Quantitative density gradient analysis by mass spectrometry

(qDGMS)

Last updated on 2020-10-17

Contents

Materials

Note: Materials with * can be acquired from a different supplier.

Reagents

Stocks and general reagents

- $MgCl_2$
- KCl
- Mannitol
- Sucrose, RNAse-free if necessary
- PMSF
- BSA
- Methanol
- Triton X-100
- L-Lysine monohydrochloride (Sigma-Aldrich, L8662)*
- L-Arginine (Sigma-Aldrich, A8094)*
- L-Proline (Sigma-Aldrich, P5607)*
- L-Lysine-$^{13}C_6$,$^{15}N_2$ hydrochloride (Sigma-Aldrich, 608041)*
- L-Arginine-$^{13}C_6$,$^{15}N_4$ hydrochloride (Sigma-Aldrich, 608033)*
- cOmplete, EDTA-free proteinase inhibitor cocktail (Roche)*
- Pierce Bovine Serum Albumin Standard Ampules, 2 mg/mL (Thermo Fischer Scientific, 23209)*
- Protein quantification reagents, e.g. DC Protein Assay Reagents Package (Bio-Rad, 5000116)*
- Protein electrophoresis and immunoblotting reagents

In-house ready-made solutions

- Ultrapure water
- 1x PBS
- 1 M Tris-HCl, pH 7.4
- 1 M HEPES, pH 7.8
- 5 M NaCl
- 0.5 M EDTA
- 20% SDS

Cell culture

- Cell line of choice (e.g. HEK 293, HeLa, 143B, HAP1)
- Cell culture vessels (e.g. 150 mm dishes, T175 flasks, multi-layer flasks)
- Vacuum Filter System, 500 mL capacity, PES 0.22 µm membrane (Corning, 431097)*
- DMEM for SILAC (Thermo Fischer Scientific, A33822)*
- Dialyzed fetal bovine serum (Sigma-Aldrich, F0392)*
- Penicillin-Streptomycin, 5000 U/mL (Thermo Fischer Scientific)*

Mass spectrometry

- Ethanol
- Acetonitrile
- Formic acid
- Trypsin
- Compressed nitrogen cylinder

Solutions

- Proline stock (200 mg mL^{-1}) in PBS. Store at -20 ºC as 500 µL aliquots.
- Light lysine stock (145.8 mg mL^{-1}) in PBS. Store at -20 ºC as 500 µL aliquots.
- Light arginine stock (69.3 mg mL^{-1}) in PBS. Store at -20 ºC as 500 µL aliquots.
- Heavy lysine stock (152.1 mg mL^{-1}) in PBS. Store at -20 ºC as 500 µL aliquots.
- Heavy arginine stock (87.8 mg mL^{-1}) in PBS. Store at -20 ºC as 500 µL aliquots.
- Hypotonic buffer: 20 mM HEPES pH 7.8, 5 mM KCl, 1.5 mM $MgCl_2$, 1 mg mL^{-1}, 1x protease inhibitor. Store at 4 ºC.
- Homogenization buffer: 600 mM mannitol, 10 mM Tris-HCl pH 7.4, 1 mM EDTA.
- Homogenization buffer + BSA: 600 mM mannitol, 10 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM PMSF, 0.1% BSA. Store at 4 ºC.
- 1x MSH buffer: 20 mM HEPES pH 7.8, 210 mM mannitol, 70 mM sucrose, 2 mM EDTA, 1x protease inhibitor. Store at 4 ºC.
- 2.5x MSH buffer: 20 mM HEPES pH 7.8, 525 mM mannitol, 175 mM sucrose, 5 mM EDTA, 1x protease inhibitor. Store at 4 ºC.
- 1.5 M sucrose cushion: 10 mM HEPES pH 7.8, 5 mM EDTA pH 8.0, 1.5 M sucrose.
- 1.0 M sucrose cushion: 10 mM HEPES pH 7.8, 5 mM EDTA pH 8.0, 1.0 M sucrose.
- 0.5 M sucrose cushion: 10 mM HEPES pH 7.8, 5 mM EDTA pH 8.0, 0.5 M sucrose.
- Lysis buffer: 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1x protease inhibitor. Aliquot and store at -20 ºC.
- TNM buffer: 50 mM Tris-HCl pH 7.4, 100 mM NaCl, 20 mM $MgCl_2$.
- 50% (w/v) sucrose TNM: Dissolve 5 g of sucrose per 10 mL of TNM buffer. Sterilize by filtration and store at 4 ºC.
- 30% (w/v) sucrose TNM: Mix 6 mL of 50% (w/v) sucrose TNM with 4 mL of TNM buffer.
- 10% (w/v) sucrose TNM: Mix 2 mL of 50% (w/v) sucrose TNM with 8 mL of TNM buffer.

