



VERSION 12  
FEB 27, 2024

OPEN ACCESS



DOI:  
[dx.doi.org/10.17504/protocols.io.6qpvro85bvmk/v12](https://doi.org/10.17504/protocols.io.6qpvro85bvmk/v12)

**Protocol Citation:** Ying-Yu Hu  
2024. Total RNA and DNA from  
Microalgae (12 samples per  
microplate) . **protocols.io**  
<https://dx.doi.org/10.17504/protocols.io.6qpvro85bvmk/v12> Version  
created by Ying-Yu Hu

**License:** This is an open  
access protocol distributed under  
the terms of the [Creative  
Commons Attribution License](#),  
which permits unrestricted use,  
distribution, and reproduction in  
any medium, provided the original  
author and source are credited

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

**Created:** Mar 15, 2023

**Last Modified:** Feb 27, 2024

**PROTOCOL integer ID:** 78848

## Total RNA and DNA from Microalgae (12 samples per microplate) V.12

Ying-Yu Hu<sup>1</sup>

<sup>1</sup>Dalhousie University

Marine Microbial Macroecology Lab

Tech. support email: [ruby.hu@dal.ca](mailto:ruby.hu@dal.ca)



Ying-Yu Hu  
Dalhousie University

### ABSTRACT

Presented herein is a protocol for the extraction and quantification of bulk RNA and DNA from microalgae, adapted from the methodology outlined by Berdalet et al. (2005). RNA and DNA are extracted from microalgae samples and quantified using the fluorochrome SYBR Green II. To ensure accuracy, the concentrations of RNA and DNA standards are determined via absorbance measurements at 260 nm and 320 nm. This additional step aids in maintaining the consistency of the standard curve coefficients (i.e., the slope). The method demonstrates a sensitivity range of approximately 20-700 ng/ml for RNA and 10-700 ng/ml for DNA in the assay.

### CITATION

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*.

LINK

<https://doi.org/10.3989/scimar.2005.69n11>

### CITATION

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*.

LINK

<https://doi.org/10.3989/scimar.2005.69n11>

**Keywords:** RNA, DNA, SYBR Green II, DNase, RNase, microalgae, fluorescence

**Funders Acknowledgement:**

Simons Foundation  
Grant ID: 549937  
Simons Foundation  
Grant ID: 723789

## GUIDELINES

### (1) Estimation of RNA/DNA in the collected microalgae samples:

L-LOD for RNA and DNA is 20 and 10 ng/mL, respectively. Common dilution from sample collected on the filter to assay is 1/40. The minimum RNA and DNA per filter requires to be no less than 1 and 0.5 ug/filter, respectively. Under replete condition, RNA and DNA is about 5.7% and 1% in total dry mass, while Chl-a is bout 1.1% in total dry mass. Therefore, RNA\_ug/L = Chl-a\_ug/L X (5.7/1.1), DNA\_ug/L = Chl-a\_ug/L X (1/1.1).

(2) Verification of DNA and RNA standard concentrations ensures highly consistent assay results, facilitating comparisons of RNA and DNA data across researchers within the same group or across different groups. Below is an example showcasing the RNA and DNA values of Triplos obtained on different processing days, with sample replicates collected from various bottles. It's important to note that due to the large cell volume, cell counting of Triplos exhibits higher variation compared to small cells that can be counted using machines. This variation contributes to the standard deviation observed in RNA and DNA values.

	Date of assay	RNA (pg/cell)	SD of RNA	DNA (pg/cell)	SD of DNA	RNA:DNA	SD of the ratio
	20240110	221.48	62.22	342.65	63.05	0.64	0.07
	20240108	219.90	50.71	355.13	58.78	0.61	0.05
	20240105	214.58	46.92	377.48	60.65	0.57	0.03
	20240104	197.81	61.88	324.47	73.52	0.60	0.08

## PROTOCOL MATERIALS

☒ Calcium chloride solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #21115-100ML** Step 66.1

☒ N-Lauroylsarcosine sodium salt solution (20% RNase/DNase free) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #L744-50mL**

Step 18.1

☒ EDTA buffer (0.5M DNase/RNase free) **Bioshop Catalog #EDT333.100** Step 18.2

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

Step 47

☒ Magnesium chloride solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #63069-100ML** Step 64.1

