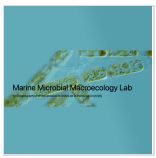

6

Nov 29, 2021

Total RNA and DNA in Microalgae V.6

Yingyu Hu[1], Zoe V Finkel[1]

[1]Dalhousie University

1

protocol .

Marine Microbial Macroecology Lab
Tech. support email: **ruby.hu@dal.ca**

Yingyu Hu
Dalhousie University

Here we describe a protocol for extracting and quantifying bulk RNA and DNA from microalgae, which is adapted from Berdalet E. et al. (2005).

RNA and DNA are extracted from microalgae samples and then quantified by fluorochrome SYBR Green II.

Berdalet E, Roldán C, Olivar MP, Lysnes K. Quantifying RNA and DNA in planktonic organisms with SYBR Green II and nucleases. Part A. Optimisation of the assay. Scientia Marina.
https://doi.org/10.3989/scimar.2005.69n11

Berdalet E, Roldán C, Olivar MP. Quantifying RNA and DNA in planktonic organisms with SYBR Green II and nucleases. Part B. Quantification in natural samples. Scientia Marina.
https://doi.org/10.3989/scimar.2005.69n117

Yingyu Hu, Zoe V Finkel 2021. Total RNA and DNA in Microalgae. **protocols.io**
https://protocols.io/view/total-rna-and-dna-in-microalgae-b2e8qbhw
Yingyu Hu

RNA, DNA, SYBR Green II, DNase, RNase, microalgae, fluorescence

protocol ,

Nov 29, 2021

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The optimized linear range in this assay for DNA is 0~100 ng/mL and RNA is 0~300 ng/mL in the microplate, respectively. Samples are diluted to 1/40 in the assay, therefore, maximum DNA and RNA required in the assay is around 4 ug and 12 ug per sample. Assuming total DNA and RNA is 1% and 5.7% of total biomass at replete condition, carbon per sample required in this assay is about 80 ug.

Microalgae samples are collected on polycarbonate filter and kept frozen under -80 ºC.

STEP MATERIALS

☒ Nuclease decontamination solution **IDT Catalog #11-05-03-01** Step 2

☒ Ribonuclease A from bovine pancreas **Sigma Aldrich Catalog #R6513-50MG** Step 6.1

☒ DEOXYRIBONUCLEASE1 RNase and Protease Free **Bioshop Catalog # DRB002.10** Step 7.1

☒ Magnesium chloride solution **Sigma Aldrich Catalog #63069-100ML** Step 40.1

☒ Calcium chloride solution **Sigma Aldrich Catalog #21115-100ML** Step 42.1

☒ SYBR™ Green II RNA Gel Stain, 10,000X concentrate in DMSO **Thermo Fisher Catalog #S7564** Step 58.2

☒ Tris(hydroxymethyl)aminomethane hydrochloride 1M pH 8.0 RNase free **Fisher Scientific Catalog #AAJ60080AK** Step 3.1

☒ Deoxyribonucleic acid from calf thymus **Sigma Aldrich Catalog #D4522-1MG** Step 5.1

☒ N-Lauroylsarosine sodium salt solution (20% RNase/DNase free) **Sigma Aldrich Catalog #L744-50mL** Step 10.1

☒ EDTA buffer (0.5M DNase/RNase free) **Bioshop Catalog #EDT333.100** Step 10.2

☒ UltraPure™ DNase/RNase-Free Distilled Water **Thermofisher Catalog #10977023** Step 3.2

☒ E. coli Total RNA **Thermo Fisher Scientific Catalog #AM7940** Step 4.1

Day 1: Freeze-dry samples

1 Freeze dry samples and blank filters. Freeze at **-80 °C** until processed.

1. Freeze-drying should be as short as possible to reduce sample degradation.
2. The exact duration of freeze-drying depends on size of filter, quantity of sample and the size of container.