Equipment and consumables

- Sterile capped tubes and microtubes
- 2.5 mL syringes
- Syringe needles, 23G
- Protein electrophoresis and immunoblotting equipment
- Tight-fitting 15 mL glass-glass and 3 mL glass-Teflon Dounce homogenizers, or Balch homogenizer (Isobiotech)
- Benchtop refrigerated centrifuge (5804 R, Eppendorf), equipped with fixed-angle rotor F-34-6-38 (Eppendorf)*
- Benchtop refrigerated microcentrifuge (5430 R, Eppendorf), equipped with fixed-angle rotor FA-45-30-11 (Eppendorf)*
- Optima L-100 XP ultracentrifuge (Beckman Coulter)
- Optima MAX-XP ultracentrifuge (Beckman Coulter)

- SW 40 Ti, Swinging bucket (Beckman Coulter)
- TLS-55, Swinging bucket (Beckman Coulter)
- Ultracentrifuge tubes, Thinwall, Ultra-Clear (Beckman Coulter)
- Gradient Station (Biocomp Instruments), including marker block, magnabase and rate zonal caps for TLS-55 rotor tubes
- Protein electrophoresis and immunoblotting equipment
- In-line nanoscale liquid chromatography system, e.g. Proxeon EASY-nLC1000 system equipped with an Acclaim PepMap 2 micron C18 reverse-phase column (Thermo Scientific), 50 µm internal diameter, 150 mm length
- Electrospray tandem mass spectrometer capable of analyzing high complexity peptide mixtures with high resolution, for example Q-Exactive Plus Orbitrap mass analyzer (Thermo Scientific)

Software tools

- Xcalibur software (Thermo Fisher Scientific)
- Proteome Discoverer™ version 1.4 (Thermo Fisher Scientific)
- R, ComPrAn package (https://github.com/Scavetta/ComPrAn)

ComPrAn input files description

- **Peptide file** – plain tab delimited .txt file with data organized in columns, first row contains column headers. Following columns must be in the file (column data format specified):
  - "PSM Ambiguity" – character/factor; rows in which "PSM Ambiguity" value is "Rejected" will be excluded from analysis
  - "Precursor Area" – numeric; column containing quantification of peptides
  - "# Protein Groups" – integer; specifies to how many protein groups a specific peptide was assigned
  - "Rank" – integer; rank assigned to the peptide by the upstream programs used
  - "Confidence Level" - character/factor; confidence assigned to the peptide by the upstream programs used
  - "Protein Group Accessions" - character/factor; UniProt IDs of proteins
  - "Protein Descriptions" – character; description of the protein, for proper functionality of plotting functions should contain "GN=" with gene name after the equal sign
  - "Modifications" – character; chemical modifications of the peptide, including labeling by heavy amino acids, if a peptide has multiple modifications values should be separated by semicolon followed by space "; ", heavy amino acids should be noted in a format containing word "Label" in its value
  - "Charge" – integer; charge state of peptide
  - "Sequence" – character; amino acid sequence of peptide in single letter format
  - "Search ID" - character to be converted to integer; column specifying fraction in a format where "A" means fraction 1, "B" fraction 2 and so on, for samples with more than 26 fractions the values continue as "AA" for 27, "AB" for 28 etc.

