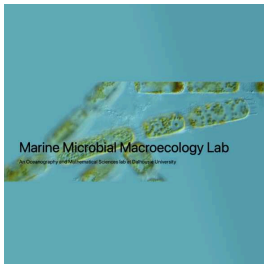
Jan 28, 2025 Version 2

Comprehensive Protocol for Total Protein Extraction, Precipitation, and Assay Measurement from Plankton Samples V.2



Version 1 is forked from Total crude protein in plankton: Pierce BCA protein assay (including the enhanced assay for low biomass)

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We use this protocol and it's working

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Abstract

This protocol outlines an optimized method for extracting total protein from plankton samples, encompassing both phytoplankton and zooplankton. The procedure involves the use of lysing matrix tubes with beads of varying sizes (0.1 mm, 1.4 mm, and 4 mm), eight homogenizing cycles, and a protein extraction buffer composed of 2% SDS, 10% glycerol, 5 mM EDTA in 100 mM Tris (pH 7). The extracted crude protein is quantified using the Pierce Micro BCA assay equivalent to bovine serum albumin (BSA), with a linear detection range of 12.5 to 1000 ug/sample.

To remove interference from chlorophyll, monosaccharides and other substances, the extracted protein undergoes precipitation using a chloroform and methanol solvent mixture, followed by re-dissolution in re-solubilization buffer composed of 1% sarcosine in 50 mM Tris (pH 7). BCA assay is then used to quantify the precipitated protein, with BSA as the standard. The absence of glycerol and the use of sarcosine instead of SDS enable a linear detection range of 5 to 2000 µg/sample.

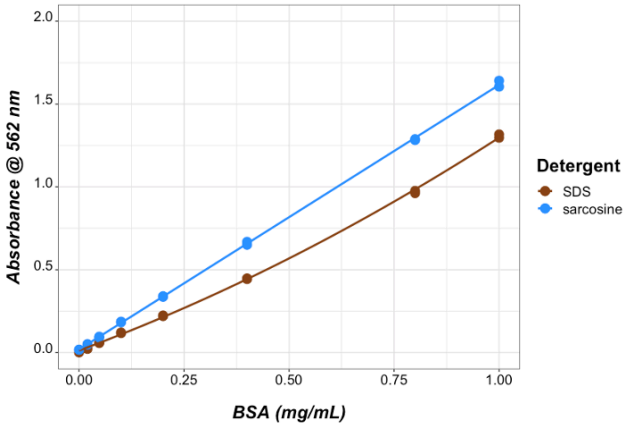
https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0011430_Pierce_BCA_Protein_Asy_UG.pdf

Guidelines

1. Comparison of protein yield between the **previous method** and the improved method. The "Ratio" column represents the protein obtained from the improved method divided by that from the previous method. All samples were collected on polycarbonate (PC) filters or directly weighed into the bead tubes.

	Species	Condition	Ratio
	Synecchococus (MITS1220)	Exponential	3.29 (0.45)
	L. fissa (CCMP2935)	Stationary	2.87 (0.33)
	L. fissa (CCMP2935)	Decline	2.23 (0.18)
	Zooplankton (size: 500 - 2k um)	field sample	1.95 (0.06)
	T. pseudonana (CCMP1335)	Exponential	1.25 (0.17)
	P. calceolata (RCC100)	Exponential	1.23 (0.08)
	P. calceolata (RCC100)	Stationary	0.98 (0.11)

2. Comparison of BCA assay response in precipitated protein dissolved in SDS vs sarcosine



3. For microplate loading:
(1) Reverse pipetting: aspirate sample right after vortexing.
(2) While dispensing, pipette tip gently touches the side of the well, avoid bending.

4. The recovery rate of protein precipitation from BSA is determined to be 84.87%. This value can be utilized to approximate corrections to the protein yield obtained from the samples.

Protocol materials

- Pierce BCA Protein Assay Kit **Thermo Fisher Scientific Catalog #**23225
- Tris Buffer 1M pH 7.0 **Fisher Scientific Catalog #**BP1756-500
- Glycerol **Bioshop Catalog #**GLY001.500
- Sodium dodecyl sulfate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**L3771
- EDTA buffer solution (0.5 M) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**4055-100ml
- N-lauroylsacosine sodium salt solution (30%) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**61747
- Chloroform (HPLC grade) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**439142-4L
- Thermo Scientific™ Pierce™ Bovine Serum Albumin Standard 2 mg/mL (50 mL) **Thermo Scientific Catalog #**Thermo Scientific™ 0023210
- Methanol (HPLC grade) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**34860-4X2L-R
- Micro BCA Protein Assay Kit **Thermo Scientific Catalog #**23235

Safety warnings

Waste from BCA assay needs to be collected into waste container and gets further treated before disposal due to the negative impact on the activity of microorganism.
Solvent from protein precipitation (1) the mixture of water and methanol (2) the mixture of methanol and chloroform shall be disposed into separated waste container.