FreeZone® 2.5 L Benchtop Freeze Dryers
Labconco® **700202000**

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Day 1: Prepare primary solutions

2 Turn on UV light in biosafety cabinet for **00:15:00** and clean working surface with decontamination solution.

Nuclease decontamination solution **IDT Catalog #11-05-03-01**

3 Prepare Tris buffer **5 mM** **8.0**

3.1 Pour **1 M** **8.0** Tris into an RNase free 15 mL Falcon tube

Tris(hydroxymethyl)aminomethane hydrochloride 1M pH 8.0 RNase free **Fisher Scientific Catalog #AAJ60080AK**

Falcon® Centrifuge Tubes
Polypropylene, Sterile, 15 mL
Corning® **352096**

3.2 Directly add **2.5 mL** **1 M** **8.0** Tris into 500 mL RNase free water in its original package.

UltraPure™ DNase/RNase-Free Distilled Water **Thermofisher Catalog #10977023**

BT Barrier Pipet Tips
Pre-Sterile
Neptune® **BT1250, BT100, BT10**

4 RNA primary standard solution (**200 ug/ml**)

4.1 In the original package, the E. Coli Total RNA is of 1 mg/mL, in which total RNA is 200 ug.

E. coli Total RNA **Thermo Fisher Scientific Catalog #AM7940**

https://assets.thermofisher.com/TFS-Assets/LSG/manuals/sp_7940.pdf

4.2 Uncap the original package of E. Coli Total RNA and directly add **800 µL** Tris buffer (**5 mM** , **8.0**) .
Cap the package and vortex for a thorough mix.

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4.3 Aliquot 30 uL by stepper with sterile tip to 600 uL RNase free microtubes. Keep frozen at **-80 °C** .

Finnpipette Stepper Pipette
Thermo Scientific™ **4540000**

Finntip stepper pipette tips
500 ul (sterile)
Thermo Scientific **Thermo Scientific™ 9404173**

Microcentrifuge Tubes
1.7 mL/0.6 mL
Axygen Scientific **MCT-175-C/MCT-060-L-C**

5 DNA primary standard solution (≈ **500 ug/ml**)

5.1 Uncap the original package of Deoxyribonucleic acid from calf thymus and add **2 mL** Tris buffer (**5 mM** , **8.0**).
☒ Deoxyribonucleic acid from calf thymus **Sigma Aldrich Catalog #D4522-1MG**

https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Product_Information_Sheet/d4522pis.pdf

5.2 Cap the package. Do not vortex or sonicate.

5.3 Keep the solution at **0 °C** ~ **4 °C** overnight to completely solubilize the DNA. Gentle reversion is recommended.

5.4 Aliquot 10 uL by stepper with sterile tip to 600 uL RNase free microtubes. Keep frozen at **-80 °C** .

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Finntip stepper pipette tips
500 ul (sterile)

Thermo Scientific	Thermo Scientific™ 9404173

5.5 Dilute 5 ul primary DNA standard solution with 95 ul Tris buffer ([M] **5 mM** , pH **8.0**) in a microtube (600 ul).
Measure DNA concentration by using µdrop plate (sample volume: 4 ul)
Use Tris buffer ([M] **5 mM** , pH **8.0**) as blank.

µDrop™ Plates

Thermo Scientific	N12391

Varioskan LUX Multimode Microplate Reader

Thermo Fisher	VL0L00D0

The dilution is to avoid saturated observation at 260 nm.

5.6 DNA concentration (µg/ml) = (Abs_{260}-$Abs_{260\ (blank)}$)x 50 µg/ml x (10mm/0.5 mm) X DF
Where, DF=20.

If the measured DNA concentration is not close to [M] **500 ug/ml** , check reverse pipetting technique.

6 RNase primary stock solution ([M] **10 mg/ml**)

6.1 Uncap the original package of Ribonuclease A from bovin pancreas and add **5 mL** Tris buffer ([M] **5 mM** , pH **8.0**).
Cap the package and vortex for a thorough mix.
Ribonuclease A from bovine pancreas **Sigma Aldrich Catalog #R6513-50MG**

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6.2 Aliquot 30 uL by stepper with sterile tip to 600 uL RNase free microtubes. Keep frozen at **-20 °C** .