☒ Ribonuclease A from bovine pancreas **Merck MilliporeSigma (Sigma-Aldrich) Catalog #R6513-50MG**

Step 13.1

☒ DEOXYRIBONUCLEASE1 RNase and Protease Free **Bioshop Catalog # DRB002.10** Step 14.1

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

Step 10.1

☒ UltraPure™ DNase/RNase-Free Distilled Water **Thermofisher Catalog #10977023** Step 10.2

☒ E. coli Total RNA **Thermo Fisher Scientific Catalog #AM7940** Step 11.1

☒ Deoxyribonucleic acid from calf thymus **Merck MilliporeSigma (Sigma-Aldrich) Catalog #D4522-1MG**

Step 12.1

## SAFETY WARNINGS

**!** No data is available addressing the mutagenicity or toxicity of SYBR® Green II Nucleic Acid Gel Stain. Because this reagent binds to nucleic acids, it should be treated as a potential mutagen and used with appropriate care. The DMSO stock solution should be handled with particular caution as DMSO is known to facilitate the entry of organic molecules into tissues. We strongly recommend using double gloves when handling the DMSO stock solution. As with all nucleic acid stains, solutions of SYBR Green II Nucleic Acid Gel Stain should be poured through activated charcoal before disposal or collected in waste container to be treated later. The charcoal must then be incinerated to destroy the dye.

## Sample collection

- 1 Filter microalgae in liquid media onto polycarbonate filters, using gentle vacuum pressure (130 mmHg).
- 2 Rinse filter tunnel with filtered artificial seawater (nutrient free) to avoid sample loss.
- 3 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) Fold once more, halving its length, so that sample is secured.
- 4 Place folded filter in 2 mL cryogenic vial.
- 5 Blank is not required in this measurement.
- 6 Flash-freeze tubes with liquid nitrogen and store at  -80 °C
- 7 Freeze-dry samples. Store at  -80 °C .

**Note**

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.

**Equipment****FreeZone® 2.5 L Benchtop Freeze Dryers**

NAME

Labconco®

BRAND

700202000

SKU

**Primary solutions****8** Turn on UV light in biosafety cabinet for  00:15:00**9** Clean working surface with decontamination solution.**10** Prepare Tris buffer  5 mM **10.1** Pour  1 M  Tris into an RNase free 15 mL Falcon tube Tris(hydroxymethyl)aminomethane hydrochloride 1M pH 8.0 RNase free **Fisher Scientific Catalog #AAJ60080AK**

## Equipment

**Falcon® Centrifuge Tubes**

Polypropylene, Sterile, 15 mL

NAME

Corning®

TYPE

352096

BRAND

SKU

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

 [UltraPure™ DNase/RNase-Free Distilled Water Thermo Fisher Catalog #10977023](#)

## Equipment

**BT Barrier Pipet Tips**

Pre-Sterile

NAME

Neptune®

TYPE

BT1250, BT100, BT10

BRAND

SKU

- 11** RNA primary standard solution ( **200 ug/ml** )

- 11.1** In the original package, the **frozen** 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](#)

## Note

[https://assets.thermofisher.com/TFS-Artifacts/LSG/manuals/sp\\_7940.pdf](https://assets.thermofisher.com/TFS-Artifacts/LSG/manuals/sp_7940.pdf)

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

## Note

Be aware of the package. If there is a conical bottom vial insert, add 400 ul Tris buffer first, cap the package, invert for a thorough mix.  
 Transfer the solution to a 2 mL RNase free tube.  
 Add another 400 ul Tris buffer, cap the package, invert for a thorough mix.  
 Transfer and combine the solutions together in the 2 mL tube.

