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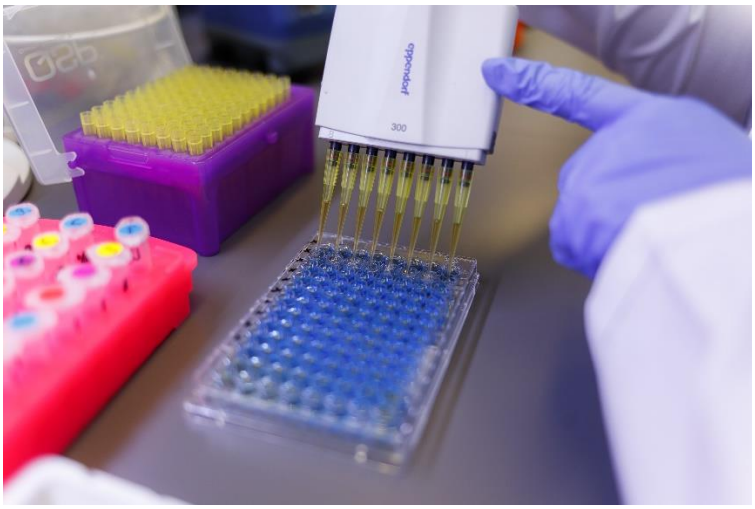
#3 PROTEIN EXTRACTION, QUANTIFICATION, ENZYMATIC DIGESTION AND SAMPLE DESALTING FOR PROTEOMIC STUDIES

PROTEIN EXTRACTION FROM BIOFLUIDS FOR PROTEOME PROFILING BY MASS SPECTROMETRY

Research Center for Advanced Medicine – Medfuture (UMFIH) can receive and process various biological samples, including liquid biopsies like blood, urine and saliva samples, but also solid biopsies meaning mainly tissue samples. All samples need to be collected, processed, aliquoted and stored according to #9 COLLECTION AND PROCESSING OF SAMPLES FOR PROTEOMIC STUDIES



Prior analysis, proteins need to be extracted from the biological samples. One way to extract proteins from biofluids is by precipitation with organic acids. The principle behind the method relies on the ability of the organic acid to alter the solubility of the proteins. Thus, in the presence of an organic acid the proteins will be precipitated and can be separated by centrifugation from the biofluid and further analyzed. Several organic acids and protocols can be used, the one with trichloric acid and cold acetone is presented here and can be applied on all biofluids.

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1.1. Protein extraction from biofluids by precipitation with trichloric acid and cold acetone

- Aliquotes of biofluids are directly processed after collection and preparation or after long term storage at -80°C
- Bring sample at room temperature or taw on ice if previously stored at -80°C
- Mix sample thoroughly by vortexing
- Centrifuge at 13000*g*, at 4°C, for 2min to remove any cell debrits
- Transfer the supernatant for protein extraction in a new protein low binding tube
- Measure the total volume of your sample
- Add up to 15% trichloroacetic acid solution (100%)
- Mix thoroughly by vortexing 2x20sec or 2 min by 1400rpm in the labmixer
- Immerse tubes in the ice and incubate for 60min
- Centrifuge at 13000x*g*, 4°C, 30 min-> a white pellet will be visible
- Take the supernatant to organic solvents waste recipient
- Wash 2 times with cold acetone (-20°C)
- Centrifuge 2x at 13000 x *g*, 4°C, for 10min
- Take the supernatant to organic solvents waste recipient
- Keep the tubes open under the fume hub for 5 min or speedvac for 2 min
- Check that the residual organic solvent evaporates completely
- Resuspend the pellet in 8M urea /2M thiourea buffer
- Mix thoroughly by shaking at 1400rpm, for 20min in the labmixer or until the sample is completely dissolved
- Freeze samples at -80°C until further analysis

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PROTEIN EXTRACTION FROM FRESH FROZEN TISSUE SAMPLES FOR PROTEOME PROFILING BY MASS SPECTROMETRY