Finntip stepper pipette tips
500 ul (sterile)
Thermo Scientific **Thermo Scientific™ 9404173**

Finntip™ Stepper Pipette Tips
500 ul (Sterile)
Thermo Scientific **21-377-149**

7 DNase primary stock solution (**5 mg/ml** = 10,000 U/mL)

7.1 Uncap the original package of Deoxyribonuclease1 and add **1 mL** Tris buffer (**5 mM** , **8.0**)
.
Cap the package and vortex for a thorough mix.
DEOXYRIBONUCLEASE1 RNase and Protease Free **Bioshop Catalog # DRB002.10**

7.2 Aliquot 100 uL by stepper with sterile tip to 600 uL RNase free microtubes. Keep frozen at **-20 °C**
.

Finntip stepper pipette tips
1250 ul (sterile)
Thermo Scientific **Thermo Scientific™ 9404183**

Day 2: Exact RNA and DNA

8 Turn on UV light in biosafety cabinet for **00:15:00** and clean working surface with decontamination solution.

9 Prepare falcon tubes and tube rack in biosafety cabinet

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A	B
Volume of tube (mL)	**Contents in the tube**
5	0.5 M EDTA
5	20% sarcosine
15	5 mM Tris
15	1% STEB

Falcon® Centrifuge Tubes
Polypropylene, Sterile, 15 mL
Corning® **352096**

Falcon® Centrifuge Tubes
Polypropylene, Sterile, 50 mL
Corning® **352070**

10 Prepare STEB ([M] **1 %**)

Use the following formula to determine the total volume of 1% STEB required:
(# samples + # blank filters) X (500 ul) + (500 ul) = total volume of 1% STEB required

10.1 Pour sarcosine ([M] **20 %**) into an RNase free 5 mL falcon tube.
☒ N-Lauroylsarosine sodium salt solution (20% RNase/DNase free) **Sigma Aldrich Catalog #L744-50mL**

10.2 Pour EDTA ([M] **0.5 M**) into an RNase free 5 mL falcon tube.
☒ EDTA buffer (0.5M DNase/RNase free) **Bioshop Catalog #EDT333.100**

10.3 Pour Tris buffer ([M] **5 mM** , pH **8.0**) into an RNase free 15 mL falcon tube.

10.4 Mix **500 µL** sarcosine ([M] **20 %**), **10 µL** EDTA ([M] **0.5 M**) and **9 mL** + **490 µL** Tris buffer ([M] **5 mM** , pH **8.0**) to obtain STEB ([M] **1 %**).

11 Prepare ice bath

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12 Remove freeze-dried samples from -80°C freezer and place them **On ice** .

13 Add **500 µL** Tris buffer (**5 mM** , **8.0**) and **500 µL** STEB (**1 %**) to the bead tube. Place tubes **On ice** .

LYSING TUBES
MATRIX D 2 mL/15 mL
MP BIOMEDICALS **116913500/116933050**

14 Rinse forceps by **70 % volume** ethanol and air dry.

Filter forceps
blunt end, stainless steel
Millipore **XX6200006P**

15 Transfer sample/blank filter into the bead tube by using clean forceps.

16 Vortex immediately then put back **On ice** . 20s

VWR ANALOG VORTEX MIXER
VWR **10153-838**
With tube insert

17 Disrupt samples on the bead mill at 6.5 m/s. 30s

Fastprep-24 5G™ Sample Preparation Instrument
MP Biomedicals **116005500**

18 Keep tubes **On ice** . Check the label on each tube, restore the label if it fades. 1m 30s

19 Disrupt samples on the bead mill at 6.5 m/s. 30s

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20 Keep tubes **On ice** . Check the label on each tube, restore the label if it fades. 1m 30s

21 Disrupt samples on the bead mill at 6.5 m/s 30s

22 Keep tubes **On ice** . Check the label on each tube, restore the label if it fades. 1m 30s

23 Disrupt samples on the bead mill at 6.5 m/s. 30s

24 Continuously shake homogenate in a multi-head vortex at the highest speed for **01:00:00** 1h
Room temperature

Votex mixer should be able to remain stable on the bench under this vortex speed.

25 In the biosafety cabinet, aliquot 150 uL of homogenates into RNase free 600 uL tubes.

Finntip™ Stepper Pipette Tips
2.5 mL (sterile)

Thermo Scientific	**Thermo Scientific™ 9404193**

26 Freeze at **-80 °C** until analyzed.

Day 3: Run the assay

27 Prepare ice bath.

28 Turn on UV light in biosafety cabinet for **00:15:00** and clean working surface with decontamination solution.

29 Prepare falcon tubes, microtubes and tube racks in biosafety cabinet
* Maximum number of samples (including blanks) per assay is 20.

