

**Protocol and Guide
for Estimating Nucleic Acids
in Larval Fish
Using a Fluorescence
Microplate Reader**

by

**Elaine M. Caldarone, Melissa Wagner,
Jeanne St. Onge-Burns, and Lawrence J. Buckley**

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Table of Contents

A.	Introduction.....	1
B.	Background Information on Analytical Methods	1
C.	Essential Trials.....	3
	1. Recovery of Spikes	3
	2. Precision.....	3
	3. Residual and Autofluorescence	4
	4. Subsample Variability.....	4
	5. Contribution of Gut Contents	4
D.	Nucleic Acid Protocol.....	4
	I. Flowchart.....	5
	II. Detailed Instructions	6
	a. Sample Preparation	6
	b. Nucleic Acid Standards	6
	c. Sample Dilution	7
	d. Microplate Loading and Processing	8
	e. Calculations.....	11
E.	Reagents.....	12
	Preparation.....	12
	Sources.....	15
F.	Technical Notes	16
	Microplate Fluorometer Information.....	16
	Comments and Answers to FAQ	18
G.	Literature Cited.....	21

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A. Introduction

Analysis of bulk ribonucleic acids (RNA) and deoxyribonucleic acids (DNA) provides several useful indices for estimating size, condition and growth of fish (Bulow 1970, 1987, Buckley et al. 1999). Two of the most widely used indices are RNA concentration ($\mu\text{g}/\text{mg}$ tissue) and the ratio of RNA to DNA (R/D). RNA, which comprises much of the cell's machinery for protein synthesis, fluctuates in response to food availability and the demand for protein synthesis. DNA content is an index of cell number or biomass, and remains relatively constant during periods of starvation.

A variety of methods has been used to estimate nucleic acid levels in larval fish [e.g. Buckley and Bulow (1987), Clemmesen (1988), Caldarone and Buckley (1991), and Westerman and Holt (1988)]. Most work prior to 1988 was based on UV absorption by purified hydrolysates. Since the late 1980s, fluorometric methods using various fluorescent dyes have been employed. Although the fluorometric methods offer several advantages, including the ability to analyze the smallest individual larvae, they can present many challenges to the analyst. In particular, problems can arise from the sensitivity of fluorometric methods to procedural details and the choice of standards.

Here we present a well-tested, detailed, fluorometric procedure for the measurement of RNA and DNA in fish larvae. We provide background information on analysis of nucleic acids in fish tissue, point out some of the problem areas, and attempt to answer questions that may arise when initiating a study of nucleic acid levels in fish. The method described utilizes nucleases, the fluorophore ethidium bromide, and a 96-well format. The protocol is a modification of methods found in the following papers: Bente et al. (1981), Caldarone and Buckley (1991), Nacci et al. (1994), and Wagner et al. (1998).

B. Background Information on Analytical Methods

From a procedural perspective, the most commonly used UV-based nucleic acid analysis method (Buckley and Bulow 1987) is fairly straightforward. Through a series of washes with cold perchloric acid (HClO_4), free nucleotides, amino acids, and other low molecular weight compounds are removed from a homogenate of the larval fish or tissue of interest. Next, RNA is hydrolyzed by the addition of potassium hydroxide, and separated from DNA and protein by the addition of cold HClO_4 . Then DNA is both hydrolyzed and separated from the remaining protein by the addition of hot HClO_4 . RNA and DNA are estimated from the absorbance of the appropriate hydrolysate at 260 nm using an extinction coefficient of $1A_{260} \text{ unit}/\text{ml} = 33.33 \mu\text{g}/\text{ml}$ hydrolyzed RNA or DNA. Since the nucleic acids are hydrolyzed to a common configuration (nucleotides), and an established extinction coefficient is used to calculate the concentrations, researchers can be reasonably assured that the nucleic acid values obtained from a variety of species, stages and tissues, are comparable between laboratories. This method also has the advantage of allowing the researcher to directly use the published UV-based R/D growth models for larval fish (Buckley 1984).

