Standard Operating Procedures (SOP) for cell culture metabolomics at the Metabolomics and Proteomics Core Facility

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1. Introduction to metabolomics

Metabolomics investigates all metabolites in a biological system (cell, tissue, or organism) under a given set of conditions and provides a versatile functional phenotyping.
Generally, metabolomics analyses can be divided into two approaches, non-targeted and targeted. Non-Targeted Metabolomics aims to detect as many metabolites as possible. Despite being dependent on the analytical platform and the sample collection procedure, non-targeted metabolomics can provide a detailed assessment of the metabolites in a sample by revealing a wide range of metabolites of different chemical classes. Thus, non-targeted metabolomics can generate new hypotheses and drive the next step of research. However, it is not yet possible to obtain all metabolite classes simultaneously, because many factors influence the metabolite detection (e.g., composition of extraction solvent, column chemistry, ionization techniques). In addition, non-targeted metabolomics does not allow absolute quantification, but only permits semi-quantitative quantification. Detection of a large number of unknown metabolites not yet annotated in metabolite databases is a common phenomenon in non-targeted metabolomics. Targeted Metabolomics is used to quantify the absolute concentrations of a predefined set of metabolites. This technology is based on standard calibration curves for the selected analytes and requires the use of stable isotope-labeled or other suitable internal standards. Thus, targeted metabolomics methods are validated for analytical performance. Due to absolute quantification of the metabolites in the sample, the results are comparable to other quantitative data. A drawback of targeted metabolomics is the limited set of simultaneously quantified metabolites, with the possibility of overlooking some biological processes.

The analysis of cultured cells by metabolomics can complement data obtained from body fluids or other biological matrices. Cultured cell metabolomics has shown its usefulness in many areas such as toxicity, pharmacology, cell culture monitoring, and researches on diseases.

The Metabolomics and Proteomics Core Facility offers the following assays to their collaborators for cell culture based samples:

Non-Targeted Metabolomics	Targeted Metabolomics
• Non-targeted Metabolomics using the technology from Metabolon Inc.	• Absolute *IDQ*™ p150 Kit (Biocrates) • Absolute *IDQ*™ p180 Kit (Biocrates) • MxP Quant® 500 Kit (Biocrates) • Lipidizer (Sciex) • Newborn Screen (ChromSystems)

Information on which metabolites are covered by the different assays can be found on the web page of the Metabolomics and Proteomics Core Facility:
https://www.helmholtz-muenchen.de/gac/platforms/metabolomics/portfolio/index.html

The following issues need special attention:

- Cases in which the analysis of samples with several techniques is desired (e.g., analyzing one sample with non-targeted metabolomics and an additional specific targeted metabolomics assay). In these cases, the samples need to be collected according to the SOP for harvesting cells for Non-Targeted analyses and it has to be considered that two analytical runs from one sample require more sample volume. Please contact one of the authors of this SOP for advice in study design and sample collection.

- The content of this SOP and the protocols for sample collection are based on our experiences with several but not all established cell lines of human and animal origin. Further cell lines, cell types, or cell strains might require special conditions and adapted sample harvesting protocols for metabolomics. Please contact one of the authors of this SOP for advice on study design and harvesting procedures.

- A method enabling data normalization should be considered and performed prior to the metabolomics measurements. Especially for adherent cell samples we recommend the Hoechst assay [1] with which the DNA content and thus indirectly the cell number in the metabolomics sample can be detected. Please contact one of the authors of this SOP if you wish the Hoechst assay to be performed on your samples.

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[1] Muschet, C., Möller, G., Prehn, C., Hrabě de Angelis, M., Adamski, J., Tokarz, J.: Removing the bottlenecks of cell culture metabolomics: fast normalization procedure, correlation of metabolites to cell number and impact of the harvesting method. Metabolomics (2016) 12: 151. Doi:10.1007/s11306-016-1104-8)

2. Special materials

2.1. Tubes for cell sample collection

Cells for metabolomics analyses have to be collected into special tubes featuring a screw cap with seal. Regular Eppendorf reaction tubes are not suited and the Metabolomics and Proteomics Core Facility cannot process samples collected in these tubes.
The different metabolomics methods offered by the Metabolomics and Proteomics Core Facility require different sample volumes. Non-Targeted metabolomics requires the usage of tubes with up to 2.0 mL, while Targeted Metabolomics assays can be performed with lower sample amount. Please refer to the chapters on harvesting cells for the different metabolomics assays for guidance on which tube to use for sample collection.

