Protocol

Preparation of Intracellular Metabolite Extracts from Liquid *Schizosaccharomyces pombe* Cultures

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The success of metabolomic analysis relies heavily on the sample preparation protocol. Here we present a protocol for intracellular metabolite extraction from liquid fission yeast cultures based on rapid quenching in pure methanol at -40°C , bead-beating in 50% methanol for cell disruption, and 10 kDa cutoff ultrafiltration for removal of proteins. Samples are concentrated by vacuum evaporation and resuspended in 50% acetonitrile for mass spectrometric analysis. This protocol is optimal for extraction of polar metabolites such as amino acids, organic acids, nucleotides, sugars, or sugar-phosphates. Its implementation requires <6 h and allows preparation of multiple samples in parallel.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous material used in this protocol.

Reagents

4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES) in 1 mM stock solutions

These compounds are used as internal standards, not as buffers. Other small molecules can also be applied as internal standards, provided that they are exogenous, stable compounds that can be reliably detected using the selected detection method.

Acetonitrile (HPLC-grade or better) in 50% solution (v/v)

Methanol (HPLC-grade or better) in 100% and 50% solutions (v/v)

NaOH (0.1 N)

Schizosaccharomyces pombe cell culture (e.g., 2×10^8 cells in EMM2 liquid medium)

*50 mL of mid-log phase culture is a good starting amount for the first metabolome experiment. In practice, a much smaller amount of cells may suffice, depending on the sensitivity of the LC-MS system and the abundance of target metabolites. For advice on *S. pombe* culture, see Introduction: **Growth and the Environment of Schizosaccharomyces pombe** (Petersen and Russell 2016).*

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Equipment

Bead-beating system for cell disruption (e.g., Multi-beads Shocker [Yasui Kikai] or Geno/Grinder [SPEX SamplePrep])
 Centrifuge tubes (50-mL, screw-cap)
 Centrifugal filters with 10 kDa cutoff, compatible with 50% methanol solvent (e.g., Amicon Ultra-4 [Millipore UFC801096])
 Deep freezer (-40°C)
 Forceps
 Glass beads (acid-washed [Sigma-Aldrich G8772])
 High-speed centrifuge capable of -20°C

A centrifuge with a swinging rotor is preferable to a fixed-angle rotor. Both 50- and 15-mL tubes are used.

Microcentrifuge
 Microcentrifuge tubes (1.5-mL)
 Microscope
 Needle (26-gauge)
 Rotary evaporator
 Vacuum filtration equipment consisting of a vacuum pump, collection flask (e.g., Millipore XX1004744), filter holder (e.g., Millipore XX1004730), and methanol-resistant membrane filters of 1- μm pore size (e.g., Millipore JAWP04700)
 Vortex

METHOD

This protocol was previously published (Pluskal et al. 2010) and has been widely applied. The workflow is briefly schematized in Figure 1. Samples are prepared from cell cultures in <6 h; the 10 kDa cutoff filtration and solvent evaporation are the most time-consuming steps. Using this protocol, one individual can prepare ~ 16 samples in one day. When higher samples numbers are required, split sample preparation over multiple days by storing the quenched cells after Step 9.

Sample Preparation

1. For each culture sample, prepare a 50-mL centrifuge tube containing 25 mL of 100% methanol. Store the tubes at -40°C .
2. Prepare one tube containing 0.5 mL of 50% methanol (v/v) per sample. Store at -40°C .
3. For each culture sample, prepare a tube for the bead-beating system (according to the instrument type) and fill with acid-washed glass beads. Store on ice.

FIGURE 1. Workflow of metabolomic sample preparation. (Reproduced from Pluskal et al. [2010] with permission of The Royal Society of Chemistry.)

4. Prerinse the 10 kDa cutoff filters according to the manufacturer's instructions.

If the filters are not prerinsed, strong signals of preservatives such as glycerol will be detected in samples. Typically, one round of washing with 0.1 N NaOH followed by another round with H₂O is sufficient. Filters should not be allowed to dry out.