Sample collection

1 Microalgae samples

1.1 Calculate the volume to obtain enough biomass for the assay:

If using 500 uL extraction buffer, the minimum sampling volume (mL) = 750/(Chl-a_ug/L)
If using 1000 uL extraction buffer, the minimum sampling volume (mL) = 2X750/(Chl-a_ug/L)

1.2 Filter microalgae in liquid media onto filters, using gentle vacuum pressure (130 mmHg).

> Note
>
> Samples collected on GF/F filters yield higher protein recovery compared to those on polycarbonate filters by using the same protocol.

1.3 Rinse filter tunnel with filtered artificial seawater (nutrient free) to avoid sample loss.

1.4 Fold the filter with two tweezers:
(1) Fold in half along its diameter, creating a semicircular shape;
(2) Fold once more in the same direction, resulting in a long strip;
(3) For polycarbonate filter, fold once more, halving its length, so that sample is secured

1.5 Place folded filter in 2 mL cryogenic vial.

1.6 Filter blank media (without cells) through polycarbonate filter as blank.

1.7 Flash-freeze tubes with liquid nitrogen and store at -80 °C

1.8 Freeze-dry samples before extraction.

2 Zooplankton samples

2.1 Grind freeze-dried samples in metal grinding tube (need dry ice) 5s

Equipment

Metal lysing matrix tube	NAME
MPBio	BRAND
116992006	SKU

Equipment

CoolPrep™ adapter for 24 × 2 mL tube holder on FastPrep-24	NAME
MPBio	BRAND
116002528	SKU

Equipment

FastPrep-24 5G	NAME
Bead-beater	TYPE
MP Biomedicals	BRAND
116005500	SKU
https://uk.mpbio.com/fastprep-24-5g-instrument	LINK

JPG

2.2 Transfer ground sample into Lysing matrix tube (containing 1.4 mm ceramic spheres, 0.1 mm silica spheres and one 4 mm glass bead), weigh the biomass and log into sampling sheet.

Equipment

Lysing matrix E tube	NAME
2 mL	TYPE
MPbio	BRAND
116914050-CF	SKU

Equipment

Prefilled tubes	NAME
100 µm and 4 mm silica and 1.4 mm zirconia acid washed beads	TYPE
Cole-Parmer	BRAND
2303-MM3	SKU

2.3 Flash-freeze tubes with liquid nitrogen, store at -80 °C .

Reagent

3 [M] 100 mM pH 7 Tris buffer

Dilute 1 part 1M pH 7 Tris with 9 part MilliQ.

Tris Buffer 1M pH 7.0 **Fisher Scientific Catalog #**BP1756-500

4 Protein extraction buffer (PEB, 4X)

4.1 Use a transfer pipet to add 40 g glycerol into a 100 mL reagent bottle.

Glycerol **Bioshop Catalog #**GLY001.500

4.2 Weigh 8 g SDS and transfer to the same reagent bottle

Sodium dodecyl sulfate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**L3771

4.3 Add 0.5 M EDTA 4 mL

EDTA buffer solution (0.5 M) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**4055-100ml

4.4 Top to 100 g with [M] 100 mM pH 7 Tris buffer.

4.5 Shake at 200 rpm, 37°C for complete dissolution.

Note

PEB (4X) might precipitate at room temperature due to the high concentration of glycerol and SDS. Redissolve by shaking at 37 degree.

5 [M] 50 mM pH 7 Tris buffer

Dilute five part 1M pH 7 Tris with 95 part MilliQ.

6 20% Sarcosine
Dilute two part 30% sarcosine with one part 50 mM pH 7 Tris buffer.

