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Chloroform-Methanol Protein Extraction with Bead Beating for Yeast

Forked from [Chloroform-Methanol Protein Extraction with Zymolyase Treatment for Yeast \(High Throughput\)](#)

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Protocol status: Working
We use this protocol and it's working

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ABSTRACT

We adapted a high-throughput sample preparation workflow for Gram-negative bacteria to work with yeast. It consists of a bead-beating step, cell lysis, protein precipitation, protein resuspension, protein quantification, and normalization of protein concentration followed by standard bottom-up proteomic procedures of reducing and blocking cysteine residues and tryptic digestion.

This protocol was adapted from the manual sample preparation method found in Chen, Y., et al. "Automated "Cells-To-Peptides" Sample Preparation Workflow for High-Throughput, Quantitative Proteomic Assays of Microbes." *Journal of proteome research* 18.10 (2019): 3752-3761.

Keywords: Proteomics,
Sample preparation, Protein
quantification, Protein
extraction, Trypsin digestion,
Yeast, Bead-beating

GUIDELINES

- All centrifuge steps use an Eppendorf 5810R centrifuge.
- Cells were lysed in a Qiagen TissueLyser II system.
- A Molecular Devices Spectramax 250 microplate reader is used for the protein quantification assay measurement.
- Tryptic digestion is accomplished in an AB Sciex Veriti 96-well thermocycler.

Notes:

- For fewer than 30 samples PCR strips are easier to handle than plates, but once the number of samples is greater than 30 we find that a plate is a better choice.
- A multi-channel pipette is recommended for large numbers of samples.
- Measuring the amount of cells by multiplying the OD of the culture by the volume of the culture provides a good estimate for most applications, but the amount of cells can be determined more accurately from dry cell weight (DCW) or cell counting methods.
- We typically extract over 100 ug of protein from 2.0 OD*mLs of cells, so adjust the starting amount of cells for your organism or culturing conditions.

MATERIALS

MATERIALS

Corning™ 96-Well Solid Black Polystyrene Microplates (Costar 3915) Fisher Scientific Catalog #07-200-590
Pierce™ Bovine Serum Albumin Standard Pre-Diluted Set Thermo Fisher Catalog #23208
Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) Merck MilliporeSigma (Sigma-Aldrich) Catalog #C4706
Iodoacetamide Merck MilliporeSigma (Sigma-Aldrich) Catalog #11149
Methanol LC-MS grade B&J Brand VWR International Catalog #BJLC230-2.5
Chloroform for HPLC Merck MilliporeSigma (Sigma-Aldrich) Catalog #34854
Water LC-MS grade B&J Brand VWR International Catalog #BJLC365-2.5
Ammonium Bicarbonate LC-MS grade VWR International Catalog #BJ40867-50G

DC Protein Assay Reagent A Bio-Rad Laboratories Catalog #500-0113
DC Protein Assay Reagent B Bio-Rad Laboratories Catalog #500-0114
8-strip PCR Tubes with Caps Axygen Catalog #14-222-251
Trypsin Merck MilliporeSigma (Sigma-Aldrich) Catalog #T6567-1MG
PCR Plate 96-well non-skirted Thermo Fisher Scientific Catalog #AB0600
Thermo Scientific Autosampler Vial Kit Thermo Fisher Scientific Catalog #03-06016
Eppendorf Snap-Cap Microcentrifuge Flex-Tube Tubes Amber Fisher Scientific Catalog #05-402-31
Hard-Shell 96-Well PCR Plates low profile thin wall skirted white/clearBio-Rad Laboratories Catalog #HSP9601
0.5 mm Zirconia/Silica Beads Bio Spec Products Inc. Catalog #11079105z

Equipment		
TissueLyser II		NAME
Qiagen		BRAND
85300		SKU

SAFETY WARNINGS

Chloroform is used in this protocol so please follow the appropriate safety guidelines for handling and disposing of halogenated solvents at your institution and use a fume hood for steps involving chloroform.

Wear proper PPE (gloves, safety goggles, and lab coat) for safety and to minimize contamination of samples.

Prepare solvents in a chemical fume hood.

Store organic solvents in a flammable storage cabinet when not in use.