Research Center for Advanced Medicine – Medfuture (UMFIH) can receive and process various biological samples, including liquid biopsies like blood, urine and saliva samples, but also solid biopsies meaning mainly tissue samples. All samples need to be collected, processed, aliquoted and stored according to #9 COLLECTION AND PROCESSING OF SAMPLES FOR PROTEOMIC STUDIES

Prior analysis, proteins need to be extracted from the biological samples. Proteins are inside the cells in tissue samples. Thus, their extraction implies aggressive and mechanical methods in order to disrupt the tissue structure and the cell walls and extract the proteins. One way to disrupt the membranes is to lize the membrane by using a lysis buffer which contains detergents and several other reagents, to freeze in liquid nitrogen and taw the sample for several times and to apply mechanical forces like bead mill. One possible protocol is presented here.

1.2. Protein extraction from fresh frozen tissue samples by bead mill extraction

- Aliquotes of tissues are directly processed after collection and preparation or after long term storage at -80°C
- Bring sample at room temperature or taw on ice if previously stored at -80°C
- Wash tissue samples with cold PBS to remove any cell debrids or blood

- Centrifuge at 13000x*g*, 4°C, 2 min
- Take the supernatant to biological waste recipient
- Repeat 2 times
- Take the desired tissue amount and place it in the bead mill container together with the appropriate number of beads
- Immerse in liquid nitrogen
- Screw the container and carefully place the lid and the base in liquid nitrogen
- Caution: Take care while keeping the container base so that the beads do not come out
- add a minimum volume of lysis buffer directly on the tissue, hold once in liquid litrogen and screw the cap.
- fix the container with tissue in the bead mill and spin at the desired speed according to the type of tissue processed
- defrost the tissue powder and rinse out with lysis buffer
- collect into protein low binding tubes (ex. 500µl each)
- put the samples on ice and vortex
- sonicate the samples in the sonicator according to the type of sample
- centrifuge at 13000 x *g*, 4°C, for 1h
- Take the supernatant in a new protein low binding tube
- Freeze samples at -80°C until further analysis

PROTEIN EXTRACTION FROM CELL CULTURE SAMPLES FOR PROTEOME PROFILING BY MASS SPECTROMETRY

Research Center for Advanced Medicine – Medfuture (UMFIH) can receive and process various biological samples, including liquid biopsies like blood, urine and saliva samples, but also solid biopsies meaning mainly tissue samples. Even more, cell culture samples can be also processed. All samples need to be collected, processed, aliquoted and stored according to #9 COLLECTION AND PROCESSING OF SAMPLES FOR PROTEOMIC STUDIES

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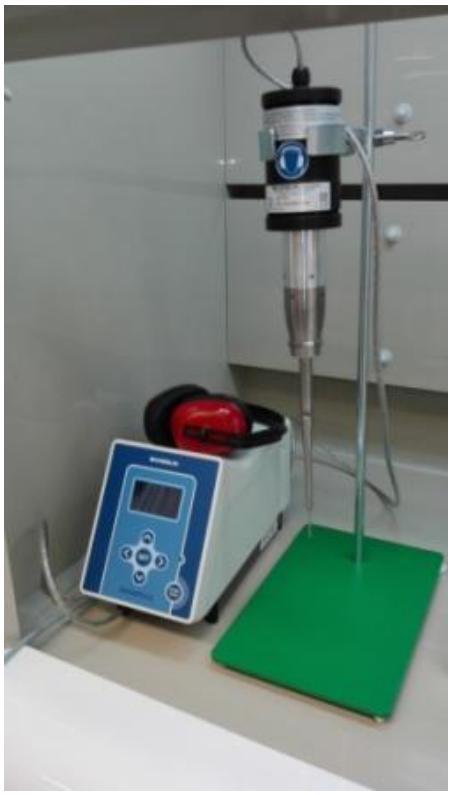


Prior analysis, proteins need to be extracted from the biological samples. Proteins are inside the cells in cell culture samples. Thus, their extraction implies aggressive methods in order to disrupt the cell walls and extract the proteins. One way to disrupt the membranes is to lize the membrane by using a lysis buffer which contains detergents and several other reagents, to freeze in liquid nitrogen and taw the sample for several times and to apply ultrasounds. One possible protocol is presented here.