Problems with this UV method can arise, however, due to either incomplete digestion of RNA or DNA, or contamination of the DNA fraction by proteins which also absorb at 260nm (Buckley and Bulow 1987). Other disadvantages are the somewhat long analysis time, and the minimum sample size requirement (0.6 mg dry weight). Small larvae must be pooled to obtain sufficient tissue biomass to run the analysis. We have found that spectrofluorometric nucleic acid analysis methods are more sensitive, more rapid, and as precise as the UV method. However, they require more attention to procedural details (e.g. choice of standards and extraction protocol) due to the binding characteristics of fluorophores. The methods most commonly used with larval fish employ the fluorophore ethidium bromide (EB: 3,8-diamino-6-phenyl-5-ethylphenanthridinium bromide). EB binds by intercalation (insertion of a molecule between two planar or stacked aromatic rings, such as adjacent bases) and therefore is specific to double-stranded polynucleotides. For single-stranded nucleic acids such as RNA, the secondary and tertiary structures will dictate the amount of EB which will bind. When EB is bound to a nucleic acid, its fluorescence is enhanced 20- to 30-fold. The fluorescence increase is not proportional to nucleic acid concentration but to the amount of double-stranded structure (Le Pecq 1971).

Typically, EB is used to measure total fluorescence of nucleic acids in a larval fish sample. RNase is then added and the fluorescence of the second reading is attributed to DNA. RNA fluorescence is calculated from the difference between the two readings. Spectrofluorometric methods require construction of standard curves to calculate concentrations. The choice of a commercial preparation to be used as a standard is critical and historically has differed among laboratories. Final estimates of RNA and DNA content can vary by a factor of two or more simply by changing the source of the standards (Caldarone and Buckley 1991). Use of common standards would significantly advance the ability to compare data generated by different laboratories, and would reduce the need for time-consuming intercalibrations. The main criteria for a DNA standard should be the absence of partial denaturation and the absence of RNA contamination. Electrophoretic gels of commercial DNA preparations from herring testes, herring sperm, and high molecular weight salmon testes showed the former two to be highly degraded and the latter to contain variable amounts of RNA (Caldarone, unpub). Only recently have highly purified commercial preparations of calf-thymus DNA become available. For example, Hoefer-Pharmacia markets a highly polymerized calf-thymus DNA preparation (now manufactured by Sigma, D-4764) as a standard for their fluorescent DNA detector. This preparation is very consistent in its fluorescence yield from batch to batch, and is guaranteed to be RNA-free.

The classes of RNA molecules that carry out protein synthesis include ribosomal RNAs (rRNA- the nucleic acid component of ribosomes), messenger RNAs (mRNA - the templates for individual proteins translated by the ribosomes), and transfer RNAs (tRNA- carry the specific amino acids to the active ribosome). Under steady state conditions, rRNAs comprise approximately 75 to 80% of total cell RNA (Brandhorst and McConkey 1974, Dahlberg 1989). Since the binding of EB to RNA relies on the secondary and tertiary structures, which can vary with base sequence and environmental conditions, the ideal RNA standard for EB use would be identical to the mixture of RNAs being analyzed. Preparation of this RNA mixture is impractical for most fishery laboratories, so an alternative is necessary. We have run electrophoretic gels with our fish larvae and commercial RNA preparations from *E. coli*, calf liver, brewer's yeast, and

18S- and 28S-rRNA. The E. coli, calf liver, and brewer's yeast RNA preparations were highly degraded, while the fish larvae and rRNA mixture displayed distinct bands with molecular weights corresponding to 18S and 28S (Caldarone, unpub). Since the majority of the bulk RNA measured in the fluorometric assays is ribosomal, we believe that the best commercial RNA standard currently available is 18S- and 28S-rRNA (eukaryotic rRNA Sigma R-0889). This product, manufactured for molecular biological research, is very pure and of consistently good quality from batch to batch.

When preparing samples for fluorometric analysis, it is necessary to include a step that will dissociate proteins from the nucleic acids. Histones will block binding of EB to nucleic acids by as much as 40% (Morgan et al. 1979 Part II). As with standards, a wide variety of dissociation/purification methods is currently used by different laboratories. Any number of methods are satisfactory, the main criteria being complete and reproducible dissociation of the proteins from the tissues under investigation, and blanks at acceptable levels. Some of the treatments currently in use include N-lauroylsarcosine (Caldarone and Buckley 1991), proteinase K (Westerman and Holt 1988), heparin (Karsten and Wollenberger 1977), and phenol extraction (Clemmesen 1993).

