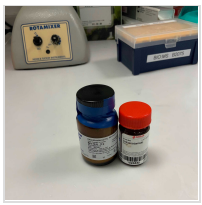

Aug 17, 2022

Reduction and alkylation of protein lysates for LC-MS (proteomics) using dithiothreitol (DTT) and iodoacetamide (IAM)

In 1 collection

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dx.doi.org/10.17504/protocols.io.6qpvr6573vmk/v1

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ABSTRACT

This protocol details the procedure of the reduction and alkylation using dithiothreitol (DTT) and iodoacetamide (IAM).

ATTACHMENTS

iiaebptmp.docx

DOI

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PROTOCOL CITATION

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COLLECTIONS

Researcher led sample preparation for LC-MS using the BioMS research core facility

KEYWORDS

Reduction, Alkylation, Iodoacetamide, Dithiothreitol, in-solution digestion, peptide desalting proteomics, mass spectrometry, LC-MS, proteomics, Cysteine, Cystine, Disulfides, Sulfhydryl Compounds

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PARENT PROTOCOLS

Part of collection

Researcher led sample preparation for LC-MS using the BioMS research core facility

GUIDELINES

- Reduction and alkylation of cysteine bonds is a prerequisite for LC-MS sample preparation for two reasons:

1. Cysteine bonds are part of the proteins secondary structure. By reducing (breaking) them, it allows better access of trypsin or other digestion enzymes for the complete conversion of protein to peptide.
2. It is not straightforward to identify cysteine containing peptides using LC-MS, because they usually are two peptides, linked by at least one cysteine bond. This will not be matched when the data is searched against the database, and will limit protein sequence coverage in the results.

- It is a sequential reaction. Dithiothreitol (DTT) is used to reduce the cysteine bonds present in the protein. After a short incubation, iodoacetamide (IAM) is added to modify the free cysteines. After another short incubation, DTT is added again to quench any free IAM.

Initial assumptions and preparation:

- Allow approximately **01:30:00** reduction and alkylation.
- You have protein lysates in Eppendorf tubes in a known volume of S-Trap lysis buffer.

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MATERIALS TEXT

Locate the following buffers, consumables, and reagents:

A	B
Location	Buffer/reagent
Fridge 02	DTT (PN# BP172-5,Fisher) pre-weighed aliquots – pink box – top shelf IAM (PN# I1149,Sigma Aldrich) pre-weighed aliquots – pink box – top shelf
Bench	Eppendorf tubes, 1.5 mL volumes depending on sample volume. LC-MS grade water tinfoil
Freezer	N/A

Identify the following equipment that you will use:

- **20 µL** pipette, **200 µL** pipette, **1 mL** pipette and pipette tips.
- Kern fine balance.

Dithiothreitol **Fisher Scientific Catalog #BP1725**

Iodoacetamide **Millipore Sigma Catalog #I1149**

ThermoMixer® C
Thermoblock
Eppendorf **5382000031**

BEFORE STARTING

Initial preparation:

Before you begin:

Locate the Eppendorf Thermomixer, and attach the appropriate heating block depending on whether your samples are in **0.5 mL** or **1.5 mL** tubes. Set the temperature to **60 °C** and a speed of **500 rpm** .

Before you begin

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1 Locate the Eppendorf Thermomixer, along with the thermoblock you will be using. The Thermoblock "clicks" into place onto the Thermomixer unit. Set the Thermomixer to **60 °C** and a speed of **500 rpm** . 5m

PCR 96 Thermoblock

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Use the PCR 96 thermoblock for **0.75 mL** Eppendorf tubes

1.5 mL thermoblock

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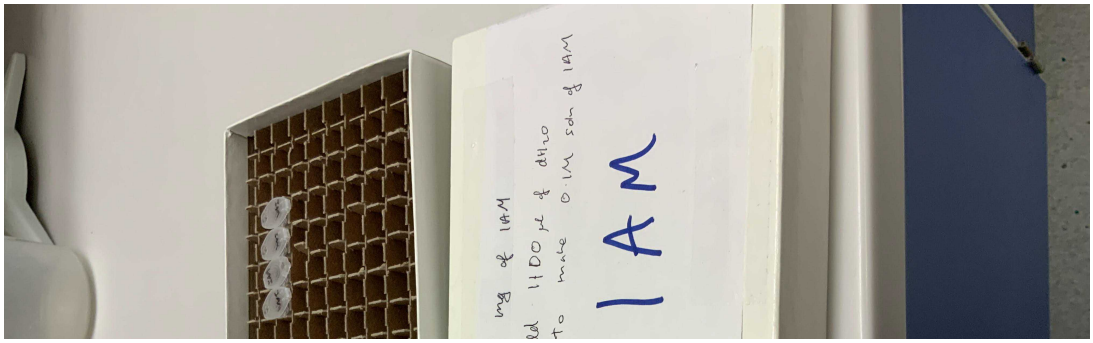
Use the 1.5 mL thermoblock for **1.5 mL** Eppendorf tubes.

2 Remove the DTT and IAM aliquots from fridge 2. 5m

Fridge location, pre-weighed aliquots are on bottom shelf

3 They are pre-weighed in 1.5 mL Eppendorf tubes. 1m



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Pre-weighed aliquots

4 Take one of each for your preparation. Place the boxes back in the fridge. 1m

5 10m

To make a [M]**100 millimolar (mM)** solution of DTT and IAM, add the volume of water indicated on the box from which you took the pre-weighed aliquot.