Procedure

A. Metabolic labelling

1. Thaw two 50 mL aliquots of dialyzed serum, two aliquots of PenStrep, two aliquots of proline stock, one aliquot of light lysine stock, one light arginine stock, one heavy lysine stock, and one heavy arginine stock.
2. Prepare SILAC media by pouring 500 mL of DMEM for SILAC, 50 mL of dialyzed FBS, 5 mL of PenStrep, and 500 µL of proline stock into two filter systems, and adding 500 µL of light lysine and light arginine stocks to one of them (light SILAC medium), and 500 µL of heavy lysine and heavy arginine stocks to the other (heavy SILAC medium). Supplements should be added at this point equally to both media.
3. Sterilize the media by low pressure filtration. SILAC media can be stored at 4 ºC.
4. Passage the cells of interest 1:1. Culture one half with light SILAC medium, and the other with heavy SILAC medium.
5. Expand cell populations for at least 7 cell generations in SILAC media, using appropriate vessels (e.g. 150 mm dishes), until ~90% confluent. Renew SILAC media every 2 days. Avoid letting the cells reach 100% confluence as this may create artefacts due to contact inhibition. If necessary (e.g. if metabolic labelling has not been studied before in the cell line in use), collect a small number of cells for quality control by mass spectrometric evaluation of heavy-label incorporation. In case cells require some treatment (e.g. doxycycline induction), this should be performed timely according to the expansion and doubling time, and all cell populations should be subjected to the same conditions. The amount of required material depends on the object of the investigation and biological source.

B. Preparation

1. Wash 2.2 mL ultracentrifuge and microcentrifuge tubes with 10% methanol. Dry these in an oven, if necessary.
2. Place PBS, the Dounce or Balch homogenizer, and the SW 40 Ti and TLS-55 rotors at 4 ºC, overnight.
3. On the day of the experiment, refrigerate a benchtop centrifuge, a benchtop microcentrifuge, Optima L-100 XP ultracentrifuge and Optima MAX-XP benchtop ultracentrifuge to 4 ºC.
4. Warm PBS in a water bath at 37 ºC. Prepare reagents for the dissociation of the cell layer if required.
5. Thaw protein quantification standard and lysis buffer on ice.

C. Cell harvest

1. Discard the culture medium from the vessels.
2. Detach cells by pipetting 10 mL of warm PBS directly on the cell layer, using dissociation reagents or scraping.
3. Collect the suspension in a centrifuge tube. Collect cells from each condition into separate tubes, e.g. for an experiment where control (c) and sample (s) were reciprocally labeled with heavy (H) and light (L) amino acids: cL, sH, cH, sL.

4. Wash the emptied dishes with additional warm PBS to recover any leftover cells.
5. Repeat steps C2 to C4 for all dishes, and for all conditions.
6. Centrifuge the cell suspensions at 500 *g* for 3-5 min at 4 ºC.
7. Discard the supernatant and wash the cell pellet twice more with cold PBS. Culture medium contains proteins that may reduce the sensitivity of the mass spectrometric analysis if not removed appropriately.

D. Protein quantification

1. Resuspend well each cell pellet in 10 mL of PBS and collect 10 µL to a new tube. Place the remaining cell suspension on ice.
2. Briefly spin the tubes to pellet the cells.
3. Discard the supernatant and add 10-20 µL of lysis buffer.
4. Aid lysis by vigorously pipetting the suspension up and down. Avoid forming bubbles.
5. Clarify the lysate by benchtop centrifugation, at 4 ºC, at maximum speed for 5 min.
6. Transfer the supernatant to a new tube. Do not collect pelleted debris or chromatin.
7. Determine the total protein concentration in all samples.
8. Identify the sample with the lowest total protein quantity for the samples that will make up each mix (i.e. cLsH and cHsL), and determine the volume that contains the same quantity of total protein.
9. Collect a small volume (10 µL) of the heavy-labeled cell suspensions. Pellet cells, add 10 µL of lysis buffer and store at -80 ºC. These will later be used for measurement of heavy isotope incorporation by mass spectrometry.
10. Mix cell suspensions so that total protein quantities from each control and sample cell type are the same.
11. Spin the cell mixes at 500 *g* for 3-5 min at 4 ºC.
12. Discard the supernatant.