We recommend using the following tubes:

Tube	Provider, Order Number	Recommended Sample Volume (mL)
Micro Tube 0.5 mL PP	Sarstedt, 72.730.005	0.3 - 0.5
Micro Tube 2.0 mL PP	Sarstedt, 72.694.005	1.0 – 1.8

It is possible to label the caps of the tubes. However, the label on the cap should not be the only label for the sample, because during homogenization, the instrument sometimes removes cap labels. Besides, samples could be mixed-up at opening of the tubes. Therefore, we recommend to provide all relevant sample information on a label placed on the sample tube.

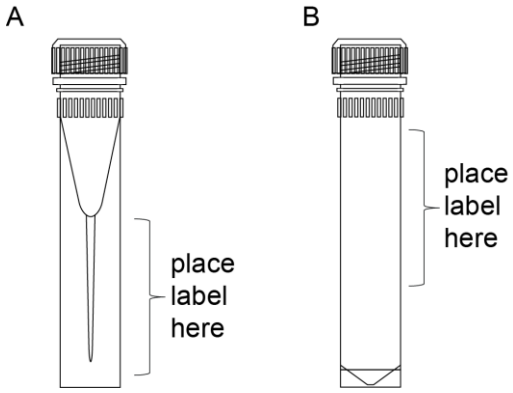

Figure 1: Sketch of Micro Tubes (Sarstedt) with volumes of 0.5 mL (A) and 2.0 mL (B). The correct position of the label is indicated.

Place the labels in a way that the sample inside the tube remains visible to the experimenter. For 0.5 mL tubes, place the label at the bottom of the tube (Fig. 1 A). For 2.0 mL tubes, place the label at the top of the tube; however, below the striation (Fig. 1 B).

Several labeling techniques are possible (place the labels in the correct area of the tube):

- Hand writing: Use a permanent marker, which is suitable for labeling plastic material and completely cover the label by transparent adhesive tape. Ensure readability.
- Print-outs on normal paper: Print your label on normal paper and fix it onto the tube using transparent adhesive tape. Make sure that the adhesive tape covers the label completely.
- Stick-on label: Print the sample description on stick-on labels and place the label in the correct area of the tube. Please make sure to use labels that stay adhesive upon freezing at -80 °C (otherwise cover labels completely with transparent adhesive tape).

2.2. Tubes for cell supernatant collection

Cell supernatants (i.e., culture media) can be collected in regular reaction tubes, like for example Eppendorf tubes. Safe lock tubes are highly recommended.
Place the labels in a way that the sample inside the tube remains visible to the experimenter. It is possible to label the caps of the tube. However, all relevant information should be given on a label placed on the tube itself.

Several labeling techniques are possible:

- Hand writing: Use a permanent marker, which is suitable for labeling plastic material and completely cover the label by transparent adhesive tape. Ensure readability.
- Print-outs on normal paper: Print your label on normal paper and fix it onto the tube using transparent adhesive tape. Make sure that the adhesive tape covers the label completely.
- Stick-on label: Print the sample description on stick-on labels and place the label in the correct area of the tube. Please make sure to use labels that stay adhesive upon freezing at -80 °C (otherwise cover labels completely with transparent adhesive tape).

2.3. Cell scraper for harvest of adherent cells

For scraping adherent cells off the culture plate, cell scrapers are needed. In principle, any cell scraper is possible. We have good experiences with the following two scraper types:

Cell Scraper Length	Blade Length	Provider, Order Number	Characteristics
Cell Scraper 25 cm	17 mm	Sarstedt, 83.1830	Blade is flexible
Cell Scraper S, 24 cm	13 mm	TPP, 99002	Blade is rather rigid

3. Procedures for Non-Targeted Metabolomics

3.1. Preparation of Extraction Solvent for Harvesting and Collection of Adherent Cells for Non-Targeted Metabolomics

1. **Purpose:**
   This SOP provides instructions for the preparation of extraction solvent for the harvesting and collection of cells for Non-Targeted Metabolomics analyses.

