min
NB: Volume might slightly change based on number of cells/pellet dimension
Tips: To keep the acetone cold, put a glove around the bottle before putting it in an ice bath.

7 Dry pellet, first removing most of the volume by pipetting, then the rest evaporating on the bench for few minutes
NB: The pellet colour will turn to yellow or white

8 **Digestion**

8.1 Prepare digestion buffer 1 M Guanidine HCl (in 100 mM HEPES, see also Materials section)

8.2 Add 25 µl digestion buffer per sample
Tips: This volume will also wet the 1,5 mL tube
NB: If the protein amount is < 25 µg, add anyway 25 µl. This is the minimum volume!

8.3 Biorupt (5 cycles, 60sec on, 30sec off, 20°C)

8.4 Add 0.1 µg/µl LysC. Calculate the exact volume in µl in order to have an enzyme:protein ration 1:100
NB: If the samples is more concentrated, then add a concentrated enzyme

8.5 Incubate at 37 °C for 4 h and 650rpm (first hour 1000rpm) in the Thermo Mixer C

NB: LysC digestion is already over after 30 min, 4h treatment is a safety measurement to allow a 100% complete digestion. If after LysC something is still floating, the trypsin will destroy it

8.6 Add 25 µl of water and 0.1 µg/µl trypsin. Calculate the exact volume of trypsin in µl in order to have an enzyme:protein ration 1:100
NB: Trypsin has to be diluted in its own buffer, to avoid auto-digestion
NB: If the samples is more concentrated, then add a concentrated enzyme

8.7 Incubate at 37 °C and 650rpm in the Thermo Mixer C overnight

DAY 3

9 **Peptide clean-up with Oasis**
NB: Use this when protein amount is less than 100 µg

9.1 Acidify samples with 10% TFA. Add a volume of TFA that is ca. 1/20 of the sample volume
NB: Check acidification on paper! Color should be turning to red (pH 1 or 2)
NB: Acidification will inactivate trypsin
NB: TFA is in an aluminum wrapped bottle because it is light sensitive
Tips: A multi-pipette with combitip to acidify in series can be used, using labelled, using labelled tips 1-4 µL

9.2 Prepare PCR collection tubes
NB: Label the tubes on top and on side with sample name, initials, date and amount of digested proteins
Tips: If few samples, use PCR tubes with connected lids, if a lot of samples, use those with separated lids. One can use the tubes with attached lid if using rows or columns on the outer side of the Oasis 96 well plate

9.3 Place waste collection tray inside the manifold, assemble the manifold and place the Oasis 96-wells plate on top. Mark the wells to be used with marker pen
NB: Take care not to spill solvent on the upper surface of the plate
NB: Start the Speed-Vac in order to make it warm up for the drying step

9.4 Condition the wells with elution buffer using 3× 100 µL Buffer B (80% (v/v) acetonitrile; 0.05% (v/v) formic acid) under slow vacuum (approx. ~ 1 drop per sec)

9.5 Equilibrate the wells so that they can bind peptides with 3× 100 µL Buffer A (0.05% (v/v) formic acid in milliQ water) under slow vacuum (approx. ~ 1 drop per sec)

Tips: Use plastic trays for dispensing the solvents with multichannel pipettes. Be careful not to mix the solvents!

9.6 Add 100 µL Buffer A to the wells and don't run through with the vacuum

9.7 Load sample into well and allow to run through under slow vacuum

9.8 Wash the wells to desalt the samples with 3× 100 µL Buffer A

9.9 Replace the waste tray with the collection vessels. (Stack 3 96-well holder to reach the right height for PCR tubes).
NB: Ensure that the PCR Tubes are right below the PCR tray and in the right order. Press well the manifold and check if the vacuum is working

9.10 Elute samples using 2×50 µL of Buffer B with slow vacuum. After no liquid in the wells can be seen, a higher vacuum can be applied to ensure full recovery
NB: When taking up the HLB plate, be sure to collect all the drops and not lose them or spill into the next collection tube

9.11 Dry the samples with the SpeedVac (45°C, ca. 30 – 50 min)
NB: samples can be stored at -20°C after peptide clean up before further sample preparation
NB: Discard the collected waste in the special waste bottle labeled Phenol/Chloroform waste (=halogenated solvents) because of the TFA!

DAY 4 (or the same day)

10 Reconstitute in MS Buffer A (5% (v/v) acetonitrile, 0.1% (v/v) TFA in water) to a final concentration of 1 µg/µl

11 Biorupting (3 cycles, 60sec on, 30sec off)

12 For Data Indipendent Analysis (DIA) sample preparation: for 10 µL sample (1 µg/µl) add 0,5 µL iRT (1:2 diluted).
NB: To prepare diluted iRT, follow the manufacturer manual:
- Add 50 µL of iRT buffer (blue cap vial) to the 2x iRT mix (red vial) → 10x iRT Kit
- Vortex
- Sonicate for 3-5 minutes
- Take the amount of iRT mix needed and dilute it 1:2 with MS Buffer A

- Add 0,5 µL to each insert, then add 10 µL of sample

Samples are now ready to run on a Mass Spec. You can keep the prepared samples for approx. 1-2 weeks at 4°C. Freeze leftover samples at -20°C.

Protocol references

Muntel et al., 2019; Ombrato et al., 2019; Sacramento et al., 2019