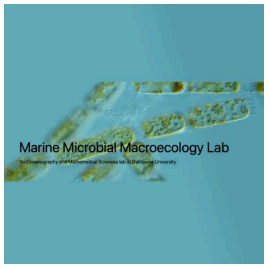
Dec 12, 2022 Version 3

Total crude protein in plankton: Pierce BCA protein assay (including the enhanced assay for low biomass) V.3



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

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Grant ID: 549937

Abstract

Here we describe a protocol for extracting total crude protein from phytoplankton and zooplankton, and quantifying by Pierce BCA protein assay. Chlorophyll, phospholipids and sucrose in crude protein could interfere the BCA assay.

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

Guidelines

1. Working range of Pierce BCA assay is 20-2000 ug/ml protein.
2. Enhanced assay has working range of 5 to 200 ug/mL protein.
3. Minimum sampling volume for microalgae (mL) = 750/(Chl-a_ug/L), if protein is extracted by only 500 ul extraction buffer instead of 1 mL.
4. One extra step of using microspin centrifuge filter is required for protein from zooplankton or microalgae samples with fine debris .

Protocol materials

- Tris base **Bioshop Catalog #**TRS001.500
- Tris HCl **Bioshop Catalog #**TRS002.500
- Lithium Dodecyl Sulphate **Bioshop Catalog #**LDS701.25
- Glycerol **Bioshop Catalog #**GLY001.500
- EDTA buffer solution (0.5 M) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**4055-100ml
- 4-(2-2-aminoethyl)-benzenesulfonyl fluoride HCL (AEBSF, Pefabloc) **Bioshop Catalog #**AEB602.100
- Pierce BCA Protein Assay Kit **Thermo Fisher Scientific Catalog #**23225
- Pierce BCA Protein Assay Kit **Thermo Fisher Scientific Catalog #**23225
- Thermo Scientific™ Pierce™ Bovine Serum Albumin Standard 2 mg/mL (50 mL) **Thermo Scientific Catalog #**Thermo Scientific™ 0023210
- BSA 2 mg/mL standard **Thermo Fisher Scientific Catalog #**23209
- Thermo Scientific™ Pierce™ Bovine Serum Albumin Standard 2 mg/mL (50 mL) **Thermo Scientific Catalog #**Thermo Scientific™ 0023210
- BSA 2 mg/mL standard **Thermo Fisher Scientific Catalog #**23209

Troubleshooting

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.

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 polycarbonate filters, using gentle vacuum pressure (130 mmHg).

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

1.4 Place sample filters in 2 mL Cryogenic Vials.

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

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

2 Zooplankton samples

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

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

2.2 Transfer ground sample into Lysing matrix tube, weigh the biomass and log into sampling sheet.

Equipment

Lysing matrix tube I	NAME
MPBio	BRAND
116918100	SKU

2.3 Flash-freeze tubes with liquid nitrogen, store at -80 °C until further processing

3 Freeze dry samples before processed.

Bead tube test for Microalgae samples

4 Bead size and lysing cycles have impact on protein extraction efficiency.

4.1 Bead size

Note

- Use 2 ml lysing matrix tube for 25 mm filter; 15 ml Teenprep tube for 47 mm filter

	Lysing tube	Bead size (mm)	Composition	2 mL tube SKU
	Matrix B	0.1	Silica spheres	116911050-CF
	Matrix Y	0.5	Yttria-stabilized zirconium oxide beads	116960050-CF
	Matrix C	1	Silica spheres	116912050-CF
	Matrix D	1.4	Zirconium-Silica spheres	116913050-CF

4.2 Lysing cycles
Compare protein yield by using four, six and eight cycles

4.3 Use the optimized bead size and lysing cycles to process protein samples.

Prepare protein solubilization buffer (PSB)

5

Citation

Ni G, Zimbalatti G, Murphy CD, Barnett AB, Arsenault CM, Li G, Cockshutt AM, Campbell DA
(2017)
. Arctic Micromonas uses protein pools and non-photochemical quenching to cope with temperature restrictions on Photosystem II protein turnover..
Photosynthesis research.

https://doi.org/10.1007/s11120-016-0310-6 LINK

6 In order to obtain compatible results, prepare sufficient PSB so that the same PSB can be used for sample extraction, blank filter extraction and standard solutions
(1) Extract all samples: Each sample requires 0.25 mL PSB
(2) Extract all blank filters: Each filter requires 0.25 mL PSB
(3) Each standard solution (500 ul) requires 0.125 mL PSB