11.3 Aliquot 30 uL by stepper with sterile tip to 600 uL RNase free microtubes. Store at -80 °C

## Equipment

## Finnpipette Stepper Pipette

NAME

Thermo Scientific™

BRAND

4540000

SKU

## Equipment

## Finntip stepper pipette tips

NAME

500 ul (sterile)

TYPE

Thermo Scientific

BRAND

Thermo Scientific™ 9404173

SKU

12 DNA primary standard solution ( $\approx$  [M] 500 ug/ml )

12.1 Uncap the original package of Deoxyribonucleic acid from calf thymus and add 2 mL Tris buffer ( [M] 5 mM ), 8.0 ).

Deoxyribonucleic acid from calf thymus **Merck MilliporeSigma (Sigma-Aldrich) Catalog #D4522-1MG**

## Note

[https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Product\\_Information\\_Sheet/d4522pis.pdf](https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Product_Information_Sheet/d4522pis.pdf)

12.2 Cap the package. Do not vortex or sonicate.

12.3 Keep the solution in the fridge overnight to completely solubilize the DNA. Gentle reversion is recommended.

12.4 Aliquot 10 uL by stepper with sterile tip to 600 uL RNase free microtubes. Store at -20 °C

## Equipment

## Finnipet pipette tips

NAME

500 ul (sterile)

TYPE

Thermo Scientific

BRAND

Thermo Scientific™ 9404173

SKU

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

13.1 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 invert for a thorough mix.

Ribonuclease A from bovine pancreas **Merck MilliporeSigma (Sigma-Aldrich) Catalog #R6513-50MG**

13.2 Aliquot 30 uL by stepper with sterile tip to 600 uL RNase free microtubes. Store at -20 °C

## Equipment

<b>Finntip stepper pipette tips</b>	NAME
500 ul (sterile)	TYPE
Thermo Scientific	BRAND
Thermo Scientific™ 9404173	SKU

## Equipment

<b>Finntip™ Stepper Pipette Tips</b>	NAME
500 ul (Sterile)	TYPE
Thermo Scientific	BRAND
21-377-149	SKU

14 DNase primary stock solution ( [M] 5 mg/ml = 10,000 U/mL)

14.1 Uncap the original package of Deoxyribonuclease1 and add 1 mL Tris buffer ( [M] 5 mM ,  $\text{pH}$  8.0 ). Cap the package and invert for a thorough mix.

DEOXYRIBONUCLEASE1 RNase and Protease Free Bioshop Catalog # DRB002.10

14.2 Use reverse pipetting to precisely aliquot 60 uL into **5 mL** RNase free tube. Store at -20 °C .

## Note

The 5 mL tube will be used as WS-A in the assay directly.  
One package of DNase can be used for 16 assays.

## Total RNA and DNA extraction

15 Turn on UV light in biosafety cabinet for  00:15:00

16 Clean working surface with decontamination solution.

17 Prepare falcon tubes and tube rack in biosafety cabinet

Volume of the tube (mL)	Content in the tube
5	0.5 M EDTA
5	20% sarcosine
50	5 mM Tris
15 or 50	1% STEB

#### Equipment

##### Falcon® Centrifuge Tubes

NAME

Polypropylene, Sterile, 15 mL

TYPE

Corning®

BRAND

352096

SKU

#### Equipment

##### Falcon® Centrifuge Tubes

NAME

Polypropylene, Sterile, 50 mL

TYPE

Corning®

BRAND

352070

SKU

18 Prepare STEB (  $[M]$  1 % )

**Note**

Use the following formula to determine the total volume of 1% STEB required:  
# samples X (500  $\mu$ L) + (500  $\mu$ L) = total volume of 1% STEB required

**18.1** Pour sarcosine ( [M] 20 % ) into an RNase free 5 mL falcon tube.

☒ N-Lauroylsarcosine sodium salt solution (20% RNase/DNase free) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #L744-50mL**

**18.2** 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**

**18.3** Pour Tris buffer ( [M] 5 mM ,  $\text{pH}$  8.0 ) into an RNase free 50 mL falcon tube.

**18.4** Mix the following ingredients to obtain STEB ( [M] 1 % ):

sarcosine ( [M] 20 % ):  $\Delta$  500  $\mu$ L

EDTA ( [M] 0.5 M ):  $\Delta$  10  $\mu$ L

Tris buffer ( [M] 5 mM ,  $\text{pH}$  8.0 ):  $\Delta$  9 mL +  $\Delta$  490  $\mu$ L

**19** Prepare ice bath

**20** Remove freeze-dried samples from -80°C freezer and place them  $\text{at}$  On ice .

**21** Add  $\Delta$  500  $\mu$ L Tris buffer ( [M] 5 mM ,  $\text{pH}$  8.0 ) and  $\Delta$  500  $\mu$ L STEB ( [M] 1 % ) to the bead tube. Place tubes  $\text{at}$  On ice .