We have found extraction with N-lauroylsarcosine (a detergent) to be a very simple, effective, and highly reproducible technique (Caldarone and Buckley 1991). Recoveries of spikes of nucleic acid standards from N-lauroylsarcosine-extracted homogenates are similar to those reported for more labor-intensive solvent extractions (Clemmesen 1993). This procedure has the added benefit of not requiring a homogenization step for larvae up to ~10mm (live length). Again, standardization of extraction and dissociation procedures would facilitate comparisons among laboratories.

C. Essential Trials

Regardless of which fluorometric method or protein dissociation method is chosen, the methods must be thoroughly evaluated for each different species, stage, and tissue type. At a minimum, the influence of the following components MUST be determined before the assay is routinely used.

1. **Recovery of Spikes:** A set of control homogenates (see reagents) should be spiked with RNA and DNA standards before the protein dissociation step to determine the recovery rates of the nucleic acids, and to reveal any problems with the quenching of fluorescence. Be sure to spike with concentrations that are functionally detectable (see microplate fluorometer information - linear range and detection limits). Our recovery rates average $98\pm 3\%$, and to date we have not observed any fluorescence quenching.
2. **Precision:** Replicate samples of the same homogenate should be extracted and analyzed to determine whether the reproducibility of the method is within acceptable limits. The reproducibility of our control homogenate averages 4-6%.
3. **Residual and Autofluorescence:** If you choose to use a method employing RNase, both RNase and DNase **must** be added to a portion of the samples to determine if the residual

fluorescence (fluorescence remaining after treatment with both enzymes) is negligible. It can **NOT** be assumed that after the addition of RNase, the remainder of the fluorescence is due **solely** to DNA - there are other compounds that fluoresce at the wavelengths used. The residual fluorescence in muscle from winter flounder juveniles is 6-26% in EB (Caldarone and Buckley 1991, Canino and Caldarone 1995); however, we have not found significant residual fluorescence with any of the larval fish species we have analyzed (winter flounder, tautog, cod, pollack, haddock). If the residual fluorescence is significant (>7%), DNase (with the appropriate cofactors of Mg^{2+} and Ca^{2+}) can be routinely added to the samples to determine the true DNA content.