7 For each 10 g PSB

7.1 Use anti-statics weighing dish to weigh the following chemicals (one chemical one dish):

Equipment

Antistatic weighing dish	NAME
Fisherbrand	BRAND
08-732-112	SKU

(1) 0.136 g Tris base

Tris base **Bioshop Catalog #**TRS001.500

(2) 0.133 g Tris HCl

Tris HCl **Bioshop Catalog #**TRS002.500

(3) 0.8 g Lithium dodecyl sulphate

Lithium Dodecyl Sulphate **Bioshop Catalog #**LDS701.25

7.2 Place a plastic beaker on the top of the scale surface

7.3 Remove the cap of a 15 mL tube and sit it in the beaker

Equipment

Falcon® Centrifuge Tubes	NAME
Polypropylene, Sterile, 15 mL	TYPE
Corning®	BRAND
352096	SKU

7.4 Tare the total weight of beaker and tube

7.5 Transfer all chemicals weighed in go to step #7.1 into the tube, rinse the dish with small amount of MilliQ water to make certain all of the solutes is transferred into the tube

7.6 Use a transfer pipet to add 4 g glycerol into the tube

Glycerol **Bioshop Catalog #**GLY001.500

7.7 Add 40 µL [M] 0.5 Molarity (M) EDTA into the tube

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

7.8 Top to 10 g with MilliQ water

7.9 Vortex until all solutes are completely dissolved.

Note

It takes some time to have solutes dissolved, especially LDS. Prepare in advance.

Prepare Pefabloc solution

8 4-(2-2-aminoethyl)-benzenesulfonyl fluoride HCL (AEBSF, Pefabloc) **Bioshop Catalog #**AEB602.100

Note

Pefabloc is a protease inhibitor, and it loses activity over 24 hours.

9 Add 20.86 mL MilliQ into 100 mg Pefabloc to obtain a final concentration of [M] 20 millimolar (mM) .

10 Aliquot into 2.5 mL portions and keep frozen at -20 °C

11 The solution can be frozen~thawed multiple times.

Assay Day 1: Extract protein

12 Prepare protein extraction buffer (PEB):

Each 1 mL PEB contains

250 ul PSB
20 ul 20 mM Pefabloc
730 ul MilliQ water

13 Prepare ice-bath, keep all samples in the ice-bath

14 Rinse forceps with 70% ethanol and air dry

Equipment

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

15 Label bead tubes and use clean forceps to transfer samples and blank filters into its corresponding bead tube.

Note

Bead tube type is selected by go to step #4

16 Reverse pipet 1 mL PEB onto the filter.

When using 15 mL Teenprep tube, horizontally shake the tube to bury filter into beads before adding PEB, which makes filter easy to be homogenized.

Note

Volume of PEB varies due to the actual biomass collected (see guideline)

17 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

18 Check the cap of each tube to make certain cap is tightly screwed. Organize the tubes in order, take notes of the position of each tube, in case the labels get rubbed out during extraction.

19 Run 00:01:00 at 6.5 m/s 1m

20 Keep tubes On ice for 00:01:00

21 Check labels. Put tubes back into FastPrep.

22 Run 00:01:00 at 6.5 m/s 1m

23 Keep tubes On ice for 00:01:00

24 Check labels. Put tubes back into FastPrep.

25 Run 00:01:00 at 6.5 m/s 1m

26 Keep tubes On ice for 00:01:00

27 Check labels. Put tubes back into FastPrep.

28 Run 00:01:00 at 6.5 m/s 1m

29 Keep tubes On ice for 00:01:00

30

Note

More cycles might be required go to step #4

31 De-foam by centrifuging the extract. 10m
2 mL Lysing Matrix tubes at 13000 rpm, Room temperature, 00:05:00
15 mL Teenprep tube at 3200 x g, Room temperature, 00:05:00

32 Transfer extract

32.1 **Microalgae** samples in QuickPrep tube (2 mL) 5m

(1) Transfer all supernatant to a 2 mL microtube
(2) Centrifuge at 13000 rpm, Room temperature, 00:05:00 to spin down debris
(3) Transfer only clear supernatant to a new microtube.