N-lauroylsacosine sodium salt solution (30%) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**61747

7 Protein extraction buffer (PEB, 1X): 2% SDS, 10% glycerol, 5 mM EDTA in 100 mM pH 7 Tris buffer:

7.1 Shake the 4X PEB at 37 °C until the SDS is fully dissolved back into the solution.

7.2 one part Protein extraction buffer (PEB, 4X)
three part 100 mM pH 7 tris buffer

Protein extraction

8 Remove samples out of the ULT and keep them On ice .

9 Reverse pipet 1 mL PEB (1X) into each bead tube.

10 Prepare samples for homogenizing

Zooplankton samples are already in the bead tubes.
Phytoplankton samples need to be transferred from cryogenic tubes to bead tubes by following the steps below:

10.1 Rinse forceps with 95% ethanol and air dry

Equipment

Filter forceps	NAME
blunt end, stainless steel	TYPE
Millipore	BRAND
XX6200006P	SKU

10.2 Label Lysing matrix tubes (containing 1.4 mm ceramic spheres, 0.1 mm silica spheres and one 4 mm glass bead) and use clean forceps to transfer samples and blank filters into its corresponding tube.

10.3 Prior to transferring:

(1) For filters folded into half-strip, unfold once to return to a strip.
(2) For filters folded into quarter-circles, unfold once to return to a half-circle shape, then fold once along the dimension to form a strip.

(2) For filters haphazardly into a compact mass, carefully unfold with two tweezers (avoiding losing biomass), fold once into a half-cricle shape, then fold once more along the dimension to form a strip

10.4 Insert the strip into beads mixture.

10.5 Place the bead tubes On ice .

11 Turn on FastPrep

Equipment

FastPrep-24 5G	NAME
Bead beater	TYPE
MP Biomedicals	BRAND
116005500	SKU
https://uk.mpbio.com/fastprep-24-5g-instrument	LINK

12 Turn on the refrigerate centrifuge, set temperature at 4 °C

Equipment

CENTRIFUGE 5430 R	NAME
Eppendorf	BRAND
MP2231000510	SKU

13 Check the cap of each tube to ensure cap is tightly closed. Organize the tubes in order, log the position of each tube, in case the labels get rubbed out during extraction.

14 Run 00:01:00 at 6.5 m/s (1/8) 1m

15 Keep tubes On ice for 00:01:00

16 Check labels. Put tubes back into FastPrep.

17 Run 00:01:00 at 6.5 m/s (2/8) 1m

18 Keep tubes On ice for 00:01:00

19 Check labels. Put tubes back into FastPrep.

20 Run 00:01:00 at 6.5 m/s (3/8) 1m

21 Keep tubes On ice for 00:01:00

22 Check labels. Put tubes back into FastPrep.

23 Run 00:01:00 at 6.5 m/s (4/8) 1m

24 Keep tubes On ice for 00:01:00

25 Check labels. Put tubes back into FastPrep.

26 Run 00:01:00 at 6.5 m/s (5/8)

27 Keep tubes On ice for 00:01:00

28 Check labels. Put tubes back into FastPrep.

29 Run 00:01:00 at 6.5 m/s (6/8)

30 Keep tubes On ice for 00:01:00

31 Check labels. Put tubes back into FastPrep.

32 Run 00:01:00 at 6.5 m/s (7/8)

33 Keep tubes On ice for 00:01:00

34 Check labels. Put tubes back into FastPrep.

35 Run 00:01:00 at 6.5 m/s (8/8)

36 De-foam by centrifuging the extract. 5m
20000 rcf, 4°C, 00:05:00

37 Transfer extract to 2 mL microtubes.

38 Centrifuging the extract. 5m
20000 rcf, 4°C, 00:05:00

39 Aliquot

Protein extraction

39.1 For microBCA assay
Transfer 12 uL supernatant to 2 mL microtube

39.2 For protein precipitation
Transfer 100 uL supernatant to 2 mL microtube in duplicate for protein precipitation.

Protein extraction

40 Freeze aliquoted extract and the remaining extract at -80 °C .

Crude protein quantitation by microBCA

41 Micro BCA Protein Assay Kit **Thermo Scientific** **Catalog #**23235

42 Thaw protein extract (go to step #39.1)

43 Add 288 µL MQ into each extract.

44 Organize eleven 2 mL microtubes in the tube rack, label the tubes from C1 to C11 (C denotes crude).

45 Thermo Scientific™ Pierce™ Bovine Serum Albumin Standard 2 mg/mL (50 mL) **Thermo Scientific** **Catalog #**Thermo Scientific™ 0023210

46 4% PEB

Mix:
1.6 mL PEB (1X)
38.4 mL MilliQ

47 PEB and BSA: transferred by reverse pipetting

	Standard	Source	Source (uL)	PEB (1X, uL)	MilliQ (uL)	4% PEB (uL)	Conc. (ug/mL)
	C1	2 mg/mL BSA	400	80	2X760	**0**	400
	C2	C1	800	0	0	800	200
	C3	C2	800	0	0	800	100
	C4	C3	960	0	0	640	60
	C5	C1	160	0	0	2X720	40
	C6	C4	800	0	0	800	30
	C7	C5	800	0	0	800	20
	C8	C7	800	0	0	800	10
	C9	C8	800	0	0	800	5
	C10	C9	800	0	0	800	2.5
	C11	C10	800	0	0	800	1.25
	C12	4% PEB	0	0	0	100*	0

*C12 is loaded directly in the microplate.