Number of tubes	Type of tubes	Contents

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6	5 mL falcon tubes	1 M MgCl2, 1 M CaCl2, Working solution A (WS-A), Working solution B (WS-B), Working solution C (WS-C), Sybr Green II working solution (SG-II WS)
2	15 mL falcon tubes	5 mM Tris buffer, 0.05% STEB
3	600 uL RNase free tubes	RNase working solution, 900 mM MgCl2, 900 mM CaCl2
2	1.7 mL RNase free tubes	Secondary RNA standard solution, Secondary DNA standard solution
33	1.7 mL RNase free tubes	RNA standard solutions for RNA standard curves, DNA standard soutions for DNA standard curves
N= total number of samples and blanks	1.7 mL RNase free tubes	Samples and blanks
3XN	1.7 mL RNase free tubes	Diluted samples and blanks

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5	Microtube racks	Tubes of 600 uL and 1.7 mL
1	Tube racks	Falcon tubes

Screw-Cap Centrifuge Tube
5 mL
VWR 10002-738

30 Organize and label the tubes as shown below

Set 1:
In microtube rack, label 1.7 mL tubes for samples and blanks to be further diluted.

1	2	3	4	5	6	7	8	9	…	Blank

Set 2:
In microtube rack, label 1.7 mL tubes for RNA (marked in pink) and DNA (marked in blue) standard solutions to be used as standard curves.
Tubes A is for standard solutions treated with working solution A (WS-A)
Tubes B is for standard solutions treated with working solution B (WS-B)
Tubes C is for standard solutions treated with working solution C (WS-C)

Tubes A	R1A	R2A	R3A	R4A	R5A	R6A	D1A	D2A	D3A	D4A	D5A
Tubes B	R1B	R2B	R3B	R4B	R5B	R6B	D1B	D2B	D3B	D4B	D5B
Tubes C	R1C	R2C	R3C	R4C	R5C	R6C	D1C	D2C	D3C	D4C	D5C

Set 3:
In microtube rack, label 1.7 mL tubes for diluted samples and blanks.
Tubes A is for diluted samples and blanks treated with working solution A (WS-A)
Tubes B is for diluted samples and blanks treated with working solution B (WS-B)
Tubes C is for diluted samples and blanks treated with working solution C (WS-C)

Tubes A	1A	2A	3A	4A	5A	6A	7A	8A	9A	...	BlankA
Tubes B	1B	2B	3B	4B	5B	6B	7B	8B	9B	...	BlankB
Tubes C	1C	2C	3C	4C	5C	6C	7C	8C	9C	...	BlankC

31 Label tubes for reagents as following.
Follow the sheet, add Tris buffer ([M] **5 mM** , pH **8.0**) to the reagent tubes:

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Reagent	5 mM Tris (uL)
0.05% STEB	9X1000+500
RNase	190
900 mM $MgCl_2$	20
900 mM $CaCl_2$	20
WS-A	2X1000+820
WS-B	2X1000+820
WS-C	2X1000+940
RNA	990+495
DNA	998

32 Add **900 µL** Tris buffer (**5 mM** , **8.0**) to each tube in **Set 1**

33 Follow the sheet, add Tris buffer (**5 mM** , **8.0**) to each tube in **Set 2**. The unit of volume is uL.

Tubes A	650	640	625	600	550	500	640	630	610	580	550
Tubes B	650	640	625	600	550	500	640	630	610	580	550
Tubes C	600	590	575	550	500	450	590	580	560	530	500

34 Follow the sheet, add Tris buffer (**5 mM** , **8.0**) to each tube in **Set 3**. The unit of volume is uL.

Tubes A	650	650	650	650	650	650	650	650	650	…	650
Tubes B	650	650	650	650	650	650	650	650	650	…	650
Tubes C	600	600	600	600	600	600	600	600	600	…	600

35 Prepare STEB (**0.05 %**)

Add **500 µL** STEB (**1 %**) to 0.05% STEB tube, and vortex.