E. Isolation of mitochondria

a Using a Dounce homogenizer

1. Resuspend each cell pellet in homogenization buffer + BSA, at a ratio of 2 mL per 150 mm dish.
2. Transfer the suspension to a glass-glass Dounce homogenizer and perform 30 strokes on ice.
3. Prepare another glass-glass homogenizer or wash the same homogenizer with deionized water, 70% ethanol, and deionized water again. Cool the homogenizer on ice.
4. Repeat steps Ea1 and Ea2 for the other cell mix.
5. Transfer the homogenates to new tubes and centrifuge at 400 *g* for 10 min at 4 ºC. If debris are not successfully pelleted, transfer the supernatant to a new tube and repeat the centrifugation.
6. Transfer the supernatants to new tubes and keep on ice.
7. Resuspend the pellet obtained in step Ea5 with 2 mL of homogenization buffer + BSA.
8. Transfer the suspension to a Teflon-glass Dounce homogenizer and perform 30 strokes on ice.
9. Transfer the homogenates to new tubes and centrifuge at 400 *g* for 10 min at 4 ºC.

10. Collect the supernatant and pool with that collected in step Ea6 for each mix.
11. Centrifuge the supernatants at 10000 *g* for 10 min at 4 ºC.

b Using a Balch homogenizer

1. Weigh the wet cell mass, using an empty tube as reference.
2. Gently resuspend the cell pellet in hypotonic buffer, at a ratio of 3 mL of buffer per 1 g of cells.
3. Incubate on ice for 10 min.
4. Meanwhile, place the assembled Balch homogenizer on ice and wash its chamber with hypotonic buffer, using two 2.5 mL syringes. Optimization may be required to select the correct size of ball bearing. The clearance space must be slightly smaller than the size of the cells when in suspension. For HEK 293T, use a ball bearing that allows 12 µm clearance. Ensure that there is no air in the syringes or in the homogenization chamber.
5. Take 2 mL of cell suspension in an empty 2.5 mL syringe, and expel any air.
6. Pass the cell suspension though the homogenization chamber (3 times for HEK 293T). Wear safety goggles as there is a high risk of contamination with biological material. The weakest points of the assembly are the connections between the homogenizer and the syringes. Optimization may be required to determine the required number of passages. To do this, inspect the suspension after each passage for intact cells under a light microscope.
7. Transfer the homogenate into a new tube on ice and immediately add 1.33 mL of 2.5x MSH.
8. Repeat steps Eb5 to Eb7 until all cells of the current mix have been homogenized.
9. Wash the homogenization chamber with 2.5 mL of 1x MSH and transfer the collected wash to the collection tube.
10. Wash the homogenizer with deionized water, 70% ethanol, and deionized water again. Cool the assembled homogenizer on ice.
11. Repeat steps Eb4 to Eb7 for the other cell mix.
12. Centrifuge the homogenates at 1000 *g* for 20 min at 4 ºC. If debris are not successfully pelleted, transfer the supernatant to a new tube on ice and repeat the centrifugation.
13. Transfer the supernatant to a new tube on ice.
14. Centrifuge at 10000 *g* for 20 min at 4 ºC.
15. Discard the supernatant and resuspend the crude mitochondrial pellet in 1 mL of 1x MSH.