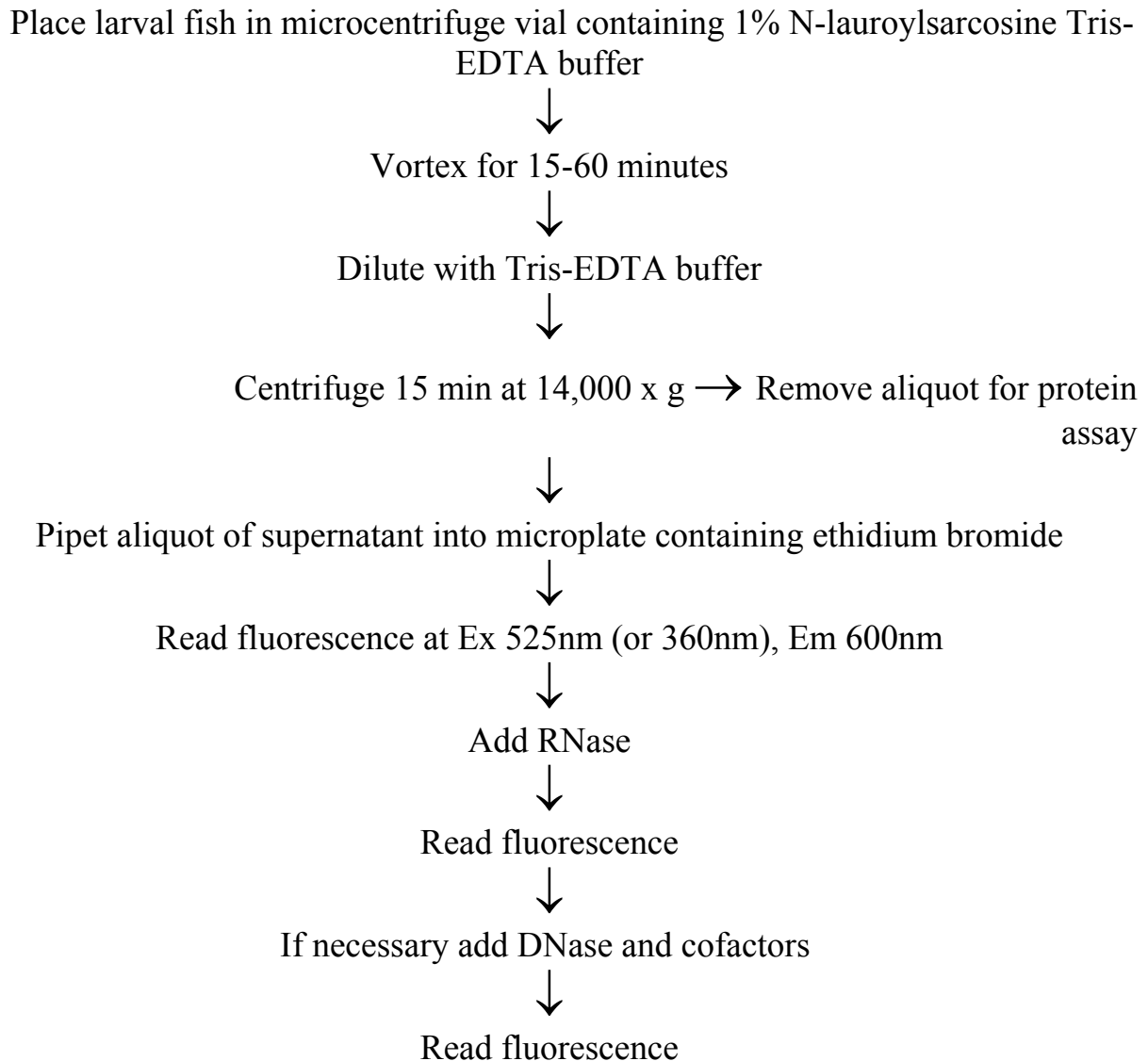
Samples should also be checked for autofluorescence (fluorescence with no EB added). We determined that inland silverside juveniles had an autofluorescence equal to 12% of the fluorescence observed in the presence of EB (Canino and Caldarone 1995). If autofluorescence is significant, the fluorescence of the samples should be read before the addition of the dye, and those values subtracted from the sample DNA fluorescence before calculating the DNA concentration.

4. **Subsample Variability:** If the fish is subsampled, you must determine whether the R/D values from different subsamples within one fish are consistent. We have found that the R/D of muscle tissue sampled from juvenile cod may vary depending on the location in the fish (Peck, unpub).
5. **Contribution of Gut Contents:** If a whole fish is analyzed, the contribution of the gut content to the R/D should be investigated. If the R/D of a full gut differs significantly from the R/D of the remaining tissues, and contributes a significant portion of the total nucleic acids, the gut should be excised before analyzing the fish to ensure comparable results between fish.

D. Nucleic Acid Protocol

The following section describes in detail a spectrofluorometric protocol for analyzing nucleic acids in larval fish. The method uses N-lauroylsarcosine to dissociate proteins from the nucleic acids, and the fluorophore EB to measure total nucleic acids. Fluorescence is detected using a 96-well fluorescence microplate reader. RNase is added to differentiate RNA from DNA, and when necessary, DNase is also added. Standard curves are constructed from genomic ultrapure calf thymus DNA, and molecular grade 18S- and 28S- rRNA.

I. Flowchart



II. Detailed Instructions

a. Sample Preparation

Sources for bold underlined **chemicals**, and directions for preparing bold underlined **reagents**, are at the end of the protocol (pages 12-16).

- Remove a vial of **control homogenate** from the -80°C freezer. Remove 77 fish samples, which have been stored individually in 1.5 ml eppendorf vials (microtubes), from the -80°C freezer. Place the vials in a rack on ice.
- Add 150 μl of **1% sarcosyl Tris-EDTA buffer** (STEB) to each vial. Add 75 μl of **2% STEB** to the control homogenate vial. Since the STEB solutions are fairly viscous, use a positive displacement pipette to dispense. Wipe the pipette tip before dispensing the liquid into the vials, and verify that the fish is submerged in the liquid.
- Cap all vials and vortex, preferably on a multihead vortexer (Fig. 1), at an intermediate speed for 15 – 60 minutes (until the tissue is visually completely disintegrated).