2. **Materials:**
   - HPLC grade methanol (e.g., ThermoFisher Optima)
   - Ultrapure water
   - Dried standard compounds*: tridecanoic acid, DL-2-fluorophenylglycine, d6-cholesterol, and DL-4-chlorophenylalanine

3. **Preparation of Standard Stock Solutions:**
   - Dissolve each of the dry standards in its glass vial with methanol, to yield stock solutions with concentrations of:
     - 2.5 mg/mL for tridecanoic acid
     - 0.25 mg/mL for DL-2-fluorophenylglycine
     - 2.5 mg/mL for d6-cholesterol
     - 1.0 mg/mL for DL-4-chlorophenylalanine

4. **Preparation of Extraction Solvent (80% (v/v) methanol + standards):**
   - To prepare 50 mL extraction solvent, pipet
     - 500 µL of stock 2.5 mg/mL tridecanoic acid in methanol (1% (v/v) of total volume)
     - 500 µL of stock 0.25 mg/mL DL-2-fluorophenylglycine in methanol (1% (v/v) of total volume)
     - 500 µL of stock 2.5 mg/mL d6-cholesterol in methanol (1% (v/v) of total volume)
     - 500 µL of stock 1.0 mg/mL DL-4-chlorophenylalanine in methanol (1% (v/v) of total volume)

     into a volumetric flask, and vortex to mix.
   - Add 38 mL of HPLC grade methanol (determined by using a glass measuring cylinder) and vortex to mix.
   - Bring up to the final volume by adding 10 mL of ultrapure water (determined by using a glass measuring cylinder) and invert several times to mix.

Note: The volume of the extraction solvent can be varied depending on the amount desired or needed, as long as the above described standard to solvent ratios are kept constant. The final concentration of methanol in the solution should be 80% (v/v).

5. Information on Stability and Storage Conditions

- Dry standard compounds should be stored at 4 °C and are stable for several months.
- Standard stock solutions (see Step 3) should be stored at 4 °C and are stable for approximately 1 month.
- Final Extraction Solvent (see Step 4) should be stored at 4 °C and is stable for approximately 2 weeks.

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*The dry standard compounds are available at the Metabolomics and Proteomics Core Facility. Please contact Anna Artati (email: anna.artati@helmholtz-muenchen.de, phone: +49-89 3187 3229) for further information.

3.2. Harvesting and Collection of Adherent Cells for Non-Targeted Metabolomics

Adherent cells for Non-Targeted Metabolomics should be grown in cell culture plates (e.g., 6 or 12-well) and not in flasks.
The suitable cell number for a Non-Targeted Metabolomics analysis depends on the cell line and should be evaluated prior to the according experiment. However, 1 x 10^6 to 5 x 10^6 cells per sample is a good starting point. To reach these cell numbers, it might be necessary to pool wells.
Sample volumes needed for the Non-Targeted analytical method are 1 mL for cells in extraction solvent and 0.5 mL for medium.
The volumes given below for cell harvesting are for a single well of a 6-well plate. At use of 12-well plates, the volumes should be scaled down.
Furthermore, a method enabling data normalization should be considered and performed prior to the metabolomics measurements (for example the Hoechst assay [1] with which the DNA content and thus indirectly the cell number of the metabolomics sample can be detected). Please contact one of the authors of this SOP if you wish the Hoechst assay to be performed on your samples.

Sample collection of medium for Non-Targeted Metabolomics analysis (Skip this step, if analysis of medium is not desired)**:**

- Harvest the medium well by well.
- Collect 1 mL of medium into a 1.5 mL or 2.0 mL safe lock Eppendorf tube.
- Centrifuge the medium at 1000 x g and room temperature for 10 minutes.
- Carefully transfer 0.5 mL of the cell-free supernatant into a fresh 1.5 mL or 2.0 mL safe lock Eppendorf tube and immediately place the tube on dry ice.

For control purposes, please provide blank medium, which has been as well incubated but without cells.

After harvesting is finished, immediately store samples at -80 °C until metabolomics analysis is performed.

Sample collection of cells for Non-Targeted Metabolomics analysis (on the example of a 6-well plate well)**:**

- Harvest the cells well by well.
- Remove the (remaining) medium.
- Wash the cells two times with PBS (37 °C). Use at least 1 mL more PBS than culture medium to ensure proper washing of the well (i.e., if the growth medium volume for the 6-well is 2 mL, then use 3 mL PBS for washing). Remove the PBS after the second washing step as completely as possible.