**Equipment**

<b>Lysing tube matrix D</b>	NAME
2 mL	TYPE
MP biomedical	BRAND
116913500	SKU

**Note**

Use 2 mL bead tube for both 25 mm and 47 mm size filter.

**22** Rinse forceps by [M] 70 % volume ethanol and air dry.

**Equipment**

<b>Filter forceps</b>	NAME
blunt end, stainless steel	TYPE
Millipore	BRAND
XX6200006P	SKU

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

Prior to placing sample filter into the bead tube:

**Note**

- (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-circle shape, then fold once more along the dimension to form a strip

**24** Invert the tube then put back [I] On ice .

20s

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

Equipment

CENTRIFUGE 5430 R

NAME

Eppendorf

BRAND

MP2231000510

SKU

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

30s

Equipment

Fastprep-24 5G™ Sample Preparation Instrument

NAME

MP Biomedicals

BRAND

116005500

SKU

27 Keep tubes On ice. Check the label on each tube, restore the label if it fades.

1m 30s

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

30s

29 Keep tubes On ice. Check the label on each tube, restore the label if it fades.

1m 30s

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

30s

- 31 Keep tubes  . Check the label on each tube, restore the label if it fades. 1m 30s
- 32 Disrupt samples on the bead mill at 6.5 m/s. 30s
- 33 Continuously shake homogenate in a multi-head vortex at the highest speed for  01:00:00  Room temperature 1h
- Note**
- Vortex mixer should be able to remain stable on the bench under this vortex speed.
- 34 Centrifuge extracted samples  10000 x g, 4°C, 00:04:00 4m
- 35 In the biosafety cabinet, prepare 3XN 2 mL RNase free microtubes, N = number of samples.
- 36 In the biosafety cabinet, transfer all extract to their corresponding microtubes.
- 37 Centrifuge extracted samples  10000 x g, 4°C, 00:04:00 4m
- 38 In the biosafety cabinet, transfer supernatant as much as possible to their corresponding microtubes, avoid disturbing the debris.
- 39 In the biosafety cabinet, transfer supernatant for the assay.

**39.1** Invert the tube and mix thoroughly, then aspire  100 µL of supernatant using the reverse pipetting technique.

**39.2** Dispense this 100 uL into the 2 mL microtube.

**39.3** Return any remaining extract in the tip back to the extraction tube.

**39.4** Place the aliquot and the remaining extract into two separate boxes, i.e., one for aliquot only, the other one for remaining extract.

**40** Store both boxes of samples at  -80 °C .

### Assay (Be prepared: it is a full working day procedure)

**41** Prepare ice bath.

**42** Turn on UV light in biosafety cabinet for  00:15:00

**43** Clean working surface with decontamination solution.

**44** Prepare falcon tubes, microtubes and tube racks in biosafety cabinet

Number of tubes	Type of tubes	Contents
4	5 mL falcon tubes	1 M MgCl2
		1 M CaCl2
		Working solution B (WS-B)
		Working solution C (WS-C)
1	50 mL falcon tube	5 mM Tris buffer
1	15 mL falcon tubes	0.05% STEB
6	2 mL RNase free tubes	RNase working solution
		RNA tertiary standard solution
		DNA tertiary standard solution
		900 mM MgCl2
		900 mM CaCl2
		Sybr green working solution (SG-II WS)
24	2 mL RNase free tubes	RNA standard solutions for RNA standard curves
		DNA standard solutions for DNA standard curves
3XN (N = Number of samples)	2 mL RNase free tubes	Diluted samples
4	Microtube racks	Tubes of 2 mL in Set 1
		Tubes of 2 mL in Set A
		Tubes of 2 mL in Set B
		Tubes of 2 mL in Set C
1	Tube racks	Falcon tubes

## Equipment

### Screw-Cap Centrifuge Tube

NAME

5 mL

TYPE

VWR

BRAND

10002-738

SKU

- 45 Organize and label the tubes as shown below, log sample numbers into the tube rack layout.