Discard used solvents and buffers in appropriate waste containers according to your institution's protocols.

BEFORE START INSTRUCTIONS

This protocol consists of steps for:

- Protein extraction from yeast cells
- Protein quantification
- Tryptic digestion

For this protocol you will need:

- an Eppendorf 5810R centrifuge with S-4-104 rotor or similar centrifuge
- a Qiagen RNeasy Lysis system
- a Molecular Devices Spectramax 250 microplate reader or similar plate reader
- an AB Sciex Veriti 96-well thermocycler or a similar incubator

Bead-beating treatment

30m

- 1 Thaw cells at Room temperature

Note

Note: If transferring directly from active cultures, omit this step. Adapt as needed for your specific organism and culturing conditions.

- 2 Transfer 2-4 OD*mLs of cells to 8-Strip PCR tubes (Axygen, Cat.#14-222-251) or a 96-well PCR plate (ThermoFisher, Cat.#AB0600).

A strip of PCR tubes filled 30-40% full of cell pellet is approximately 10-20 OD*mLs of cells.

3

Centrifuge at 4000 rpm, 25°C, 00:01:00 then remove the supernatant. 0 μ L

4

Add 60 μ L of LC-MS grade Methanol (VWR Scientific, Cat.#BJLC230-2.5). Pipet to resuspend well.

Note

Note: You can combine step #4 and step #5 by adding 120 μ l 1:1 methanol:chloroform together.

5

Add 60 μ L of Chloroform (Sigma-Aldrich, Cat.#34854). Pipet/vortex to mix.

Safety information

Use a fume hood when handling and pipetting chloroform.

- 6 Add 50 μ L of beads (BioSpec 0.5mm Zirconia/Silica) to each PCR tube.

Note

Note: You can also transfer resuspended cells into a new set of PCR tubes that is pre-loaded with beads.

- 7 Bead beat for 5 cycles of 1 minute at 30 Hz (max. frequency) in TissueLyser with 30 seconds on ice in between cycles.

- 8 Gently spin samples to bring all liquid to the bottom of the tube

Note

Important: Spin before adding water/inducing phase separation.

Protein extraction

30m

- 9 Transfer supernatant (as much as possible) into new PCR tubes.

- 10 Add 50 μ L of LC-MS grade Water (VWR Scientific, Cat.#BJLC365-2.5). Pipet/vortex to mix.

Note

Note: This makes the supernatant now roughly 1:1:1 methanol:chloroform:water, if we assume ~20 μ L lost within the beads; about 150 μ L total volume remains in the PCR tubes.

11

Centrifuge at 4000 rpm, 25°C,
00:01:00

12

Carefully remove the top layer of solvent (Methanol + Water) by pipetting.

13

Add 100 μ L of Methanol.

Note

Tip: Break up the protein pellet by piercing it with the pipet tip, and add Methanol to the bottom of the tube or plate.

14

Centrifuge at 4000 rpm, 25°C,
00:02:00

15

Carefully discard solvent (Methanol + Chloroform).

Safety information

Discard in an appropriate waste container for halogenated solvents.

16

Air-dry for 00:05:00

5m

Note

Tip: Do not dry for longer than 00:45:00 or the pellet will be difficult to resuspend.

Safety information

Dry samples in a fume hood.

17

Resuspend with 60 μ L

100 millimolar (mM) Ammonium bicarbonate in 20% Methanol

Note

Note: Samples are typically cloudy in this step. After trypsin digestion they will be nearly clear.

18

Store at -20 °C until ready for Protein Quantitation Assay.

Protein Quantitation Assay (Lowry Method)

30m

19

Dilute samples 10 fold by adding 5 μ L Protein sample, mix well right before transfer to 45 μ L Water in 8-Strip PCR tubes or 96-well plate.

Note

Note: The protein concentration can be determined by using several methods that are available in kits. We use the Bio-Rad DC Protein Assay (Bio-rad Laboratories, Cat.#500-0113, Cat.#500-0114) but the Bradford protein quantification assay is also commonly used. The accuracy of most protein concentration measurements can be variable, thus it is important to minimize differences in sample handling and to use replicates when quantifying the amount of protein in a sample.