32.2 **Microalgae** samples in TeenPrep tube (15 mL) 5m

(1) Use 200 uL tip, go straight to the bottom along the side, try to transfer all extract to a 2 mL microtube.
(2) Centrifuge at 13000 rpm, Room temperature, 00:05:00 to spin down debris
(3) Transfer only clear supernatant to a new microtube.

32.3 **Zooplankton** samples and **Microalgae** samples with cloudy extract in which debris is too fine to be centrifuged down 5m

(1) Use puncher to cut glass fibre filters into about 7 mm disks
(2) Insert centrifuge filter tube into 2 mL microtube, line the bottom with two glass fibre filter disks by using ethanol rinsed and air-dried tweezers
(3) Transfer all extract to filter tube
(4) Centrifuge at 13000 rpm, Room temperature, 00:05:00 to completely remove debris, and keep the filtrate, discard the filter tube.

Equipment

new equipment NAME

Costar® Spin-X® Centrifuge Tube Filters, Corning® BRAND

33500-692 SKU

Equipment

Microcentrifuge tube NAME

Corning BRAND

29442-590 SKU

33 Freeze at -80 °C

Assay Day 2: Prepare Bovine serum albumin (BSA) standard solutions

34 Thaw [M] 20 millimolar (mM) pefabloc and transfer 150 ul to a 600 ul microtube.

Note

Put the rest of the stock back into the freezer immediately.

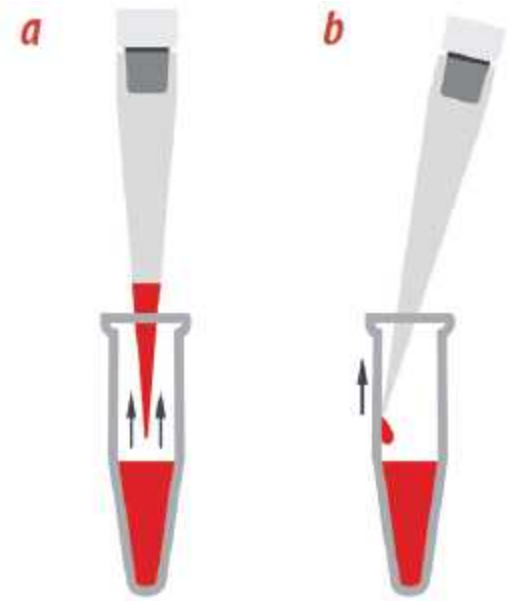
35 Thaw extract in the fridge.

36 Organize eight 2 mL microtubes in the tube rack, label the tubes from SD1 to SD8.

37 Reverse pipetting: dispense 125 µL PSB into each microtube.

Note

When aspiring solution, ensure the pipette to be held vertically. When dispensing, ensure you hold the pipette at an angle (**10-45°**). Working to these angles ensures the desired liquid amount is drawn into the tip properly and that all of the liquid is fully dispensed without leaving any residue in the tip.



https://www.americanlaboratory.com/914-Application-Notes/240482-Ten-Tips-for-Proper-Pipetting/

38 Reverse pipetting: dispense 10 µL pefabloc into each microtube

Note

Wipe or dab the liquid drop on the outside of the tip, avoid wiping the tip open before dispensing the liquid.

39 Forward pipetting: Add MilliQ into each microtube according to the sheet below:

	Standard	PSB (uL)	Pefabloc (uL)	MQ (uL)	BSA (2 mg/mL) (uL)	Final Conc. (mg/mL)
	SD1	**125**	**10**	**365**	**0**	**0**
	SD2	125	10	360	5	0.02
	SD3	125	10	353	12	0.048
	SD4	125	10	340	25	0.1
	SD5	125	10	315	50	0.2
	SD6	125	10	265	100	0.4
	SD7	125	10	165	200	0.8
	SD8	125	10	115	250	1

40 Primary BSA standard

40.1 If BSA (2 mg/mL) is in 50 mL bottle, transfer 1 mL into a microtube.

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

40.2 If BSA (2 mg/mL) is in ampule, break the ampule with ample opener.

BSA 2 mg/mL standard **Thermo Fisher Scientific Catalog #**23209

Equipment

SCIENCEWARE® Break-Safe™ Ampule Opener	NAME
Bel-Art®	BRAND
89217-378	SKU

41 Reverse pipet certain amount of BSA (2 mg/mL) into each tube according to the sheet

go to step #39

Note

Wipe or dab the liquid drop on the outside of the tip, avoid wiping the tip open before dispensing the liquid.