48 Reverse pipetting: load 4 µL of standard solution (from C9 to C2) onto microdrop plate.

Equipment

µDrop™ Plates — NAME

Thermo Scientific — BRAND

N12391 — SKU

Note

Concentrations of C10 to C12 are below detection limit.

49 Read absorbance of eight standard solutions at 205 nm.

50 Plot the Absorbance at 205 nm versus its concentration in mg/ml. If the standard curve has good Coefficient of Determination, i.e., R^2>0.99, the standard solutions are in good quality; otherwise, prepare a new series of standard solutions until the quality of standard solutions meets the requirement.

51 Reverse pipet, load 100 µL standard solutions into the microplate, in duplicate.
Be aware of the layout, C12 (the blank) is loaded first.

	A	B	C	D
	C12	C12	C4	C4
	C11	C11	C3	C3
	C10	C10	C2	C2
	C9	C9	C1	C1
	C8	C8		
	C7	C7		
	C6	C6		
	C5	C5		

52 Reverse pipet, load 100 µL diluted extract into the microplate, in duplicate.

53 Use the following formula to determine the total volume of WR required. Consider leaving several mL of extra volume:

(# standards + # samples) X 200 µL = total volume WR required

54 Prepare WR by mixing 200 parts of Micro BCA reagent A, with 192 part of Micro BCA Reagent B, and 8 part of Micro BCA reagent C in a 15 or 50 mL falcon tube.

55 Turn on incubator and preheat to 37 °C

Equipment

SHAKING INCUBATOR	NAME
71L	TYPE
Corning® LSE™	BRAND
6753	SKU

56 Using Finnpipette stepper pipette (5 mL tip), dispense 100 µL of WR into each well. Ensure that the tip does not come into contact with the solution to prevent cross-contamination of samples.

Equipment

96-Well Microplates	NAME
Polystyrene, Clear,	TYPE
Greiner Bio-One	BRAND
82050-760	SKU

57 Cover the microplate with lid, shake and incubate at 37 °C for 02:00:00 at 150 rpm. 2h

Equipment

Microplate Lids	NAME
Polystyrene	TYPE
Greiner	BRAND
656170	SKU
https://www.fishersci.ca/shop/products/high-profile-polystyrene-microplate-lids-2/07000288#clear%20microplate%20lid	LINK

58 Shake for 5 s at 600 rpm in a continuous and high force mode
Read endpoint 562 nm with a measurement time 100 ms

Equipment

Varioskan LUX Multimode Microplate Reader	NAME
Thermo Fisher	BRAND
VL0L00D0	SKU

Calculate protein content per filter

59 Subtract the average absorbance at 562 nm of the blank standard replicates from the 562 nm measurements of all other individual **standards**.

60 Subtract the average absorbance at 562 nm of the blank sample (filter) replicates from the 562 nm measurements of all other individual **samples**.

61 Create a response curve where the corrected absorbance at 562 nm for each BSA standard is plotted against its concentration in mg/mL.

62 Standard curve

62.1 For crude protein ranging from 40 to 400 ug/mL, the standard curve is quadratic.
$Abs = aConc^2 + bConc + c$
Where Abs is the blank corrected absorbance at 562 nm and $Conc$ is the concentration of protein.

62.2 For precipitated protein or crude protein ranging from 0 to 40 ug/mL, the standard curve is linear.
$Abs = aConc + b$
Where Abs is the blank corrected absorbance at 562 nm and $Conc$ is the concentration of protein.

63 Use the standard curve to determine the protein concentration of each unknown sample by using its corrected absorbance at 562 nm.

64 Actual detection limit of the assay ($L - LOD$)

64.1 Enter the standard deviation (SD) of the absorbance values from the blank as Abs in the standard curve to determine the $Conc$.

64.2 $L - LOD = 3.3 * Conc$

65 If the calculated sample concentration is lower than L-LOD, then use "<LOD" as the result, indicating the protein content is undetectable.