20

Transfer 2 replicates of each of the following to Corning 96-Well Black Polystyrene Microplate (Fisher Scientific, Cat.#07-200-590):

5 μ L Water
(Blank)

5 μ L Pierce Bovine Serum Albumin Standard Pre-Diluted Set (Std) (ThermoFisher,
Cat.#23208)

5 μ L Diluted samples, mix well right before adding to plate (Example 1-
20)

	Blank	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7				
	Blank	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7				
	1	2	3	4	5	6	7	8	9	10	11	12
	1	2	3	4	5	6	7	8	9	10	11	12
	13	14	15	16	17	18	19	20				
	13	14	15	16	17	18	19	20				

Example Plate with 20 samples

21

Add 25 μ L Bio-Rad DC Protein Assay Reagent A (Bio-rad Laboratories, Cat.#500-
0113) and
wait 00:05:00 .

22

Add 200 μ L Bio-Rad DC Protein Assay Reagent B (Bio-rad Laboratories, Cat.#500-
0114) and
wait 00:10:00 .

23

Read plate in the microplate reader (280 nm) and calculate protein concentrations.

Trypsin Digestion (5h - 16h)

5h

24

Chemicals to prepare:

15m

• **Prepare** [M] 100 millimolar (mM) Tris(2-carboxyethyl)phosphine (TCEP) **solution** by dissolving

⚗ 28.7 mg TCEP in ⚗ 1 mL 100mM Ammonium Bicarbonate

• **Prepare** [M] 200 millimolar (mM) Iodoacetamide (IAA) **solution** by dissolving

⚗ 36.8 mg Iodoacetamide in ⚗ 1 mL 100mM Ammonium Bicarbonate

• **Prepare** [M] 1 mg/mL Trypsin by adding ⚗ 1 mL 1mM HCl to ⚗ 1 mg Trypsin

Note

Store TCEP, IAA, and Trypsin in -20C.

IAA is light sensitive. Store in amber tube (Fisher Scientific, Cat.#05-402-31).

25

Dilute protein samples to [M] 2.4 $\mu\text{g}/\mu\text{L}$ in

[M] 100 millimolar (mM) Ammonium Bicarbonate (AMBIC)

Note

Mix protein well before you dilute it.

26

Mix protein with TCEP, IAA, and trypsin in

[M] 100 millimolar (mM) Ammonium Bicarbonate (AMBIC)

Note

The final concentrations will be $2\text{ }\mu\text{g}/\mu\text{L}$ protein (in 50 μL total volume)

5 mM TCEP

10 mM IAA

, and $2\text{ }\mu\text{L}$ Trypsin (1 mg/ml)

(1:50 trypsin:protein ratio). Adjust as needed for your data acquisition protocols.

add $41.67\text{ }\mu\text{L}$ protein ($2.4\text{ }\mu\text{g}/\mu\text{L}$)

add $2.5\text{ }\mu\text{L}$ TCEP (100 mM)

add $2.5\text{ }\mu\text{L}$ IAA (200 mM)

add $2\text{ }\mu\text{L}$ Trypsin (1 mg/ml)

add $1.33\text{ }\mu\text{L}$ AMBIC

up to $50\text{ }\mu\text{L}$ total volume

Note

If you do not have enough protein, you can set your final protein concentration to $1\text{ }\mu\text{g}/\mu\text{L}$.

add $20.83\text{ }\mu\text{L}$ protein ($2.4\text{ }\mu\text{g}/\mu\text{L}$)

add $2.5\text{ }\mu\text{L}$ TCEP (100 mM)

add $2.5\text{ }\mu\text{L}$ IAA (200 mM)

add $1\text{ }\mu\text{L}$ Trypsin (1 mg/ml)

add $23.17\text{ }\mu\text{L}$ AMBIC

up to $50\text{ }\mu\text{L}$ total volume

27 Incubate at 37 °C for 04:00:00 - 16:00:00 .

28 Centrifuge at 4000 rpm, 4°C, 00:15:00 .

29 Carefully pipet out clear liquid sample into plastic autosampler vials (ThermoFisherScientific, Cat.#03-060-016) or a 96-well plate (BIO-RAD, Cat.#HSP9601).

30 Store at -20 °C until ready for LC-MS/MS analysis.