F. Purification of mitochondria

1. Place 4 mL of 1.5 M sucrose cushion on a SW 40 Ti ultracentrifuge tube (~14 mL), then carefully add 4 mL of 1.0 M sucrose cushion on top, and finally 3 mL of 0.5 M sucrose cushion.
2. Layer 1 mL of crude mitochondrial suspension on top of the discontinuous sucrose density gradient, and add 1 mL of 1x MSH buffer.
3. Weigh the tubes and balance them using 1x MSH buffer.
4. Place the balanced tubes on the pre-chilled SW 40 Ti rotor.
5. Centrifuge at 85200 g_{avg} for 1 h, at 4 ºC.
6. Remove the 0.5 M and most of the 1.0 M sucrose cushions.
7. Transfer the mitochondrial band (located in the interface between the 1.0 M and 1.5 M sucrose cushions) to a new tube.

8. Add 4 vol of 1x MSH buffer and mix.
9. Pellet mitochondria by centrifugation at 10000 *g* for 10 min, at 4 ºC.
10. Discard the supernatant.

G. Sucrose density gradient

1. Add 250 µL of lysis buffer to the mitochondrial pellet.
2. Allow lysis to occur at 4 ºC for 15-20 min, with mixing.
3. Meanwhile, prepare 10% (w/v) and 30% (w/v) sucrose TNM solutions and keep on ice.
4. Mark 2 or 4 2.2 mL ultracentrifuge tubes using the marker block.
5. Place the marked ultracentrifuge tubes in the magnabase, and on the arm of the Gradient Station.
6. Fill each tube with 10% (w/v) sucrose TNM until the meniscus reaches the mark (≳1 mL).
7. Using a syringe with needle, gently deposit 30% (w/v) sucrose TNM in the bottom of the tubes until the interphase reaches the mark (≳1 mL).
8. Place the caps on the tubes, trying to avoid leaving bubbles.
9. Run the TLS-55 10-30 Sucrose Short program.
10. Slowly and carefully remove the magnabase from the Gradient Station and keep it in a cold room until use.
11. Clarify the mitochondrial lysate by centrifuging at 13000 *g* for 5 min at 4 ºC.
12. Please note: If this procedure has never been performed before, quantify total protein in the clarified mitochondrial lysate to avoid overloading the gradient. It may be convenient to split the lysate through two or more gradients, avoiding loading more than 2.5 mg of protein per gradient. Alternatively, larger scale gradients can be used with larger amounts of protein.
13. Carefully remove 100 µL of solution from the top of each gradient.
14. Layer 200 µL of clarified mitochondrial lysate on top of each gradient, by dispensing slowly from the side of the tube.
15. Check the weight of the gradients and add lysis buffer to correct any imbalances.
16. Place the gradients in a chilled TLS-55 rotor and then into a refrigerated Optima MAX-XP benchtop ultracentrifuge.
17. Centrifuge at 39000 rpm (~100000 *g*) for 2 h 15 min, at 4 ºC.
18. Carefully remove the gradients from the buckets, place them on a support. Carefully move to the nearest cold room; if not possible, fractionate one gradient at a time near the ultracentrifuge, leaving the remainder in the cooled chamber.
19. Collect 100 µL fractions form the top of the gradient into methanol-washed microcentrifuge tubes. Alternatively, a small hole can be pierced on the very bottom of the ultracentrifuge tube to allow the gradient to drain by dripping into methanol-washed microtubes.
20. Transfer 5-10 µL of each fraction into new tubes containing protein denaturing solution for SDS-PAGE.
21. Store the fractions collected in step G19 at -80 ºC until needed for mass spectrometric analysis. Avoid long storage times. If stored properly, samples can be kept at -80 ºC for months before being processed.
22. Analyze the integrity of the gradient and profile of the complex of interest by SDS-PAGE and immunoblotting, using the samples from step G20.