- Add 400 µL of the precooled ice cold (dry ice) extraction solvent 80% (v/v) methanol containing 4 recovery standard compounds* to the 6-well and ensure that all cells are covered immediately. Please refer to chapter *3.1. Preparation of Extraction Solvent for Harvesting and Collection of Adherent Cells for Non-Targeted Metabolomics* of this SOP for preparation of the extraction solvent.

- Detach the cells using a rubber-tipped cell scraper and transfer the cell suspension into precooled (dry ice) 2.0 mL screw cap micro tubes (Sarstedt Micro tube 2.0 mL, PP, Reference #: 72.694.005). The micro tubes should be not only precooled in dry ice but also handled on dry ice during the whole procedure.

- Add another 100 µL of ice cold (dry ice) extraction solvent to the well to wash the well and transfer the wash volume to the micro tube as well.

<u>As 1 mL of cell suspension per tube is needed for the Non-Targeted analytics, please pool the suspensions of two 6-wells to achieve one sample.</u>

After harvesting is finished, immediately store samples at -80 °C until metabolomics analysis is performed.

Please send samples on dry ice and in one batch to the Metabolomics and Proteomics Core Facility.

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*The recovery standard compounds are included in the extraction solvent to monitor extraction efficiency. The compounds are available at the Metabolomics and Proteomics Core Facility. Please contact Anna Artati (email: anna.artati@helmholtz-muenchen.de, phone: +49-89 3187 3229) for further information.

[1] Muschet, C., Möller, G., Prehn, C., Hrabě de Angelis, M., Adamski, J., Tokarz, J.: Removing the bottlenecks of cell culture metabolomics: fast normalization procedure, correlation of metabolites to cell number and impact of the harvesting method. Metabolomics (2016) 12: 151. Doi:10.1007/s11306-016-1104-8

3.3. Harvesting and Collection of Suspension Cells for Non-Targeted Metabolomics

Suspension cells for Non-Targeted Metabolomics should be grown in cell culture flasks.
The suitable cell number for Non-Targeted Metabolomics analysis depends on the cell line and should be evaluated prior to the according experiment. However, 1 x 10^6 to 5 x 10^6 cells per sample is a good starting point.
The sample volume needed for the Non-Targeted analytical method is 0.5 mL for medium, while cells are needed as dry pellet.
Despite suspension cells are counted prior to harvest, cell losses usually occur during aliquoting the cells and the subsequent washing steps. Therefore, a method enabling normalization of the final cell sample should be considered and performed prior to the metabolomics measurements (for example the Hoechst assay [1] with which the DNA content and thus indirectly the cell number of the metabolomics sample can be detected). Please contact one of the authors of this SOP if you wish the Hoechst assay to be performed on your samples.

Sample collection of medium for Non-Targeted Metabolomics analysis (Skip this step, if analysis of medium is not desired)**:**

- Gently mix the cell suspension.
- Transfer 1 mL of the cell suspension into a 1.5 mL or 2.0 mL safe lock Eppendorf tube.
- Centrifuge the cell suspension at 500-800 x g* and room temperature for 5-10 minutes*.
- Carefully transfer 0.5 mL of the cell-free supernatant into a fresh 1.5 mL or 2.0 mL safe lock Eppendorf tube and immediately place the tube on dry ice.

For control purposes, please provide blank medium, which has been as well incubated but without cells.

After harvesting is finished, immediately store the samples at -80 °C until metabolomics analysis is performed.

Sample collection of cells for Non-Targeted Metabolomics analysis:

- Gently mix the cell suspension in the cell culture flask.
- Count the cells in an aliquot using an appropriate cell counting method.

- Transfer the respective cell suspension volume containing the desired cell number from the flask to a 2.0 mL screw cap micro tube (Sarstedt Micro tube 2.0 mL, PP, Reference #: 72.694.005).
- Pellet the cells at 500-800 x g* and room temperature for 5-10 minutes*. Remove the supernatant as completely as possible and gently re-suspend the cell pellet in 2 mL warm PBS (37 °C). Perform two washing steps with PBS, remove the PBS of the second washing step as completely as possible, and immediately place the tube with the dry cell pellet on dry ice.

After harvesting is finished, immediately store the samples at -80 °C until metabolomics analysis is performed.