1.3. Protein extraction from cell culture samples by freeze taw

Minimum $5x10^5$ cells are needed - best would be $5x10^6$ cells – or all cells from one well of a 6 well plate! At the end of the protein extraction, 10µg protein/ sample are needed for 2 technical replicates.

- After medium removal from the plate/flask, wash cells three times with cold PBS. Remove PBS completely.
- Harvest cells in the minimum volume of 8M urea /2M thiourea buffer (start with 100µL UT for example).
- Snap freeze cell lysates by immersion in liquid nitrogen.

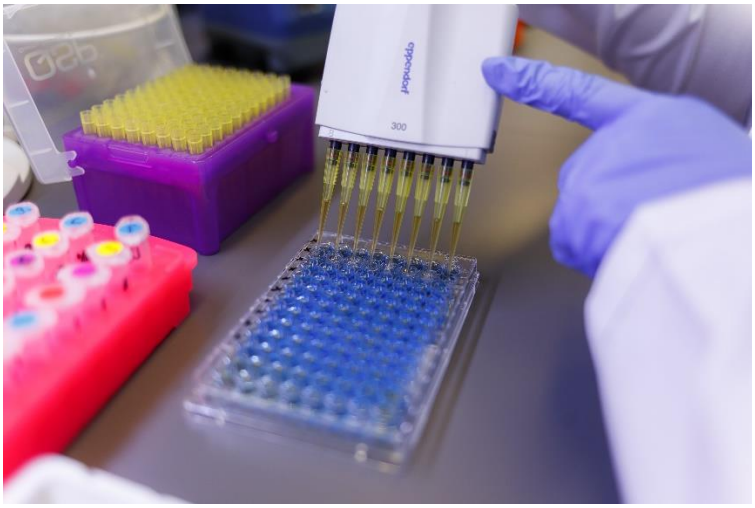
■**PAUSE POINT** -> The samples can be stored at -80°C/ shipped on dry ice.

- Incubate for 10 min at 30°C at 1400 rpm in the labmixer.
- Mix by vortexing.
- Repeat freeze and taw for a total of 5 times.
- Sonicate with 3 x 3 sec pulses 50% power
- Centrifuge the lysates at 13000*g* at 4°C for 1 hour.
- Transfer the supernatant into a protein low binding tube, discard the pellet.

■**PAUSE POINT** -> The samples can be stored at -80°C/ shiped on dry ice.

PROTEIN CONCENTRATION DETERMINATION BY BRADFORD IN MICROPLATES

Research Center for Advanced Medicine – Medfuture (UMFIH) can receive and process various biological samples, including liquid biopsies like blood, urine and saliva samples, but also solid biopsies meaning mainly tissue samples. All samples need to be collected, processed, aliquoted and stored according to #9 COLLECTION AND PROCESSING OF SAMPLES FOR PROTEOMIC STUDIES



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After protein extraction from the biological samples, a total protein concentration determination is the next stept to downstream analysis in proteomics. There are several methods to establish the protein concentration, one frequently used being Bradford method. Bradford is an organic reagent which forms with proteins a stable blue-colored complex that can be measured by spectrofotometry at 595nm. There are several kits on the market, each coming with specific protocols. In principle a standard curve needs to be done and the sample concentration will be determined against it. Modern methods use small amounts of samples, thus the microplate method will be presented further.

1.1 Protein concentration determination by Bradford in 96-well microplate

- Please refer to the Bradford kit product instruction's manual or a general protocol as following:
- Prepare a standard curve using bovine serum albumin or the protein provided by the kit by preparing 7 points of concentration, where the middle point is the expected concentration of your sample
- Plot the standard curve and the corresponding equation and r^2

- Prepare the samples and the sample blanc according to the standard curve

2. Read the samples against the standard curve and calculate the concentration
3. Coomassie Plus (Bradford) Assay Kit (Thermo Scientific Cat. No. 23236) example
    1. Albumin standard (BSA) curve preparation 1-25µg/mL

- Take 1 ampule of 2mg/mL of Albumin standard provided by the kit
- Pipette according to the kit instructions as following