42 Vortex each tube.

43 Reverse pipetting: load 4 µL of each standard solution onto microdrop plate.

Equipment

µDrop™ Plates	NAME
Thermo Scientific	BRAND
N12391	SKU

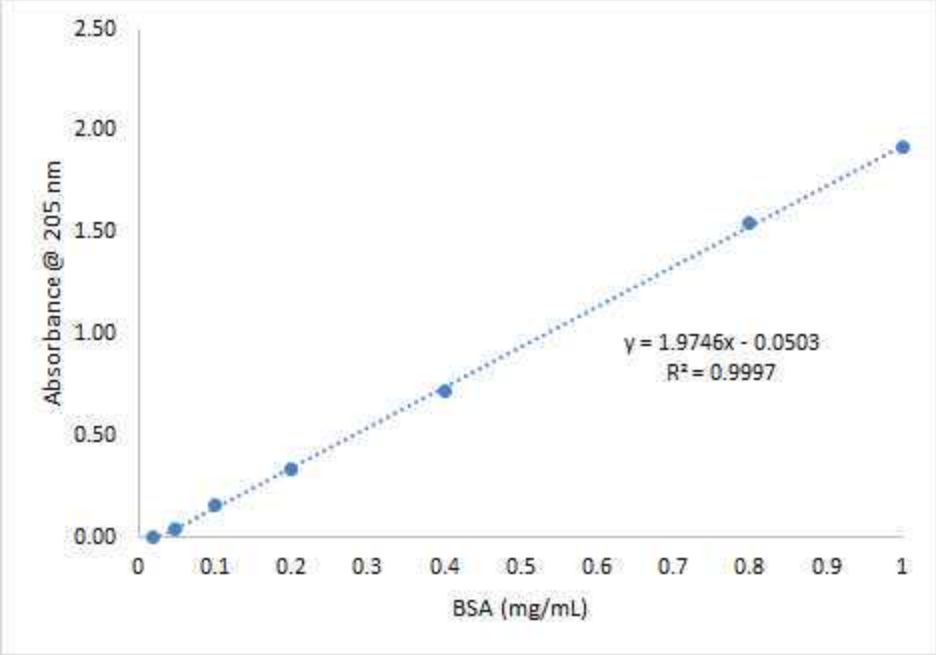
44 Read absorbance of eight standard solutions at 205 nm

Equipment

Varioskan LUX Multimode Microplate Reader	NAME
Thermo Fisher	BRAND
VL0L00D0	SKU

45 Subtract absorbance at 205 nm of blank standard from the 205 nm measurements of all other standard solutions

46 Plot the blank-corrected 205 nm measurement for each standard solution versus its concentration in mg/ml.



Example of BSA standard curve: Absorbance read at 205 nm versus concentration (mg/mL)

47 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.

48 Standard solutions can be kept at Room temperature .

49 Organize eight 2 mL microtubes in the tube rack, label the tubes from SD1 to SD8.
Reverse pipet 100 µL standard solution into its corresponding tube.

Assay Day 2: Prepare BCA working reagent (WR)

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

(# standards + # samples + # blank filters) X (800 µL) = total volume WR required

51 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

Equipment	
Falcon® Centrifuge Tubes	NAME
Polypropylene, Sterile, 50 mL	TYPE
Corning®	BRAND
352070	SKU

Assay Day 2: Pierce BCA assay 2h

52 Turn on incubator and preheat to 37 °C

Equipment

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

53 Keep thawed extract On ice

54 Organize 2 mL microtubes in the tube rack, label the tubes for blanks and samples

55 Vortex and then use reverse pipetting: transfer 100 µL extract of blanks or samples into the corresponding tubes.

56 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.

Note

Since BCA assay is sensitive to reaction duration, although reagent is aqueous, it is more efficient to use reverse pipetting and quickly dispense reagent into all tubes, therefore the duration difference amongst standards and samples can be minimized.

57 Vortex each tube, shake and incubate at 37 °C for 00:30:00 30m

58 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

59

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1										
B	S2	S2										
C	S3	S3										
D	S4	S4										
E	S5	S5										
F	S6	S6										
G	S7	S7										
H	S8	S8										

Samples and sample blanks: 40 with duplicate

Example of organizing samples on the microplate.