#### Set 1:

This tube rack holds sample extract (100  $\mu$ L) to be further diluted.

Set 1											
-------	--	--	--	--	--	--	--	--	--	--	--

#### Set A, B and C:

In microtube rack, label 2 mL tubes for RNA (marked in pink), DNA (marked in blue) standard solutions and samples (marked in yellow)

Set A is for working solution A (WS-A) treatment, i.e. treated with DNase

Set B is for working solution B (WS-B) treatment, i.e. treated with RNase

Set C is for working solution A (WS-A) and C (WS-C) treatment, i.e. treated with DNase and RNase

Set A	R1A	R2A	R3A	R4A	R5A	D1A	D2A	D3A					
	Yellow												

Set B	R1B	R2B	R3B	R4B	R5B	D1B	D2B	D3B					
	Yellow												

Set C	R1C	R2C	R3C	R4C	R5C	D1C	D2C	D3C					
	Yellow												

#### 46 Label tubes for reagents as following.

Follow the sheet, add Tris buffer ( [M] 5 mM , pH 8.0 ) to the reagent tubes:

Content	5 mM Tris (uL)
SG-II WS	1000+250
WS-B	2X1000+820
WS-C	2X1000+940
RNAse	380
900 mM MgCl <sub>2</sub>	40
900 mM CaCl <sub>2</sub>	40
RNA tertiary	690X2
DNA tertiary	960
0.05% STEB	9X1000 + 500

#### 47 Thaw Sybr green II at room temperature

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

#### 48 Add Tris buffer ( [M] 5 mM , pH 8.0 ) to each tube in **Set A, B and C**. The unit of volume is uL.

## Note

Avoid changing the dilution for samples in this step. The total volume required for samples in Set A, B and C is already 750  $\mu$ L.

Set A	650	640	600	550	500	640	610	550					
	650	650	650	650	650	650	650	650	650	650	650	650	650

Set B	650	640	600	550	500	640	610	550					
	650	650	650	650	650	650	650	650	650	650	650	650	650

Set C	600	590	550	500	450	590	560	500					
	600	600	600	600	600	600	600	600	600	600	600	600	600

## 49 Prepare STEB ( [M] 0.05 % )

Add  $\ddagger$  500  $\mu$ L STEB ( [M] 1 % ) to 0.05% STEB tube, and invert to mix.

50 Add  $\ddagger$  250  $\mu$ L STEB ( [M] 0.05 % ) to RNA and DNA standards in **Set A, B and C** by reverse pipetting.

Set A, B, C	250	250	250	250	250	250	250	250					

51 Place sample extract, RNase and DNase primary stock solutions, RNA and DNA primary standard solutions  $\ddagger$  On ice .

## 52 Important technique for accurately preparing standards and working solutions

**Note**

Be aware of the conical shaped microtubes. The conical shaped bottom often retains a small volume of liquid due to surface tension. This residual liquid can impact the accuracy of measurements or the concentration of the solution.

**(1) For inverting mix:**

Gently invert the tube several times to ensure that any residual liquid at the bottom is mixed back into the solution.

**(2) For pipet mix:**

Place the pipette tip all the way to the bottom of the conical tube and then aspirate/dispense multiple times.

**53** Prepare DNA secondary standard solution [M] 25 ug/ml

**53.1** Add 190 µL 5 mM Tris buffer into DNA primary tube.

**54** Prepare DNA tertiary standard solution [M] 1 ug/ml

**54.1** Mix DNA secondary standard solution by aspiring up and down several times with pipet.

**Note**

Do not vortex!

**54.2** Transfer 40 µL DNA secondary solution ( [M] 25 ug/ml ) to DNA tertiary standard tube.

Keep On ice .

**55** Prepare RNA secondary standard solution [M] 25 ug/ml

**55.1** Add 210 µL Tris buffer into RNA primary tube.

56 Prepare RNA tertiary standard solution [M] 2 ug/ml

56.1 Mix RNA secondary standard solution by aspiring up and down several times with pipet.

Note

Do not vortex!