Please send samples in one batch on dry ice to the Metabolomics and Proteomics Core Facility.

* Note: The centrifugal force and centrifugation time given in this SOP are guiding values. Both parameters are dependent on the cell line/cell strain analyzed. It might be possible to use less centrifugal force and shorter centrifugation time based on own experiences with the cells; however, it has to be ensured that the cell pellet is firm enough to separate the pellet from the supernatant. The use of higher centrifugal forces should be omitted to not to destroy the cells and make them leaky.

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[1] Muschet, C., Möller, G., Prehn, C., Hrabě de Angelis, M., Adamski, J., Tokarz, J.: Removing the bottlenecks of cell culture metabolomics: fast normalization procedure, correlation of metabolites to cell number and impact of the harvesting method. Metabolomics (2016) 12: 151. Doi:10.1007/s11306-016-1104-8

4. Procedures for Targeted Metabolomics

4.1. Preparation of Extraction Solvent for Harvesting and Collection of Adherent Cells for Targeted Metabolomics

1. **Purpose:**
   This SOP provides instructions for the preparation of extraction solvent for the harvesting and collection of cells for Targeted Metabolomics analyses.

2. **Materials:**
   - HPLC grade methanol (e.g., ThermoFisher Optima)
   - Ultrapure water

3. **Preparation of Extraction Solvent (80% (v/v) methanol):**
   - To prepare 50 mL Extraction solvent, transfer 40 mL of HPLC grade methanol (determined using a glass measuring cylinder) into a glass flask
   - Add 10 mL of ultrapure water (determined using a glass measuring cylinder) and invert several times to mix.

Note: The volume of the Extraction solvent can be varied depending on the amount desired or needed, as long as the solvent ratios are kept constant. The final concentration of methanol in the solution should be 80% (v/v).

4. **Information on Stability and Storage Conditions**
   - The final Extraction Solvent (see Step 3) should be stored at 4 °C and is stable for approximately 4 weeks.
   - Prior to use, the extraction solvent is preferably stored over night at -80 °C.

4.2. Harvesting and Collection of Adherent Cells for Targeted Metabolomics

Adherent cells for Targeted Metabolomics should be grown in cell culture plates (e.g., 6 or 12-well) and not in flasks.
The suitable cell number for the Targeted Metabolomics analysis depends on the cell line and should be evaluated prior to the according experiment. However, 1 x 10^6 to 2 x 10^6 cells per sample is a good starting point.
The sample volume needed for the Targeted analytical method is 0.3 mL for cells in Extraction solvent and 0.5 mL for medium.
The volumes given below for cell harvesting are for a single well of a 12-well plate. At use of 6-well plates, the volumes should be scaled up.
Furthermore, a method enabling data normalization should be considered and performed prior to the metabolomics measurements (for example the Hoechst assay [1] with which the DNA content and thus indirectly the cell number of the metabolomics sample can be detected). Please contact one of the authors of this SOP if you wish the Hoechst assay to be performed on your samples.

Sample collection of medium for Targeted Metabolomics analysis (Skip this step, if analysis of medium is not desired)**:**

- Harvest the medium well by well.
- Transfer 1 mL of medium into a 1.5 mL or 2.0 mL safe lock Eppendorf tube.
- Centrifuge the medium at 1000 x g and room temperature for 10 minutes.
- Carefully transfer 0.5 mL of the cell-free supernatant into a fresh 1.5 mL or 2.0 mL safe lock Eppendorf tube and immediately place the tube on dry ice.

For control purposes, please provide blank medium, which has been as well incubated but without cells.

After harvesting is finished, immediately store samples at -80 °C until metabolomics analysis is performed.

Sample collection of cells for Targeted Metabolomics analysis (on the example of a 12-well plate well)**:**

- Harvest the cells well by well.
- Remove the (remaining) medium.
- Wash the cells two times with PBS (37 °C). Use at least 1 mL more PBS than culture medium to ensure proper washing of the well (i.e., if the growth medium volume for the 12-well is 1 mL, then use 2 mL

PBS for washing). Remove the PBS after the second washing step as completely as possible.

- Add 200 µL of the precooled ice cold (dry ice) extraction solvent 80% (v/v) methanol and ensure that all cells are covered immediately. Please refer to chapter *4.1. Preparation of Extraction Solvent for Harvesting and Collection of Adherent Cells for Targeted Metabolomics* of this SOP for preparation of the extraction solvent.

