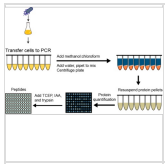

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Chloroform-Methanol Protein Extraction for Gram-negative Bacteria (High Throughput)

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2 *Works for me* dx.doi.org/10.17504/protocols.io.bfx6jpre

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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.

EXTERNAL LINK

https://pubs.acs.org/doi/abs/10.1021/acs.jproteome.9b00455

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

NAME	CATALOG #	VENDOR
Corning™ 96-Well Solid Black Polystyrene Microplates (Costar 3915)	07-200-590	Fisher Scientific
Pierce™ Bovine Serum Albumin Standard Pre-Diluted Set	23208	Thermo Fisher

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NAME	CATALOG #	VENDOR
Tris(2-carboxyethyl)phosphine hydrochloride (TCEP)	C4706	Sigma Aldrich
Iodoacetamide	I1149	Millipore Sigma
Methanol LC-MS grade B&J Brand	BJLC230-2.5	VWR Scientific
Chloroform for HPLC	34854	Sigma – Aldrich
Water LC-MS grade B&J Brand	BJLC365-2.5	VWR Scientific
Ammonium Bicarbonate LC-MS grade	BJ40867-50G	VWR Scientific
DC Protein Assay Reagent A	500-0113	Bio-rad Laboratories
DC Protein Assay Reagent B	500-0114	Bio-rad Laboratories
8-strip PCR Tubes with Caps	14-222-251	Axygen
Trypsin	T6567-1MG	Sigma Aldrich
PCR Plate 96-well non-skirted	AB0600	Thermo Fisher Scientific
Thermo Scientific Autosampler Vial Kit	03-060-016	Thermo Fisher Scientific
Eppendorf Snap-Cap Microcentrifuge Flex-Tube Tubes Amber	05-402-31	Fisher Scientific
Hard-Shell 96-Well PCR Plates low profile thin wall skirted white/clear	HSP9601	BIO-RAD

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 STARTING

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: 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).

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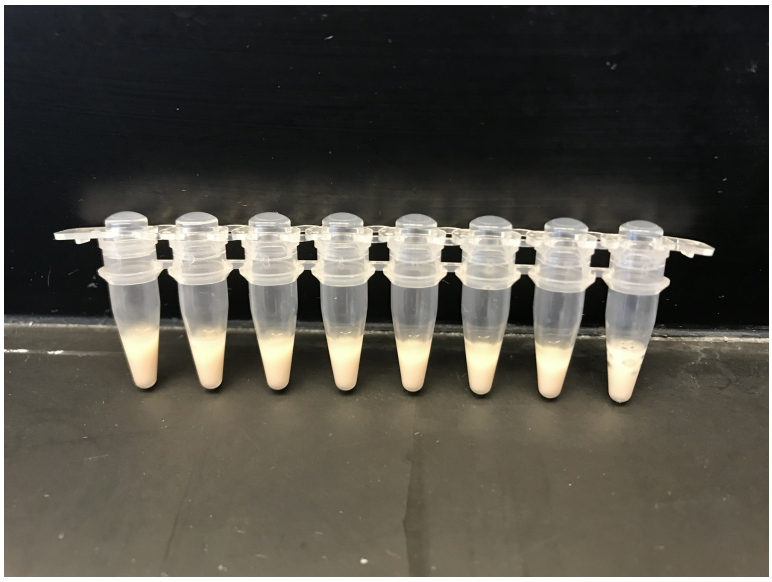

A strip of PCR tubes filled 30-40% full of cell pellet is approximately 2-5 OD*mLs of cells.

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.

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.

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

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

10 Carefully discard solvent (Methanol + Chloroform).

Discard in an appropriate waste container for halogenated solvents.

11 Air-dry for **00:05:00** . 5m

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

Dry samples in a fume hood.

12 Resuspend with **60 µl** **100 Milimolar (mM) Ammonium bicarbonate in 20% Methanol** .

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: 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.

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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 Milimolar (mM) Tris(2-carboxyethyl)phosphine (TCEP) solution** by dissolving **28.7 mg TCEP** in **1 ml 100mM Ammonium Bicarbonate**

• **Prepare [M]200 Milimolar (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**

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

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

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

Mix protein well before you dilute it.

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

The final concentrations will be [M]2 µg/µl protein (in 50 ul total volume) , [M]5 Milimolar (mM) TCEP , [M]10 Milimolar (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

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

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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.

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