Figure 1: Multihead vortexer

b. Nucleic Acid Standards

- While the samples are vortexing, prepare the nucleic acid standards. Remove the **DNA** and **RNA stock** aliquots from the -80°C freezer. **IMMEDIATELY** after they are thawed, dilute with **0.1% STEB** to the proper concentrations. The two tables below are **examples** of a standard dilution series. Your final concentrations will be different since they depend upon the initial concentrations of DNA and RNA in your stock solutions.

Example of a calibration curve for DNA (initial stock concentration of 27.5 µg/ml)

DNA	µl of stock	µl of 0.1% STEB	Conc. µg/ml
D-1	5	550	0.248
D-2	10	550	0.491
D-3	20	550	0.965
D-4	30	550	1.422
D-5	40	550	1.864
D-6	50	550	2.292

Example of a calibration curve for RNA (initial stock concentration of 21.6 µg/ml)

RNA	µl of stock	µl of 0.1% STEB	Conc. µg/ml
R-1	10	240	0.864
R-2	20	240	1.662
R-3	35	240	2.749
R-4	60	240	4.320
R-5	110	240	6.789
R-6	160	240	8.641

c. Sample Dilution

- After vortexing, add 1.35 ml **Tris-EDTA buffer** to the control homogenate vial and each sample. Mix by manually inverting 40X (a whole rack of capped vials can be done together). Centrifuge all of the vials for 15 minutes at 14,000 × g at room temperature.
- After centrifuging, the nucleic acids will be in the supernatant fraction. Supernatants derived from larger fish will need to be diluted to fall within the standard curve range. The following table may be used as a guideline for dilutions, but species and fish condition can change the size intervals listed. If the pellet becomes disturbed before or during removal of the supernatant, centrifuge again before removing the supernatant.

Fish size (mm)	Dilution factor	µl of supernatant	µl of 0.1% STEB
< 4.7	None	500	-
4.7 - 6.0	2	500	500
6.0 - 7.2	4	250	750
7.2 - 8.4	8	125	875

d. Microplate Loading and Processing

- 1) Add 75 μ l of nucleic acid standards, control homogenate, sample supernatant or diluted sample supernatant to each microplate well. Almost any flat-bottomed 96-well microplate can be used (see microplate fluorometer information – microplates. We use Falcon 3915). Nucleic acid standards are run singly; all other samples are run in duplicate. Leave the final six wells empty on the first microplate to allow for possible redilutions. See microplate loading map (Fig. 2) for suggested positions of standards and samples.

Figure 2. Example of loading map for a 96-well microplate.

	1	2	3	4	5	6	7	8	9	10	11	12	
A	R-1	D-1	sample 1	sample 1	sample 9	Sample 9	sample 17	sample 17	sample 25	sample 25	sample 33	sample 33	A
B	R-2	D-2	sample 2	sample 2	sample 10	Sample 10	sample 18	sample 18	sample 26	sample 26	sample 34	sample 34	B
C	R-3	D-3	sample 3	sample 3	sample 11	Sample 11	sample 19	sample 19	sample 27	sample 27	sample 35	sample 35	C
D	R-4	D-4	sample 4	sample 4	sample 12	Sample 12	sample 20	sample 20	sample 28	sample 28	sample 36	sample 36	D
E	R-5	D-5	sample 5	sample 5	sample 13	Sample 13	sample 21	sample 21	sample 29	sample 29	sample 37	sample 37	E
F	R-6	D-6	sample 6	sample 6	sample 14	Sample 14	sample 22	sample 22	sample 30	sample 30	for possible redilution	for possible redilution	F
G	Blank 0.1% STEB	blank 0.1% STEB	sample 7	sample 7	sample 15	Sample 15	sample 23	sample 23	sample 31	sample 31	for possible redilution	for possible redilution	G
H	Standard homogenate	standard homogenate	sample 8	sample 8	sample 16	Sample 16	sample 24	sample 24	sample 32	sample 32	for possible redilution	for possible redilution	H
	1	2	3	4	5	6	7	8	9	10	11	12	

- 2) Turn on the microplate fluorescence reader. Refer to the owner's manual to determine proper warm-up time.

NOTE: For spill containment purposes, steps 3-5, 9, and 12-13 should be carried out in a confined area that is lined with disposable lab bench paper. EB is a mutagen and contamination of a nucleic acid lab with RNase or DNase is extremely difficult to rectify. Always wear nitrile gloves whenever you are handling EB. Nitrile gloves provide better protection than latex gloves.