	Tube	Diluent (µL)	BSA (µL)	Final BSA concentration (µg/mL)
1	A	3555	45	25
2	B	6435	65	20
3	C	3970	30	15
4	D	3000	from tube B 3000	10
5	E	2500	from tube D 2500	5
6	F	1700	from tube E 1700	2.5
7	G	4000	0	0
	Total µL	25160		

*Diluent can be distilled water or a 1:30 dilution of sample buffer (900µL + 26100µL distilled water – total volume = 27000µL)

- Use immediately or aliquote and store at -20°C

2. Coomassie Plus (Bradford) Assay Reagent

- Bring the Bradford Reagent from the fridge and let it reach the room temperature
- Mix wel and aliquote a desired working solution into a 15mL tube
- Bring the Bradford Reagent back to the fridge

3. Method

- Pipette 150µL standard curve in each well in duplicates
- Sample preparation
    - Taw the sample on ice
    - Mix throughly or centrifuge at 13000*g* for 2 min if needed to remove cell debrits
    - Take 10µL of a 1:1 dilution first to see the range for the dilution needed
    - Pipette 140µL in each well in duplicates
- Add 150µL of the Bradford Reagent in each well
- Put the 96-well microplate into the microplate reader and select the Read Method at 595nm after 10min of incubation
- Calculate the sample concentration
- Discard samples

ENZYMATIC DIGESTION OF PROTEIN SAMPLES FOR PROTEOME PROFILING BY MASS SPECTROMETRY

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After protein extraction and protein concentration determination, reduction, alkylation and enzymatic digestion follows towards proteome profiling by mass spectrometry. A possible protocol is presented further.

3.1. Reduction, alkylation, and digestion by trypsin of proteins before proteome profiling by mass spectrometry

- take a 4µg protein sample volume
- fill up sample volume to end concentration of maximum 10% urea/thiourea buffer with ammoniumbicarbonate buffer (ABC) 20mM
- add dithiothreitol up to end concentration of 2.5mM)
- incubate for 60min at 60°C
- add iodoacetamide to an end concentration of 10mM
- incubate for 30min at 37°C (protect from light!)
- add trypsin 1:25 (trypsin: protein ratio)

4. incubate overnight at 37°C in the incubator
5. stop the digestion by acidifying the sample with an acid solution
6. proceed to the desired sample purification protocol prior mass spectrometry (ex. ZipTip C_{18})

DESALTING OF PEPTIDE SAMPLES FOR PROTEOME PROFILING BY MASS SPECTROMETRY

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After enzymatic digestion, sample clean-up prior mass spectrometry needs to be done in order to remove all the reagents and salts that might interfere with the mass spectromery analysis. One possible way ist o clean-up the samples by using micro-column built in tips, the so called ZipTip. One possible protocol is presented further.

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1.1 Sample desalting by μC$_{18}$ ZipTip for proteome profiling by mass spectrometry

- Follow the product instruction's manual or a general protocol as following:
- equilibrate a U C_{18} ZipTip with acetonitrile
- equilibrate a U C_{18} ZipTip stepwise with 3 decreasing concentrations of acetonitrile in mobile phase of the mass spectrometry analysis and 100% the aquous mobile phase of the mass spectrometry analysis
- bind the peptides on the ZipTip: aspirate and dispense the sample
- wash with the aquous mobile phase
- elute the peptides with the medium concentration of acetonitrile solution in the aquous mobile phase
    - pipette sample on the ZipTip and transfer to a mass spectrometry vial
- elute the peptides with the high concentration of acetonitrile solution in the aquous mobile phase
    - pipette sample on the ZipTip and transfer to a mass spectrometry vial
    - freeze the vials at -80°C and concentrate the samples by liophillization

Disclaimer: This protocol was realised with the EEA Financial Mechanism 2014-2021 financial support through the project Cooperation strategy for knowledge transfer, internationalization and curricula innovation in the field of research education at the 3rd level of study – AURORA. Its content (text, photos, videos) does not reflect the official opinion of the Programme Operator, the National Contact Point, and the Financial Mechanism Office. Responsibility for the information and views expressed therein lies entirely with the authors.