66 Protein_mg/filter = Protein_mg/mL X PEB_mL X DF
In Micro BCA assay, DF = 25
In BCA for precipitated protein, DF = 1

Protein precipitation

50m 30s

67 Protein extract from go to step #39.2

68 Turn on refrigerate centrifuge, set temperature at 4 °C

Equipment

CENTRIFUGE 5430 R	NAME
Eppendorf	BRAND
MP2231000510	SKU

69 Add 300 µL MilliQ into each tube with extract.

70 In the fume hood, vortex after adding chloroform 100 µL

Chloroform (HPLC grade) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**439142-4L

71 In the fume hood, add methanol 400 µL

Methanol (HPLC grade) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**34860-4X2L-R

72 Vortex until the mixture turns milky.

73 Gently vortex for 00:00:30 by using a tube insert 30s

Equipment

VWR ANALOG VORTEX MIXER	NAME
VWR	BRAND
10153-838	SKU
With tube insert	SPECIFICATIONS

74 Incubate in the fridge for 00:30:00 30m

75 20000 rcf, 4°C, 00:10:00 10m

76 In the fume hood, remove upper phase (about 700 uL)

Note

Do not disturb the interphase.

77 In the fume hood, add 300 µL methanol. Invert the tube gently at a slight angle to avoid letting precipitated protein and liquid touch the inside of the cap.

78 20000 rcf, 4°C, 00:10:00 10m

79 In the fume hood, remove all solvent by using 100 uL pipet tip.

Note

Watch the pipet tip closely. Do not remove protein pellets with the solvent.

80 With microtube lid open and vacuum pump running, dry pellet in vacuum desiccator for 00:15:00 at Room temperature 15m

> Note
>
> Methanol and chloroform interfere the BCA assay. However, do not dry protein pellet for too long, otherwise it might lead to difficulties in resolubilizing the pellet .

81 Store at -80 °C .

BCA assay to quantify precipitated protein 50m 30s

82 Re-solubilization buffer (RSB):
Add 500 µL 20% sarcosine (go to step #6) into 9.5 mL [M] 50 mM Tris (go to step #5)

83 Reverse pipetting, add 100 µL RSB (go to step #82) to each tube with precipitated protein (go to step #81).
Vortex for complete re-solubilization.

84 Transfer about 800 uL 2 mg/mL BSA to a 2 mL microtube.

Thermo Scientific™ Pierce™ Bovine Serum Albumin Standard 2 mg/mL (50 mL) **Thermo Scientific Catalog #**Thermo Scientific™ 0023210

85 Organize eight 2 mL microtubes in the tube rack, label the tubes from P1 to P8, P denotes precipitated.

86 BSA standard solutions

	Standards	20% sarcosine (uL)	50 mM Tris pH 7 (uL)	2 mg/mL BSA (uL)	Final Conc. (mg/mL)
	P2	25	470	5	0.02
	P3	25	463	12	0.048
	P4	25	450	25	0.1
	P5	25	425	50	0.2
	P6	25	375	100	0.4
	P7	25	275	200	0.8

	Standards	20% sarcosine (uL)	50 mM Tris pH 7 (uL)	2 mg/mL BSA (uL)	Final Conc. (mg/mL)
	P8	25	225	250	1

87 Reverse pipet 100 µL RSB (go to step #82) into P1.

88 Prepare seven 2 mL microtubes for P2 to P8.

89 Vortex and then use reverse pipetting: transfer 100 µL standard solutions (PS2 to PS8) into the new tubes.

90 Use the following formula to determine the total volume of working reagent (WR) required. Consider leaving several mL of extra volume:
(# standards + # samples) X 800 µL = total volume WR required

91 Prepare WR by mixing 50 parts of BCA reagent A with 1 part of BCA Reagent B in a 50 mL falcon tube
Pierce BCA Protein Assay Kit **Thermo Fisher Scientific Catalog #**23225

92 Use one tip and reverse pipetting: Add 800 µL WR into each tube, make sure that the tip doesn't have contact with the solution, so that samples are not cross-contaminated.

93 Vortex each tube, shake and incubate at 37 °C for 00:30:00 at 200 rpm. 30m

94 Remove samples from the incubator, and centrifuge 5m
13300 rpm, Room temperature, 00:05:00

95 Each microplate can hold eight standard solutions and forty samples+blanks, all in duplicate

Equipment

96-Well Microplates	NAME
Polystyrene, Clear,	TYPE
Greiner Bio-One	BRAND
82050-760	SKU

96 Shake for 5 s at 600 rpm in a continuous and high force mode
Read endpoint 562 nm with a measurement time 100 ms

Equipment

Varioskan LUX Multimode Microplate Reader	NAME
Thermo Fisher	BRAND
VL0L00D0	SKU

97 Calculation refers to the section: **Calculate protein content per filter**