60 Remove samples from the incubator and centrifuge 5m

13300 rpm, Room temperature, 00:05:00

61 For microplate loading:

Note

1. Reverse pipetting: aspire 200 µL sample from the middle of the solution
2. Tip gently touches the side of the well, avoid bending. Dispense 200 uL into the microplate
3. Dispose the tip
4. Use a new tip, reverse pipet another 200 µL as replicate
5. Tip gently touches the side of the well, avoid bending. Dispense 200 uL into the microplate

62 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

63 Subtract the average 562 nm absorbance measurement of the blank standard replicates from the 562 nm measurements of all other individual **standard**.

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

65 Prepare a standard curve by plotting the average Blank-corrected 562 nm measurement for each BSA standard versus its concentration in mg/ml.

66 Use the standard curve to determine the protein concentration of each unknown sample by using its blank-corrected 562 absorbance.

67 Calculate the low-limit-of-detection:
L-LOD_mg/mL=3.3*SD/slope
where SD is the mean value of standard deviation between each standard replicates.

L-Abs=L-LOD*slope - intercept

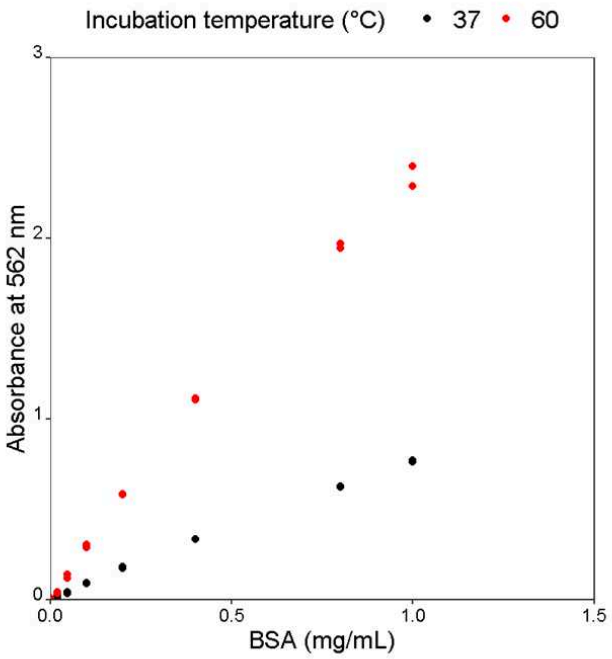
68 If the absorbance of sample is lower than L-Abs, go to Section:

Assay Day 3: Enhanced Pierce BCA assay for protein 5 to 200 ug/sample

69 Protein_mg/filter = Protein_mg/mL X PEB_mL

Assay Day 3: Enhanced Pierce BCA assay for protein 5 to 200 ug/sample

70



Response of absorbance at 562 nm to BSA concentration after 30-min incubation at 37 and 60 °C

71 Thaw [M] 20 millimolar (mM) pefabloc and transfer 150 ul to a 600 ul mcirotube.

Note

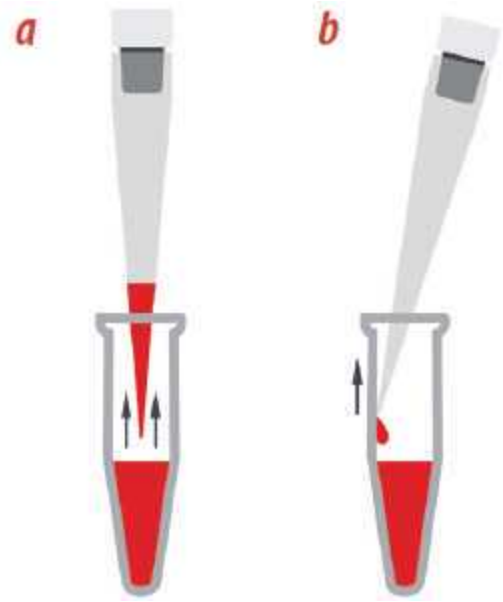
Put the rest of the stock back into the freezer immediately.

72 Organize eight 2 mL microtubes in the tube rack, label the tubes from SD1 to SD8.

73 Reverse pipetting: dispense 125 µL PSB into each microtube.

Note

When aspiring solution, hold the pipet vertically. When dispensing, ensure you hold the pipette at an angle (**10-45°**). Working to these angles ensures the desired liquid amount is drawn into the tip properly and that all of the liquid is fully dispensed without leaving any residue in the tip.



https://www.americanlaboratory.com/914-Application-Notes/240482-Ten-Tips-for-Proper-Pipetting/

74 Reverse pipetting: dispense 10 µL pefabloc into each microtube

Note

Wipe or dab the liquid drop on the outside of the tip, avoid wiping the tip open before dispensing the liquid.