36 Add **250 µL** STEB (**0.05 %**) to each tube in **Set 2**.

37 Place RNase and DNase primary stock solutions, RNA and DNA primary standard solutions and samples **On ice** .

38 Turn on refrigerated centrifuge and set the temperature to **4 °C** .

CENTRIFUGE 5430 R

Eppendorf MP2231000510

39 Turn on shaker/incubator and set temperature to **37 °C** .

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SHAKING INCUBATOR
71L
Corning® LSE™ 6753

40 Prepare [M]**900 mM** $MgCl_2$

40.1 Pour [M]**1 M** $MgCl_2$ solution into 5 mL RNase free Falcon tube

Magnesium chloride solution **Sigma Aldrich Catalog #63069-100ML**

40.2 Transfer **180 µL** [M]**1 M** $MgCl_2$ solution into 900 mM $MgCl_2$ tube

41 Add **60 µL** [M]**900 mM** $MgCl_2$ to WS-A and WS-B

42 Prepare [M]**900 mM** $CaCl_2$

42.1 Pour [M]**1 M** $CaCl_2$ solution into 5 mL RNase free Falcon tube

Calcium chloride solution **Sigma Aldrich Catalog #21115-100ML**

42.2 Transfer **180 µL** [M]**1 M** $CaCl_2$ solution into 900 mM $CaCl_2$ tube

43 Add **60 µL** [M]**900 mM** $CaCl_2$ to WS-A and WS-B

44 Prepare RNase working solution [M]**0.5 mg/ml**

Add **10 µL** RNase primary stock solution ([M]**10 mg/ml**) to RNase tube

45 Add **60 µL** [M]**0.5 mg/ml** RNase to WS-B and WS-C.

Keep WS-B and WS-C **On ice** .

46 Add **60 µL** DNase primary stock solution ([M]**5 mg/ml**) to WS-A.

Keep WS-A **On ice** .

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47 Centrifuge extracted samples **10000 x g, 4°C, 00:04:00**

48 Prepare RNA secondary standard solution **2 ug/ml**
Add **15 µL** RNA primary standard solution to RNA standard tube and vortex.
Keep **On ice** .

49 Prepare DNA secondary standard solution **1 ug/ml**
Add **2 µL** DNA primary standard solution to DNA standard tube and vortex.
Keep **On ice** .

50 Load **50 µL** WS-A to Tubes A in **Set 2** and **Set 3**.

51 Load **50 µL** WS-A to Tubes C in **Set 2** and **Set 3**.

52 Load **50 µL** WS-B to Tubes B in **Set 2** and **Set 3**.

53 Load **50 µL** WS-C to Tubes C in **Set 2** and **Set 3**.

54 Add **100 µL** centrifuged samples to its corresponding tubes in **Set 1**.
Vortex each tube.

55 From Set 1, transfer **250 µL** of diluted samples to each corresponding tubes in **Set 3**.

56 Follow the sheet:
Add RNA secondary standard to tubes (marked in pink) in Set 2.
Add DNA secondary standard to tubes (marked in blue) in Set 2.
The unit of volume is uL.

Tubes A	0	10	25	50	100	150	10	20	40	70	100
Tubes B	0	10	25	50	100	150	10	20	40	70	100
Tubes C	0	10	25	50	100	150	10	20	40	70	100

57 Vortex each tube for **00:00:02** and place all tubes into the shaker/incubator at **37 °C** , continuously shaking at 200 RPM for **00:20:00** . 20m

Read fluorescence

58 Prepare SYBR Green II working solution (SG-II WS)

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58.1 Each 96-well microplate requires 1 mL of SG-II WS.

58.2 Wrap 5 mL tube with foil, add **7 µL** SYBR Green II 10,000X concentrate to each one mL Tris buffer (**5 mM** , **8.0**) in biosafety cabinet.

SYBR™ Green II RNA Gel Stain, 10,000X concentrate in DMSO **Thermo Fisher Catalog #S7564**

58.3 Load **10 µL** SG-II WS to each well in the microplate with 0.5 mL tip of stepper.

Finntip™ Stepper Pipette Tips
500 uL
Thermo Scientific™ **9404170**

96-Well Black Microplates
Polystyrene
Greiner Bio-One **655076**

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

58.4 Adhere black film on the top of a microplate lid and cover the plate with this lid.

Black Vinyl Films for Fluorescence and Photoprotection
VWR **89087-692**

Microplate Lids
Polystyrene
Greiner Bio-One **07000288**

59 After incubation, vortex each tube for **00:00:02** and then place into the fridge to stop the reaction.

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60 Allow samples to reach **Room temperature** for **00:02:00** before loading the microplate.