H. Mass spectrometry

1. Thaw collected fractions from step G19.
2. Transfer the adequate volume from each fraction to 2 mL microtubes, previously washed with methanol. The minimum volume of sample required for analysis varies according to diverse factors, including the amount of biological material used in the gradient, and the distribution/dispersion of the proteins of interest throughout the gradient. If unsure, use as much of the fractions as possible, as the processed material can be stored, and used for additional mass spectrometric analysis, if necessary. As a rule of thumb, use 30 µL of each fraction.
3. Add 20 vol of cold absolute ethanol to each sample, and incubate overnight at -20 ºC.
4. Centrifuge the samples at 4 ºC and discard the supernatant.
5. Add 1% (w/w) trypsin in 50 mM NH_4HCO_3 to the pellets and incubate overnight at 37 ºC.
6. Fractionate the obtained peptide digests by nano-scale reverse-phase liquid chromatography using a gradient of 5-40% acetonitrile in 0.1% (v/v) formic acid over 84 min, at a flow rate of 300 nL min^{-1}. The eluate is transferred directly to the electrospray interface of the mass spectrometer.
7. Acquire data from 400 to 1600 m/z for precursor ions and set the ten most abundant multiply charged precursor ions in each spectrum to be fragmented by HCD with nitrogen. Precursor and fragment ion spectra are acquired with resolutions of 70000 and 17500 respectively.
8. Identify proteins by comparing fragment ion data to the appropriate MASCOT database.
9. Measure relative peptide quantities by comparing peak area of different extracted ion chromatograms (XICs), using Xcalibur.

I. Data analysis

1. Using the Proteome DiscovererTM Deamon Utility submit the raw mass spectrometry files for all fractions for analysis by Proteome Discoverer™.
2. Identify peptides by comparing the MS/MS spectra to the appropriate MASCOT database with Proteome Discoverer™
3. Quantify peptides using Proteome Discoverer™.
4. Open a report from all fractions.
5. Export the report of all peptides from all fractions as a single tab delimited file.
6. Use file from step I5 as an input for analysis by the ComPrAn R package.

a Analysis using the interactive app

1. Start R and load the ComPrAn package.
2. Start the app by entering the command: *>compranApp()*
3. The app will open in a new window, we recommend that you click “Open in Browser” on the top left corner of the window
4. Import the data by clicking on *Import* → *Browse…* navigate to the file produced in step I5 and click *Open*.
5. After progress bar shows “Upload complete” click *Process data*.
6. Specify names of the labeled (“heavy”) and unlabeled (“light”) samples in the appropriate boxes (these names will be used in plots later on).
7. Switch to the *Peptide-to-protein* tab.
8. *Summary* tab. Summary plots about the data sets will be shown.

9. *Filter* tab. Adjust the available setting and click *Filter the data* button. Plots summarizing total number of proteins in samples are shown.
10. *Filter* tab. Click *Select peptides* button to select representative peptides for each protein
11. *Rep Peptides* tab. On this page a list of all peptides for any given protein can be visualized with the option to highlight the peptide that was selected by the analysis software as representative of the protein.
12. *Normalize* tab. Click on *Normalize the data* button; all protein quantity values will be normalized to be between 0 and 1, normalized data can be exported in tab delimited format.
13. Switch to *Protein workflow* tab
14. *Normalized Proteins* tab. On this page the quantity of selected proteins can be compared between labeled and unlabeled samples.
15. *Heatmap* tab. Click on *Browse…* and navigate to the file with information about the protein complex of interest (file format is described in vignettes). This produces a heatmap showing the quantitative comparison of protein profiles between samples.
16. Click on *Co-migration plot*, paste UniProt IDs of interest in a box to qualitatively compare the profile of one or two protein complexes.
17. Click on *Cluster*. Proteins are assigned into clusters, separately for each sample, based on correlation of profiles. Plots showing the number of proteins per cluster and table with proteins assigned into clusters can be downloaded.

b Analysis using the R command line

1. Start R and load ComPrAn package
2. Step-by-step workflow for command line processing of data is described in ComPrAn package vignette "SILACcomplexomics.html". To load the vignette type following command:

```
>browseVignettes("ComPrAn")
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