75 Forward pipetting: Add MilliQ into each microtube according to the sheet below:

	Standard	PSB (uL)	Pefabloc (uL)	MQ (uL)	BSA (0.4 mg/mL) (uL)	Final Conc. (mg/mL)
	SD1	**125**	**10**	**365**	**0**	**0**
	SD2	125	10	360	5	4
	SD3	125	10	355	10	8

	Standard	PSB (uL)	Pefabloc (uL)	MQ (uL)	BSA (0.4 mg/mL) (uL)	Final Conc. (mg/mL)
	SD4	125	10	345	20	16
	SD5	125	10	335	30	24
	SD6	125	10	305	60	48
	SD7	125	10	240	125	100
	SD8	125	10	115	250	200

76 Prepare BSA standard: [M] 0.4 mg/mL

76.1 If BSA (2 mg/mL) is in 50 mL bottle, directly reverse pipet 300 µL BSA standard into a 2 mL microtube (do not return remaining solution back into the bottle). Forward pipet 600 µL + 600 µL Milli-Q into the tube, vortex.

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

76.2 If BSA (2 mg/mL) is in ampule, break the ampule with ample opener. Reverse pipet 300 µL BSA standard into a 2 mL microtube. Forward pipet 600 µL + 600 µL Milli-Q into the tube,

BSA 2 mg/mL standard **Thermo Fisher Scientific Catalog #**23209

Equipment

SCIENCEWARE® Break-Safe™ Ampule Opener NAME

Bel-Art® BRAND

89217-378 SKU

77 Reverse pipet certain amount of BSA ([M] 0.4 mg/ml) into each tube according to the sheet go to step #75

> Note
>
> Wipe or dab the liquid drop on the outside of the tip, avoid wiping the tip open before dispensing the liquid.

78 Vortex each tube.

79 Standard solutions can be kept at Room temperature .

80 Organize eight 2 mL microtubes in the tube rack, label the tubes from SD1 to SD8. Reverse pipet 100 µL standard solution into its corresponding tube.

81 Use the following formula to determine the total volume of WR required. Consider leaving several mL of extra volume since Finntip stepper is unable to expel the entire volume from the tip:

(# standards + # samples + # blank filters) X (800 µL) = total volume WR required

82 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

Equipment

Falcon® Centrifuge Tubes	NAME
Polypropylene, Sterile, 50 mL	TYPE
Corning®	BRAND
352070	SKU

83 Turn on dry bath and preheat to 60 °C

Equipment

Digital dry bath	NAME
LSE	TYPE
Corning	BRAND
6875SB	SKU

84 Keep extracted samples On ice

85 Organize 2 mL microtubes in the tube rack, label the tubes for blanks and samples

86 Forward pipetting: Add 800 µL WR into the tubes.

87 Reverse pipetting: transfer 100 µL extract of blanks or samples into the corresponding tubes.

Note

Aspire and mix up and down at least three time before transferring the extract

88 Vortex each tube, incubate at 60 °C for 00:30:00

89 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

90

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1										
B	S2	S2										
C	S3	S3										
D	S4	S4										
E	S5	S5										
F	S6	S6										
G	S7	S7										
H	S8	S8										

Samples and sample blanks: 40 with duplicate

Example of organizing samples on the microplate.

91 For microplate loading:

Note

1. Reverse pipetting: aspire 200 µL sample
2. Tip gently touches the side of the well, avoid bending. Dispense 200 uL into the microplate
3. Return remaining sample from the tip back to the tube
4. Dispose the tip
5. Vortex the tube
6. Use a new tip, reverse pipet another 200 µL as replicate
7. Tip gently touches the side of the well, avoid bending. Dispense 200 uL into the microplate

92 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

Citations

Step 5

Ni G, Zimbalatti G, Murphy CD, Barnett AB, Arsenault CM, Li G, Cockshutt AM, Campbell DA. Arctic Micromonas uses protein pools and non-photochemical quenching to cope with temperature restrictions on Photosystem II protein turnover.
https://doi.org/10.1007/s11120-016-0310-6