45m

61 Load **190 µL** working sample to the microplate.

Standards must be included in each microplate.
Pink area: RNA standard solutions for RNA standard curves
Blue area: DNA standard solutions for DNA standard curves
Yellow area: Samples and blanks

	1	2	3	4	5	6	7	8	9	10	11	12
A	R1A	R2A	R3A	R4A	R5A	R6A	1A	1A	1B	1B	1C	1C
B	R1B	R2B	R3B	R4B	R5B	R6B	2A	2A	2B	2B	2C	2C
C	R1C	R2C	R3C	R4C	R5C	R6C	3A	3A	3B	3B	3C	3C
D	D1A	D2A	D3A	D4A	D5A		4A	4A	4B	4B	4C	4C
E	D1B	D2B	D3B	D4B	D5B		5A	5A	5B	5B	5C	5C
F	D1C	D2C	D3C	D4C	D5C		6A	6A	6B	6B	6C	6C
G	9A	9A	9B	9B	9C	9C	7A	7A	7B	7B	7C	7C
H	Blank A	Blank A	Blank B	Blank B	Blank C	Blank C	8A	8A	8B	8B	8C	8C

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

10m

62 Shake black film covered microplate at **Room temperature** for **00:10:00**

63 Setup microplate reader:
Plate: Greiner F bottom chimney well PP 96 well;
Shake: Continuous 5s at 600 rpm
Endpoint reading: Ex 490 nm/Em 520 nm;

Varioskan LUX Multimode Microplate Reader

Thermo Fisher **VL0L00D0**

64 Read fluorescence and export data to excel sheet.

Calculate

65 RNA standard curve

65.1 Concentrations of RNA standards in the microplate

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Standard	2 ug/mL (uL)	Tris+WS (uL)	0.05% STEB (uL)	SG II WS (uL)	Final (ng/mL)
R1	0	700	250	50	0
R2	10	690	250	50	20
R3	25	675	250	50	50
R4	50	650	250	50	100
R5	100	600	250	50	200
R6	150	550	250	50	300

65.2 Slope of fluorescence in Tube A vs concentration of RNA standard gives $m_{RNA+DNase}$
Slope of fluorescence in Tube B vs concentration of RNA standard gives $m_{RNA+RNase}$

65.3 Calculate ρ

$$\rho = \frac{m_{RNA+RNase}}{m_{RNA+DNase}}$$

66 Total RNA of the samples

$$ug\ Total\ RNA\ (ml\ assay)^{-1} = 0.001 \times \frac{(RFU_A - RFU_{A_{Blank}}) - (RFU_C - RFU_{C_{Blank}})}{(1-\rho)} / m_{RNA+DNase}$$

Where,
RFU_A and RFU_C are the fluorescence in Tube A and Tube C of the same sample.
RFU_{ABlank} and RFU_{CBlank} are the fluorescence in Tube A and Tube $_C$ of the blank.

67 DNA standard curve

67.1 Concentrations of DNA standards in the microplate

Standard	1 ug/mL (uL)	Tris+WS (uL)	0.05% STEB (uL)	SG II WS (uL)	Final (ng/mL)
D1	10	690	250	50	10
D2	20	680	250	50	20
D3	40	660	250	50	40
D4	70	630	250	50	70
D5	100	580	250	50	100

67.2 Slope of fluorescence in Tube A vs concentration of DNA standard gives $m_{DNA+DNase}$
Slope of fluorescence in Tube B vs concentration of DNA standard gives $m_{DNA+RNase}$

67.3 Calculate δ

$$\delta = \frac{m_{DNA+DNase}}{m_{DNA+RNase}}$$

Total DNA of the samples

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68 $$ug\ Total\ DNA\ (ml\ assay)^{-1} = 0.001 \times \frac{(RFU_B - RFU_{B_{Blank}}) - (RFU_C - RFU_{C_{Blank}})}{(1-\delta)} / m_{DNA+RNase}$$

Where,
RFU_B and RFU_C are the fluorescence in Tube B and Tube C of the same sample
RFU_{BBlank} and RFU_{CBlank} are the fluorescence in Tube B and Tube $_C$ of the blank.

69 Dilution factor=40
If,

- Sample is extracted by 1 mL extraction reagent
- In Set 1, sample is diluted to 1/10
- In Set 3, diluted by Tris and all working solutions to 250/950
- In microplate, diluted by SG-II WS to 190/200

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