56.2 Transfer  $\ddot{\text{v}}$  120  $\mu\text{L}$  RNA secondary solution ( [M] 25 ug/ml ) to RNA tertiary standard tube and mix.

Keep  $\ddot{\text{v}}$  On ice .

57 Remove the DNA and RNA secondary out of the biosafety cabinet.

58 Before loading the samples, use pipet tip to aspire and dispense multiple times for thorough mix.

59 Use reverse pipetting:

Load 4  $\mu\text{l}$  DNA and RNA secondary onto the  $\mu\text{drop}$  plate, in duplicate.

Equipment

$\mu\text{Drop}^{\text{TM}}$  Plates

NAME

Thermo Scientific

BRAND

N12391

SKU

## Equipment

**Varioskan LUX Multimode Microplate Reader**

NAME

Thermo Fisher

BRAND

VL0L00D0

SKU

**60** Read absorbance at 260 and 320 nm.

**61** DNA\_primary concentration ( $\mu\text{g/ml}$ ) =  $(\text{Abs}_{260}-\text{Abs}_{320}) \times 50 \mu\text{g/ml} \times (10\text{mm}/0.49 \text{ mm}) \times DF$

Where, DF=20.

The DNA primary concentration should be around 500  $\mu\text{g/mL}$ .

If the DNA primary concentration is less than 400 (reading from udrop is less than 0.055) or higher than 600  $\mu\text{g/mL}$ , repeat

[go to step #53](#) to [go to step #60](#). Pay more attention to the solution mixing of primary DNA solution.

**62** RNA\_primary concentration ( $\mu\text{g/ml}$ ) =  $(\text{Abs}_{260}-\text{Abs}_{320}) \times 40 \mu\text{g/ml} \times (10\text{mm}/0.49 \text{ mm}) \times DF$

Where, DF=8.

The DNA primary concentration should be around 200  $\mu\text{g/mL}$ .

If the DNA primary concentration is less than 150 (reading from udrop is less than 0.055) or higher than 250  $\mu\text{g/mL}$ , repeat

[go to step #56](#) to [go to step #60](#). Pay more attention to the solution mixing of primary RNA solution.

**63** Turn on shaker/incubator and set temperature to  $37^\circ\text{C}$ .

## Equipment

**SHAKING INCUBATOR**

NAME

71L

TYPE

Corning® LSE™

BRAND

6753

SKU

64 Prepare [M] 900 mM MgCl<sub>2</sub>

64.1 Pour [M] 1 M MgCl<sub>2</sub> solution into 5 mL RNase free Falcon tube

☒ Magnesium chloride solution Merck MilliporeSigma (Sigma-Aldrich) Catalog #63069-100ML

64.2 Transfer 360 µL [M] 1 M MgCl<sub>2</sub> solution into 900 mM MgCl<sub>2</sub> tube

65 Add 60 µL [M] 900 mM MgCl<sub>2</sub> to WS-B

66 Prepare [M] 900 mM CaCl<sub>2</sub>

66.1 Pour [M] 1 M CaCl<sub>2</sub> solution into 5 mL RNase free Falcon tube

☒ Calcium chloride solution Merck MilliporeSigma (Sigma-Aldrich) Catalog #21115-100ML

66.2 Transfer 360 µL [M] 1 M CaCl<sub>2</sub> solution into 900 mM CaCl<sub>2</sub> tube

67 Add 60 µL [M] 900 mM CaCl<sub>2</sub> to WS-B

68 Prepare SG-II WS

68.1 Centrifuge one tube of SG-II concentrate at Room temperature 13000 rpm, 00:05:00 to deposit DMSO.

68.2 Wrap SG-II WS tube with foil, transfer **supernatant** of SYBR Green II 10,000X concentrate to SG-II WS tube in biosafety cabinet ( [M] 8.75  $\mu$ L per 1.25 mL Tris)

Note

Any step involving SYBR Green II should be operated in dark room or at least dim light. Prepare Sybr green II WS at this step to allow enough time for stabilization.

69 Check absorbance of SG-II WS:

69.1 In a transparent microplate, load

- (1) 200  $\mu$ L Tris buffer as blank
- (2) 10  $\mu$ L SG-II WS and 190  $\mu$ L Tris buffer

69.2 Read absorbance at 480 nm, the value after subtracted by blank shall be no higher than 0.21

70

Note

Lunch break!