Cell Harvesting and Quenching

5. Collect all the cells from a culture sample onto a methanol-resistant membrane filter using the vacuum filtration system. Immediately drop the filter (including the cells) into a 50-mL tube containing 25 mL of methanol (−40°C) from Step 1. Vortex the tube briefly to ensure that the filter is completely submerged and place at −40°C.

This step should be performed as quickly as possible.

6. After collecting all samples, vortex the sample tubes thoroughly one by one to ensure cells are detached from the filters, and then remove the filters using forceps.
7. Centrifuge the sample tubes at −20°C (1500g for 3 min should be sufficient), and discard the supernatant.
8. Resuspend each pellet in 0.4 mL of 50% methanol (−40°C) from the tube prepared in Step 2.
9. Add 10 µL of internal standard solution (e.g., 1 mM PIPES and HEPES) to each tube.

Quantities of internal standards can be adjusted according to the measurement method.

After this step, samples can be stored for up to several days at −40°C, until the next step is performed.

Cell Disruption and Extraction of Metabolites

10. Transfer the samples into the appropriate bead-beating tubes prepared in Step 3. Perform bead beating, preferably at low temperature (0°C).

The efficiency of cell disruption can be verified by microscopy. Alter the beating speed or duration to ensure cell disruption.

11. Collect the fluid from the beads as follows.

- i. Puncture the bottom of the bead-beating tube with a 26-gauge needle and insert the tube into a 1.5-mL microcentrifuge tube.
- ii. Centrifuge briefly (e.g., 700g for 1 min at 4°C) to expel the fluid into the lower microcentrifuge tube, while retaining the beads in the original tube.

Beads can be discarded after use, as consumption per sample is relatively small.

12. Filter the extracted fluid using a prewashed 10 kDa cutoff filter according to the filter manual (e.g., centrifuge at 4000g for 60 min at 4°C, if using a swing rotor). Use a clean 15 mL tube to collect the flowthrough. After filtration, discard the filter, including the residual sample (containing proteins and other large molecules).

13. Evaporate the solvent on a rotary evaporator.

Samples can be split into several tubes (e.g., 200 µL/tube) to achieve faster evaporation. In order to avoid chemical reactions among the metabolites during evaporation, heat should not be used. Typically the evaporation of 200 µL of solvent takes 60–90 min, depending on the instrument.

14. Resuspend the evaporated samples in 40 µL of 50% acetonitrile by gentle pipetting. Store the samples at −80°C until analysis.

The final sample volume and solvent can be adjusted according to the measurement method.

15. Analyze the samples as described in Protocol: Measurement of Metabolome Samples Using Liquid Chromatography-Mass Spectrometry, Data Acquisition, and Processing (Pluskal and Yanagida 2016).

See Troubleshooting.

TROUBLESHOOTING

Problem (Step 15): During analysis, metabolite signals cannot be detected from samples, or signal intensity is low.

Solution: Check the signals of the internal standards. If they cannot be detected properly, the problem may lie in the detection method. If the internal standards are detected properly, check the efficiency of cell disruption by microscopy. If the signal intensity is low, try increasing culture volume.

Problem (Step 15): Quantitative data are not reproducible.

Solution: Check whether the signal intensity of the internal standards is reproducible. If not, the problem might be with the detection method. If the internal standards are reproducible, make sure the quenching step is performed rapidly, and check the efficiency of cell disruption by bead beating by microscopy.

Problem (Step 15): Quantitative measurements of reduced glutathione (GSH) and oxidized glutathione (GSSG) are not reproducible.

Solution: Glutathione is easily oxidized once exposed to air. Unfortunately, there is no good solution for this problem. For the quantification of glutathione using metabolomics, specialized derivatization methods have been developed (Suh et al. 2009, 2012). However, application of these methods may distort the quantitative measurements of other metabolites.

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