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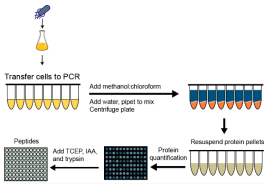
Chloroform-Methanol Protein Extraction for Gram-negative Bacteria (High Throughput)



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We use this protocol and it's working

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Abstract

Recent improvements in the speed and sensitivity of liquid chromatography-mass spectrometry systems have driven progress toward system-wide characterization of the proteome of many species. These efforts create large proteomic datasets that provide insight into biological processes and identify diagnostic proteins whose abundance changes significantly under different experimental conditions. Consequently, it is important to have reproducible sample preparation methods that consist of mixing, various centrifugation and incubation steps, and an extended tryptic digestion step. We developed a high-throughput sample preparation workflow that consists of 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.

Guidelines

- All centrifuge steps use an Eppendorf 5810R centrifuge.

- 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 ~130 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 #**I1149
- Methanol LC-MS grade B&J Brand **VWR International (Avantor) Catalog #**BJLC230-2.5
- Chloroform for HPLC **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**34854
- Water LC-MS grade B&J Brand **VWR International (Avantor) Catalog #**BJLC365-2.5
- Ammonium Bicarbonate LC-MS grade **VWR International (Avantor) 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 #**03-060-016
- 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/clear **Bio-Rad Laboratories Catalog #**HSP9601

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 gloves and appropriate PPE for safety and to minimize contamination of samples.

Before start

This protocol consists of steps for:
- Protein extraction from Gram-negative bacterial cells
- Protein quantification
- Tryptic digestion

For this protocol you will need:
- an Eppendorf 5810R centrifuge with S-4-104 rotor or similar centrifuge
- a Molecular Devices Spectramax 250 microplate reader or similar plate reader
- an AB Sciex Veriti 96-well thermocycler or a similar incubator

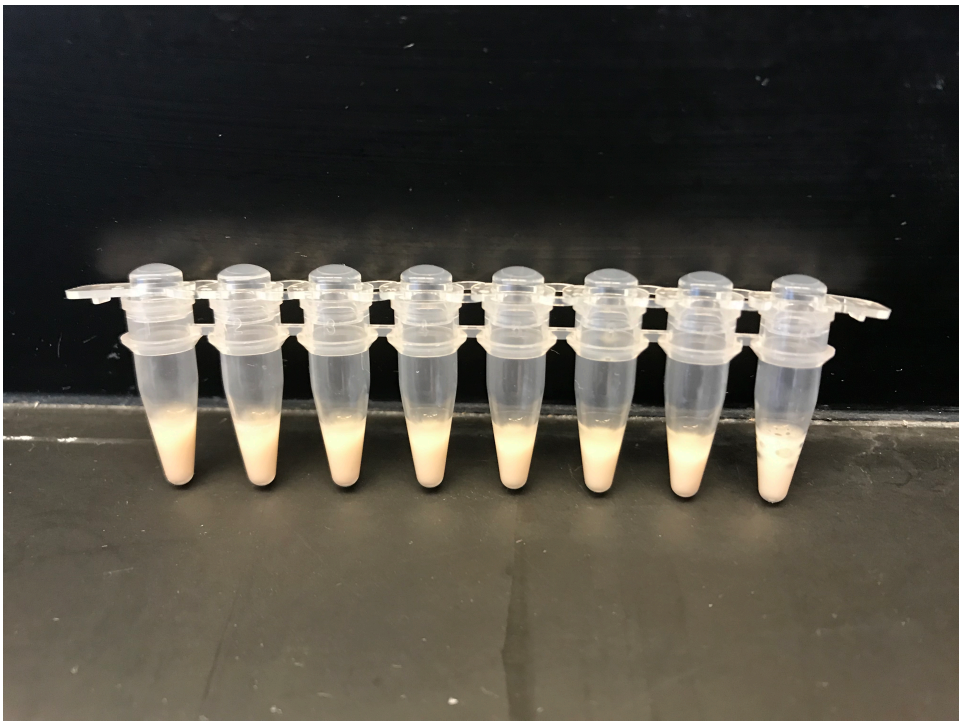
Protein extraction

30m

1 Thaw cells at On ice

> **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 2-5 OD*mLs of cells.

3 Add 80 µL of LC-MS grade Methanol (VWR Scientific, Cat.#BJLC230-2.5). Pipet to resuspend well.

4 Add 20 µL of Chloroform (Sigma-Aldrich, Cat.#34854). Pipet/vortex to mix.

Safety information

Use a fume hood when handling and pipetting chloroform.

5 Add 60 µL of LC-MS grade Water (VWR Scientific, Cat.#BJLC365-2.5). Pipet/vortex to mix.

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

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

8 Add 100 µL 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.

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

10 Carefully discard solvent (Methanol + Chloroform).

Safety information

Discard in an appropriate waste container for halogenated solvents.

11 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.

12 Resuspend with 60 µL [M] 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.

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

Protein Quantitation Assay (Lowry Method) 30m

14 Dilute samples 10 fold by adding 5 µL Protein sample, mix well right before transfer to 45 µL 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.

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

5 µL Water (Blank)

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

5 µL 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

16 Add

25 µL Bio-Rad DC Protein Assay Reagent A (Bio-rad Laboratories, Cat.#500-0113)

and wait 00:05:00 .

.

17 Add

200 µL Bio-Rad DC Protein Assay Reagent B (Bio-rad Laboratories, Cat.#500-0114)

and wait 00:10:00 .

.

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

Trypsin Digestion (5h - 16h)

5h

19 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).

20 Dilute protein samples to [M] 2.4 µg/µL in [M] 100 millimolar (mM) Ammonium Bicarbonate (AB)

Note

Mix protein well before you dilute it.

21 Mix protein with TCEP, IAA, and trypsin in [M] 100 millimolar (mM) Ammonium Bicarbonate (AMBIC) .

Note

The final concentrations will be [M] 2 µg/µL protein (in 50 ul total volume) , [M] 5 millimolar (mM) TCEP , [M] 10 millimolar (mM) IAA , and 2 µL Trypsin (1 mg/ml) (1:50 trypsin:protein ratio). Adjust as needed for your data acquisition protocols.

add 41.67 µL protein (2.4 ug/ul)
add 2.5 µL TCEP (100 mM)
add 2.5 µL IAA (200mM)
add 2 µL Trypsin (1 mg/ml)
add 1.33 µL AMBIC
up to 50 µL total volume

Note

If you do not have enough protein, you can set your final protein concentration to 1 ug/ul.

add 20.83 µL protein (2.4 ug/ul)
add 2.5 µL TCEP (100 mM)
add 2.5 µL IAA (200 mM)
add 1 µL Trypsin (1 mg/ml)
add 23.17 µL AMBIC
up to 50 µL total volume

22 Incubate at 37 °C for 04:00:00 - 16:00:00 . 4h

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

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

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