NB. It is important to wrap the [M]100 millimolar (mM) solution of IAM in tinfoil when it is prepared.
Both of these solutions have a short half-life (approximately 24 hours), and must be prepared fresh.

Reduction and alkylation: 1h 20m

6

You will need to know the volume of the protein lysate sample that you wish to alkylate. If proceeding from LE220+ sample processing, this will be approximately 130 uL for the smaller tubes, and 500 uL for the larger tubes.

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7 Reduce your protein sample.

A final concentration (Fc) of **5 millimolar (mM)** of DTT is used for reducing proteins.

7.1 If the starting concentration (Sc) of the stock DTT is **100 millimolar (mM)**, and the volume of sample (Fv) to be reduced is **130 µL**, then use the following calculation to work out how much of the stock DTT (Sv) to add = (Fc * Fv) / Sc.

7.2

For **130 µL** of lysate, add (5 * 130) / 100, or **6.5 µL** of stock **100 millimolar (mM)** DTT.

8

For the **500 µL** lysate, add **25 µL** of the stock **100 millimolar (mM)** DTT.

9 Place the tubes on the Eppendorf thermomixer and heat for **00:10:00** at **60 °C**. 10m

This reduces the cysteine bonds.

10

Alkylate your protein sample. Remove the protein samples from the thermomixer, and allow to cool to **Room temperature**. Add IAM. It is important to add at least three times the amount of IAM to DTT. So alkylate with **15 millimolar (mM)** of IAM.

10.1

For **130 µL** of protein sample, add (15 * 130) / 100 or **20 µL** of IAM to the samples, and for **500 µL** of sample, add (15 * 500) / 100 or **75 µL** of IAM to the samples.

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11 30m

Vortex mix the tubes briefly and place the protein sample tubes in the dark (a drawer) for **00:30:00** .

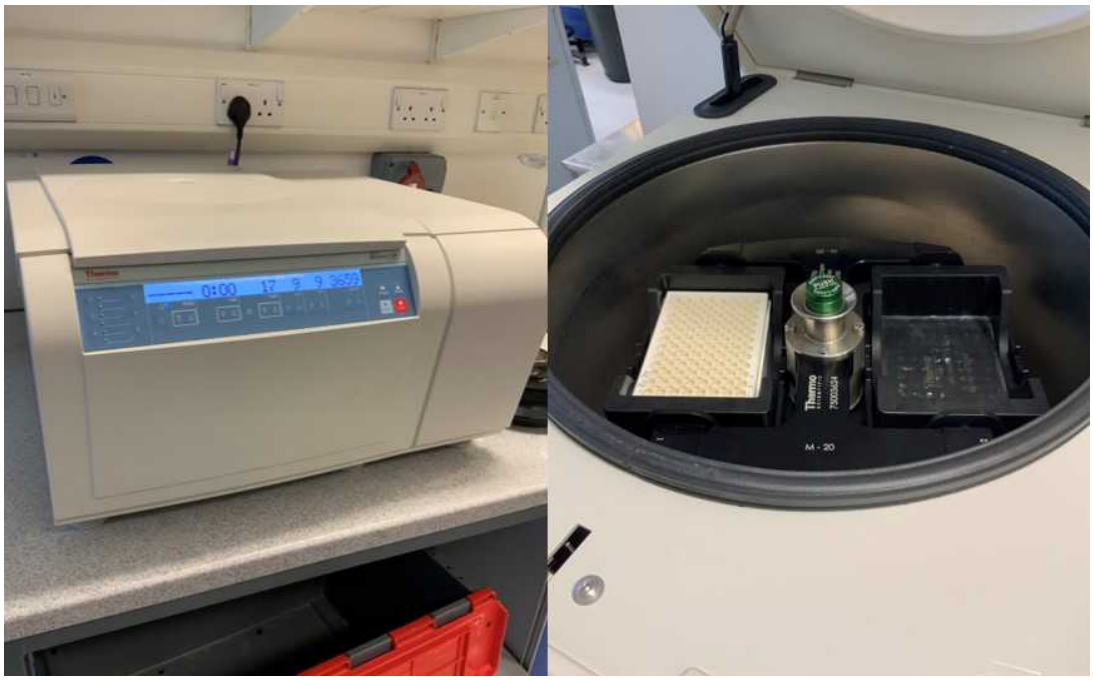
12 30m

After **00:30:00** , add DTT to quench the alkylation reaction. Add the same amount of DTT again as to what you added in the reduction step. Vortex mix briefly.

Do not incubate your protein lysate in the presence of IAM for longer than 30 minutes. Extended incubation may result in unwanted side-chain modifications of amino acids other than cysteines.

13 10m

Centrifuge your samples at **14000 rcf** for **00:10:00** using the centrifuge in room B2075. To use, switch is located at rear of machine. To swop rotors, press down on the green spindle, and lift the rotor upwards. The centrifuge is for using a plate rotors, and a tube rotor. The rotors are located to the right of the machine, along with balances.
Tubes and plates must be balanced!



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Hereus Megafuge 16R

14 10m

Remove the supernatant using a clean pipette tip to a labelled tube. This is the protein lysate.

15 You have now reduced and alkylated your protein lysate samples.

You may now freeze the samples at this stage, or proceed to protein quantitation using the Millipore Direct Detect.

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