- Detach the cells using a rubber-tipped cell scraper and transfer the cell suspension into precooled (dry ice) 0.5 mL screw cap micro tubes (Sarstedt Micro tube 0.5 mL, PP, Reference #: 72.730.005). The micro tubes should not only be precooled in dry ice but also handled on dry ice during the whole procedure.

- Add another 100 µL of ice cold (dry ice) extraction solvent to the well to wash the well and transfer the wash volume to the micro tube as well.

<u>As 0.3 mL of cells in extraction solvent per tube is needed for the targeted analytics, the harvested cell extraction solvent amount of one 12-well is sufficient for one sample.</u>

After harvesting is finished, immediately store the samples at -80 °C until metabolomics analysis is performed.

Please send samples on dry ice and in one batch to the Metabolomics and Proteomics Core Facility.

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[1] Muschet, C., Möller, G., Prehn, C., Hrabě de Angelis, M., Adamski, J., Tokarz, J.: Removing the bottlenecks of cell culture metabolomics: fast normalization procedure, correlation of metabolites to cell number and impact of the harvesting method. Metabolomics (2016) 12: 151. Doi:10.1007/s11306-016-1104-8

4.3. Harvesting and Collection of Suspension Cells for Targeted Metabolomics

Suspension cells for Targeted Metabolomics should be grown in cell culture flasks.
The suitable cell number for the Targeted Metabolomics analysis depends on the cell line and should be evaluated prior to the according experiment. However, 1 x 10^6 to 2 x 10^6 cells per sample is a good starting point.
The sample volume needed for the Targeted analytical method is 0.5 mL for medium, while cells are needed as dry pellet.
Despite suspension cells are counted prior to harvest, cell losses usually occur during aliquoting the cells and the subsequent washing steps. Therefore, a method enabling normalization of the final cell sample should be considered and performed prior to the metabolomics measurements (for example the Hoechst assay [1] with which the DNA content and thus indirectly the cell number of the metabolomics sample can be detected). Please contact one of the authors of this SOP if you wish the Hoechst assay to be performed on your samples.

Sample collection of supernatant for Targeted Metabolomics analysis (Skip this step, if analysis of medium is not desired)**:**

- Gently mix the cell suspension.
- Transfer 1 mL of the cell suspension into a 1.5 mL or 2.0 mL safe lock Eppendorf tube.
- Centrifuge the cell suspension at 500-800 x g* and room temperature for 5-10 minutes*.
- Carefully transfer 0.5 mL of the cell-free supernatant into a fresh 1.5 mL or 2.0 mL safe lock Eppendorf tube and immediately place the tube on dry ice.

For control purposes, please provide blank medium, which has been as well incubated but without cells.

After harvesting is finished, immediately store the samples at -80 °C until metabolomics analysis is performed.

Sample collection of cells for Targeted Metabolomics analysis:

- Gently mix the cell suspension in the cell culture flask.
- Count the cells in an aliquot using an appropriate cell counting method.
- Transfer the respective cell suspension volume containing the desired cell number from the flask to

a 0.5 mL screw cap micro tube (Sarstedt Micro tube 0.5 mL, PP, Reference #: 72.730.005).

- Pellet the cells at 500-800 x g* and room temperature for 5-10 minutes*. Remove the supernatant as completely as possible and gently re-suspend the cell pellet in 0.5 mL warm PBS (37 °C). Perform two washing steps with PBS, remove the PBS of the second washing step as completely as possible, and immediately store the dry cell pellet on dry ice.

After harvesting is finished, immediately store the samples at -80 °C until metabolomics analysis is performed.

Please send samples on dry ice and in one batch to the Metabolomics and Proteomics Core Facility.

* Note: The centrifugal force and centrifugation time given in this SOP are guiding values. Both parameters are dependent on the cell line/cell strain analyzed. It might be possible to use less centrifugal force and shorter centrifugation time based on own experiences with the cells; however, it has to be ensured that the cell pellet is firm enough to separate the pellet from the supernatant. The use of higher centrifugal forces should be omitted to not to destroy the cells and make them leaky.