71 Prepare RNase working solution [M] 0.5 mg/ml

Add  20  $\mu$ L RNase primary stock solution ( [M] 10 mg/ml ) to RNase tube

72 Thoroughly mix RNase tube and then transfer  60  $\mu$ L [M] 0.5 mg/ml RNase working solution to WS-B.

Keep WS-B  On ice .

73 Thoroughly mix RNase tube and then transfer  60  $\mu$ L [M] 0.5 mg/ml RNase working solution to WS-C.

Keep WS-C  On ice .

74 Label DNase tube with "WS-A", add **2X1000+820 mL** 5 mM Tris buffer into the tube.

75 Add **60 µL** **[M] 900 mM** MgCl<sub>2</sub> to WS-A

76 Add **60 µL** **[M] 900 mM** CaCl<sub>2</sub> to WS-A.

Keep WS-A **On ice**.

77 Use reverse pipetting: load **50 µL** WS-A to tubes in **Set A**.

78 Use reverse pipetting: load **50 µL** WS-A to tubes **Set C**.

79 Use reverse pipetting: load **50 µL** WS-B to tubes in **Set B**.

80 Use reverse pipetting: load **50 µL** WS-C to tubes in **Set C**.

81 Organize sample extracts (i.e., the 100 uL aliquot) into **Set 1**.

Forward pipetting, add 900 uL **[M] 5 mM** Tris buffer into each tube.

Set 1										

82 ***Thoroughly mix sample prior to transferring.***

Follow the layout below to add diluted samples, RNA tertiary and DNA tertiary into Set A, B and C. The unit of volume is uL.

Set A, B, C	0	10	50	100	150	10	40	100				
	250	250	250	250	250	250	250	250	250	250	250	250

#### Note

Forward pipetting:

(1) To avoid enzyme cross-contamination:

Renew tip between sets when dispensing the same sample or standard tertiary solution

(2) Aspire up and down for a complete dispense and thorough mix

**82.1** Load diluted samples to each corresponding tubes (marked in yellow) in **Set A, B and C**.

**82.2** Add RNA tertiary standard to tubes (marked in pink) in **Set A, B and C**.

**82.3** Add DNA tertiary standard to tubes (marked in blue) in **Set A, B and C**.

**83** Invert each tube for thorough mixing, then organize the tubes in a 96-well microtube rack following the same order as the microplates are loaded.

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

- 84 Place all tubes into the shaker/incubator at 37 °C, continuously shaking at 200 RPM for 00:20:00 . 20m

#### Note

Incubation time is critical. Temperature might be disturbed by door open/close. Don't start the timer until temperature returns to 37°C.

- 85 After incubation, Invert each tube for thorough mixing, then place them into the freezer for 2 min to stop the reaction.

## Fluorescence measurement

- 86 Remove samples out of the freezer and allow to reach Room temperature before loading the microplate.

#### Note

Since fluorescence decreases with increasing temperature, with percentage changes depending on the fluorophore (Bashford, 1987), the SG-II WS must be kept dark at RT (22°C) and the samples must be equilibrated at RT (c. 2 min).

- 87 Adhere black film on the top of a microplate lid.

**Equipment****Black Vinyl Films for Fluorescence and Photoprotection** NAME

VWR BRAND

89087-692 SKU

**Equipment****Microplate Lids** NAME

Polystyrene TYPE

Greiner Bio-One BRAND

07000288 SKU

- 88** Load 10 µL SG-II WS to each well by either 0.5 mL stepper or 10 uL pipet. Cover the plate with the black-film lid.

**Equipment****96-Well Black Microplates** NAME

Polystyrene TYPE

Greiner Bio-One BRAND

655076 SKU

- 89** Reverse pipetting: load 190 µL working samples to the microplate.

45m

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

**Note**

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

- 90 Shake black film covered microplate at Room temperature for 01:30:00

1h 30m

**Note**

The fluorescence value and the ratio between RNA and DNA stabilize within 90 minutes, contrary to the 10 minutes reported in the original paper.

- 91 Setup microplate reader:

Plate: Greiner F bottom chimney well PP 96 well;

Shake: Continuous 5s at 600 rpm

Fluorescence bandwidth: 12 nm

Excitation: 490 nm

Emission: 520 nm

**Equipment****Varioskan LUX Multimode Microplate Reader**

NAME

Thermo Fisher

BRAND

VL0L00D0

SKU

**Note**

Bandwidth at 12 nm gives more consistent results compared to 5 nm bandwidth.

**92** Read fluorescence and export data to excel sheet.

**93** In the fume hood, dispose any waste with SG-II into fluorescence stain waste container (some stain waste has DMSO solvent).

**Calculation**

**94** RNA standard curve

**94.1** Concentrations of RNA standards in the microplate: Use measured RNA primary concentration instead of 200 ug/mL:

	RNA primary conc. (ug/mL)	RNA primary (uL)	Tris (uL)	Conc. RNA secondary (ug/mL)
		30	210	

	RNA secondary conc. (ug/mL)	RNA secondary (uL)	Tris (uL)	Conc. RNA tertiary (ug/mL)
		120	1380	

	RNA standard	Tertiary (uL)	Tris (uL)	STEB (uL)	WS (uL)	Sample in microplate (uL)	SG-II (uL)	Conc in microplate (ng/mL)
	R1	0.00	650.00	250.00	50.00	190.00	10.00	0.00
	R2	10.00	640.00	250.00	50.00	190.00	10.00	~20.00
	R3	50.00	600.00	250.00	50.00	190.00	10.00	~100.00
	R4	100.00	550.00	250.00	50.00	190.00	10.00	~200.00
	R5	150.00	500.00	250.00	50.00	190.00	10.00	~300.00

**94.2** Slope of fluorescence in Set A vs concentration of RNA standard gives  $m_{\text{RNA+DNase}} (\sim 0.024)$

Slope of fluorescence in Set B vs concentration of RNA standard gives  $m_{\text{RNA+RNase}}$

**94.3** Calculate  $\rho$  ( $\leq 0.15$ )

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

**95** Total RNA of the samples

$\mu\text{g Total RNA (ml assay)}^{-1}$

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

Where,

$RFU_A$  and  $RFU_C$  are the fluorescence in Tube A and Tube C of the same sample.

$RFU_{A_{Blank}}$  and  $RFU_{C_{Blank}}$  are the fluorescence in Tube A and Tube C of the blank.

**96** DNA standard curve

**96.1** Concentrations of DNA standards in the microplate: Use measured DNA primary concentration instead of 500  $\mu\text{g/mL}$ :

	DNA primary Conc (ug/mL)	DNA primary (uL)	Tris (uL)	Conc. DNA secondary (ug/mL)
		10	190	

	DNA secondary Conc. (ug/mL)	DNA secondary (uL)	Tris (uL)	Conc. DNA tertiary (ug/mL)
		40	960	

	DNA standard	DNA tertiary (uL)	Tris (uL)	STEB (uL)	WS (uL)	Sample in microplate (uL)	SG-II (uL)	Conc. in microplate (ng/mL)
R1	0	650	250	50	190		10	0
D1	10	640	250	50	190		10	~10
D2	40	610	250	50	190		10	~40
D3	100	550	250	50	190		10	~100

**96.2** Slope of fluorescence in Set A vs concentration of DNA standard gives  $m_{DNA+DNase}$

Slope of fluorescence in Set B vs concentration of DNA standard gives  $m_{DNA+RNase}$  (~0.13)

**96.3** Calculate  $\delta$  ( $\leq 0.15$ )

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

**97** Total DNA of the samples

$\mu\text{g Total DNA (ml assay)}^{-1}$

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

Where,

$RFU_B$  and  $RFU_C$  are the fluorescence in Tube B and Tube C of the same sample

$RFU_{B_{Blank}}$  and  $RFU_{C_{Blank}}$  are the fluorescence in Tube B and Tube C of the blank.

**98** Dilution factor=40

If,

- Sample is extracted by 1 mL extraction reagent
- In Set 1, sample is diluted to 100/1000
- In Set 3, diluted by Tris and all working solutions to 250/950
- In microplate, diluted by SG-II WS to 190/200