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[1] Muschet, C., Möller, G., Prehn, C., Hrabě de Angelis, M., Adamski, J., Tokarz, J.: Removing the bottlenecks of cell culture metabolomics: fast normalization procedure, correlation of metabolites to cell number and impact of the harvesting method. Metabolomics (2016) 12: 151. Doi:10.1007/s11306-016-1104-8

5. Procedures for Lipidomics

5.1. Preparation of Extraction Solvent for Harvesting and Collection of Adherent Cells for Lipidomics

1. **Purpose:**
   This SOP provides instructions for the preparation of extraction solvent for the harvesting and collection of cells for Lipidomics analyses.

2. **Materials:**
   - HPLC grade methanol (e.g., ThermoFisher Optima)
   - Ultrapure water

3. **Preparation of Extraction Solvent (80% (v/v) methanol):**
   - To prepare 50 mL Extraction solvent, transfer 40 mL of HPLC grade methanol (determined using a glass measuring cylinder) into a glass flask
   - Add 10 mL of ultrapure water (determined using a glass measuring cylinder) and invert several times to mix.

Note: The volume of the Extraction solvent can be varied depending on the amount desired or needed, as long as the solvent ratios are kept constant. The final concentration of methanol in the solution should be 80% (v/v).

4. **Information on Stability and Storage Conditions**
   - The final Extraction Solvent (see Step 3) should be stored at 4 °C and is stable for approximately 4 weeks.
   - Prior to use, the extraction solvent is preferably stored over night at -80 °C.

5.2. Harvesting and Collection of Adherent Cells for Lipidomics

Adherent cells for Lipidomics should be grown in cell culture plates (e.g., 6 or 12-well) and not in flasks.
The suitable cell number for the Targeted Metabolomics analysis depends on the cell line and should be evaluated prior to the according experiment. However, 1 x 10^6 to 2 x 10^6 cells per sample is a good starting point.
The sample volume needed for the Targeted analytical method is 2.0 mL for cells in Extraction solvent and 1.0 mL for medium.
The volumes given below for cell harvesting are for a single well of a 6-well plate. At use of 12-well plates, the volumes should be scaled down.
Furthermore, a method enabling data normalization should be considered and performed prior to the metabolomics measurements (for example the Hoechst assay [1] with which the DNA content and thus indirectly the cell number of the metabolomics sample can be detected). Please contact one of the authors of this SOP if you wish the Hoechst assay to be performed on your samples.

Sample collection of medium for Lipidomics analysis (Skip this step, if analysis of medium is not desired)**:**

- Harvest the medium well by well.
- Transfer 1.1 mL of medium into a 1.5 mL or 2.0 mL safe lock Eppendorf tube.
- Centrifuge the medium at 1000 x g and room temperature for 10 minutes.
- Carefully transfer 1.0 mL of the cell-free supernatant into a fresh 1.5 mL or 2.0 mL safe lock Eppendorf tube and immediately place the tube on dry ice.

For control purposes, please provide blank medium, which has been as well incubated but without cells.

After harvesting is finished, immediately store samples at -80 °C until metabolomics analysis is performed.

Sample collection of cells for Lipidomics analysis (on the example of a 6-well plate well)**:**

- Harvest the cells well by well.
- Remove the (remaining) medium.
- Wash the cells two times with PBS (37 °C). Use at least 1 mL more PBS than culture medium to ensure proper washing of the well (i.e., if the growth medium volume for the 6-well is 2 mL, then use 3 mL PBS for washing). Remove the PBS after the second washing step as completely as possible.

- Alternative washing especially suitable for differentiated adipopcytes [2]: Wash the cells once with 6 mL PBS (37 °C). Remove the PBS as completely as possible.

- Add 400 µL of the precooled ice cold (dry ice) extraction solvent 80% (v/v) methanol and ensure that all cells are covered immediately. Please refer to chapter *5.1. Preparation of Extraction Solvent for Harvesting and Collection of Adherent Cells for Lipidomics* of this SOP for preparation of the extraction solvent.

- Detach the cells using a rubber-tipped cell scraper and transfer the cell suspension into precooled (dry ice) 2.0 mL screw cap micro tubes (Sarstedt Micro tube 2.0 mL, PP, Reference #: 72.694.005). The micro tubes should not only be precooled in dry ice but also handled on dry ice during the whole procedure.

- Add another 100 µL of ice cold (dry ice) extraction solvent to the well to wash the well and transfer the wash volume to the micro tube as well.

<u>As 2.0 mL of cell suspension per tube is needed for the Lipidomics analysis, please pool the suspensions of four 6-wells to achieve one sample.</u>

After harvesting is finished, immediately store the samples at -80 °C until metabolomics analysis is performed.

Please send samples on dry ice and in one batch to the Metabolomics and Proteomics Core Facility.

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[1] Muschet, C., Möller, G., Prehn, C., Hrabě de Angelis, M., Adamski, J., Tokarz, J.: Removing the bottlenecks of cell culture metabolomics: fast normalization procedure, correlation of metabolites to cell number and impact of the harvesting method. Metabolomics (2016) 12: 151. Doi: 10.1007/s11306-016-1104-8

[2] Miele, F., Möller, G., Cecil, A., Lintelmann, J., Wabitsch, M., Tokarz, J., Adamski, J., Haid, M.: Lipidomic phenotyping reveals extensive lipid remodeling during adipogenesis in human adipocytes. Metabolites (2020) 10: 217. Doi: 10.3390/metabo10060217

5.3. Harvesting and Collection of Suspension Cells for Lipidomics

Suspension cells for Lipidomics should be grown in cell culture flasks.
The suitable cell number for the Lipidomics analysis depends on the cell line and should be evaluated prior to the according experiment. However, 1 x 10^6 to 2 x 10^6 cells per sample is a good starting point.
The sample volume needed for the Lipidomics analytical method is 1.0 mL for medium, while cells are needed as dry pellet.
Despite suspension cells are counted prior to harvest, cell losses usually occur during aliquoting the cells and the subsequent washing steps. Therefore, a method enabling normalization of the final cell sample should be considered and performed prior to the metabolomics measurements (for example the Hoechst assay [1] with which the DNA content and thus indirectly the cell number of the metabolomics sample can be detected). Please contact one of the authors of this SOP if you wish the Hoechst assay to be performed on your samples.

Sample collection of supernatant for Lipidomics analysis (Skip this step, if analysis of medium is not desired)**:**

- Gently mix the cell suspension.
- Transfer 1.1 mL of the cell suspension into a 1.5 mL or 2.0 mL safe lock Eppendorf tube.
- Centrifuge the cell suspension at 500-800 x g* and room temperature for 5-10 minutes*.
- Carefully transfer 1.0 mL of the cell-free supernatant into a fresh 1.5 mL or 2.0 mL safe lock Eppendorf tube and immediately place the tube on dry ice.

For control purposes, please provide blank medium, which has been as well incubated but without cells.

After harvesting is finished, immediately store the samples at -80 °C until metabolomics analysis is performed.

Sample collection of cells for Lipidomics analysis:

- Gently mix the cell suspension in the cell culture flask.
- Count the cells in an aliquot using an appropriate cell counting method.
- Transfer the respective cell suspension volume containing the desired cell number from the flask to a 2.0 mL screw cap micro tube (Sarstedt Micro tube 2.0 mL, PP, Reference #: 72.694.005).

- Pellet the cells at 500-800 x g* and room temperature for 5-10 minutes*. Remove the supernatant as completely as possible and gently re-suspend the cell pellet in 0.5 mL warm PBS (37 °C). Perform two washing steps with PBS, remove the PBS of the second washing step as completely as possible, and immediately store the dry cell pellet on dry ice.

After harvesting is finished, immediately store the samples at -80 °C until metabolomics analysis is performed.

Please send samples on dry ice and in one batch to the Metabolomics and Proteomics Core Facility.

* Note: The centrifugal force and centrifugation time given in this SOP are guiding values. Both parameters are dependent on the cell line/cell strain analyzed. It might be possible to use less centrifugal force and shorter centrifugation time based on own experiences with the cells; however, it has to be ensured that the cell pellet is firm enough to separate the pellet from the supernatant. The use of higher centrifugal forces should be omitted to not to destroy the cells and make them leaky.

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[1] Muschet, C., Möller, G., Prehn, C., Hrabě de Angelis, M., Adamski, J., Tokarz, J.: Removing the bottlenecks of cell culture metabolomics: fast normalization procedure, correlation of metabolites to cell number and impact of the harvesting method. Metabolomics (2016) 12: 151. Doi:10.1007/s11306-016-1104-8