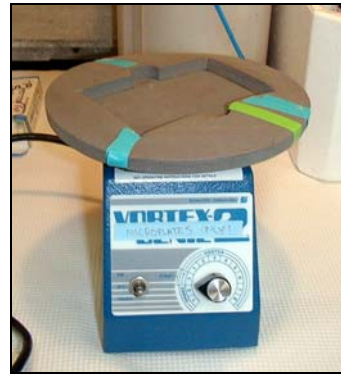
- 3) Remove **RNase** from the freezer to allow it to thaw.
- 4) Prepare a working solution of ethidium bromide (EB) (2 μ g/ml) by adding 32 μ l of the 1 mg/ml **EB stock** to 16 ml of Tris-EDTA buffer in a disposable tube. Cap and invert 20X to mix. Pour solution into a reagent trough.

- 5) Using a multi-channel pipette (Fig. 3), add 75 μ l of EB working solution to each well. Tap the microplate gently on the countertop a few times to bring down any EB droplets clinging to the sides of the wells. Shake the microplates for five minutes at a low setting using a vortexer equipped with a microplate head (Fig. 4). We use a setting of 1 or 2 out of a possible range of 1-10.

Figure 3: Multi-channel pipettes and reagent troughs.



Figure 4: Vortexer with microplate head



- 6) Take the microplate out of the vortexer and read the fluorescence using the microplate fluorescence reader. Ideally, the excitation wavelength should be set to 525 nm, and the emission wavelength to 600 nm (see microplate fluorometer information - wavelengths). Verify that the fluorescence values of the samples fall within the linear range of the fluorometer (see microplate fluorometer information - linear range and detection limits). Dilute any samples that are too concentrated and pipet the diluted samples into the empty wells in microplate #1. Treat the diluted samples with EB as in step 5. Do not save these fluorescence readings – you are just verifying that the samples have been diluted appropriately. You may skip this step if the dilutions you choose are always correct.
- 7) Return the microplate to the vortexer for an additional 10 minutes if no samples were diluted, or for 15 minutes if new dilutions have been added.
- 8) Read the fluorescence and save these values. Later in the calculations section, these will be referred to as **1st Fluorescence Reading**. Record the room temperature (see FAQ #6).
- 9) Pour the thawed and vortexed **RNase working solution** into a reagent trough. Using a multi-channel pipette, add 7.5 μ l to each well, touching the pipette tip to the side of the well. Tap the microplate to bring down any clinging droplets, place on the vortexer and shake for 20 minutes.
- 10) Read the fluorescence of the RNased samples and save these values as **2nd Fluorescence Reading**. Record the room temperature again.
- 11) If desired, freeze the remaining supernatant (or dilution, if used) from each fish or control homogenate for later protein analysis.

12) If it is necessary to add DNase to your samples (see residual fluorescence explanation on page 6) follow Plate Loading steps 1- 11 above; then:

13) Prepare the DNase working stock by mixing together:

660 μ l **0.1M MgCl₂**

660 μ l **0.08M CaCl₂**

450 μ l of **1U/ μ l DNase**

1230 μ l Tris-EDTA buffer

(this is enough for two microplates)

Note: final amounts and concentrations in each well will be:

1.125 U DNase (i.e. 6.8 U DNase/ml)

0.165 μ moles MgCl₂ (i.e. 1.0 mM MgCl₂)

0.132 μ moles CaCl₂ (i.e. 0.8mM CaCl₂)

14) Add 7.5 μ l of DNase working stock to each well, touching the pipette tip to the side of the well. Tap the microplate gently on the countertop a few times to bring down any droplets clinging to the sides of the wells. Shake the microplates on a vortexer for 15 minutes at a setting of 1 or 2.

15) Place a cover on the microplate and incubate in a drying oven at 37° C for 1 hour.

16) With the cover still on, cool the microplate to room temperature (~30 minutes).

17) Remove the microplate cover, read the fluorescence of the DNased samples and save these values as **3rd Fluorescence Reading**. Record the room temperature.

18) Waste solutions from the microplates and reagent troughs can be safely collected for disposal with the vacuum apparatus shown in Figure 5.

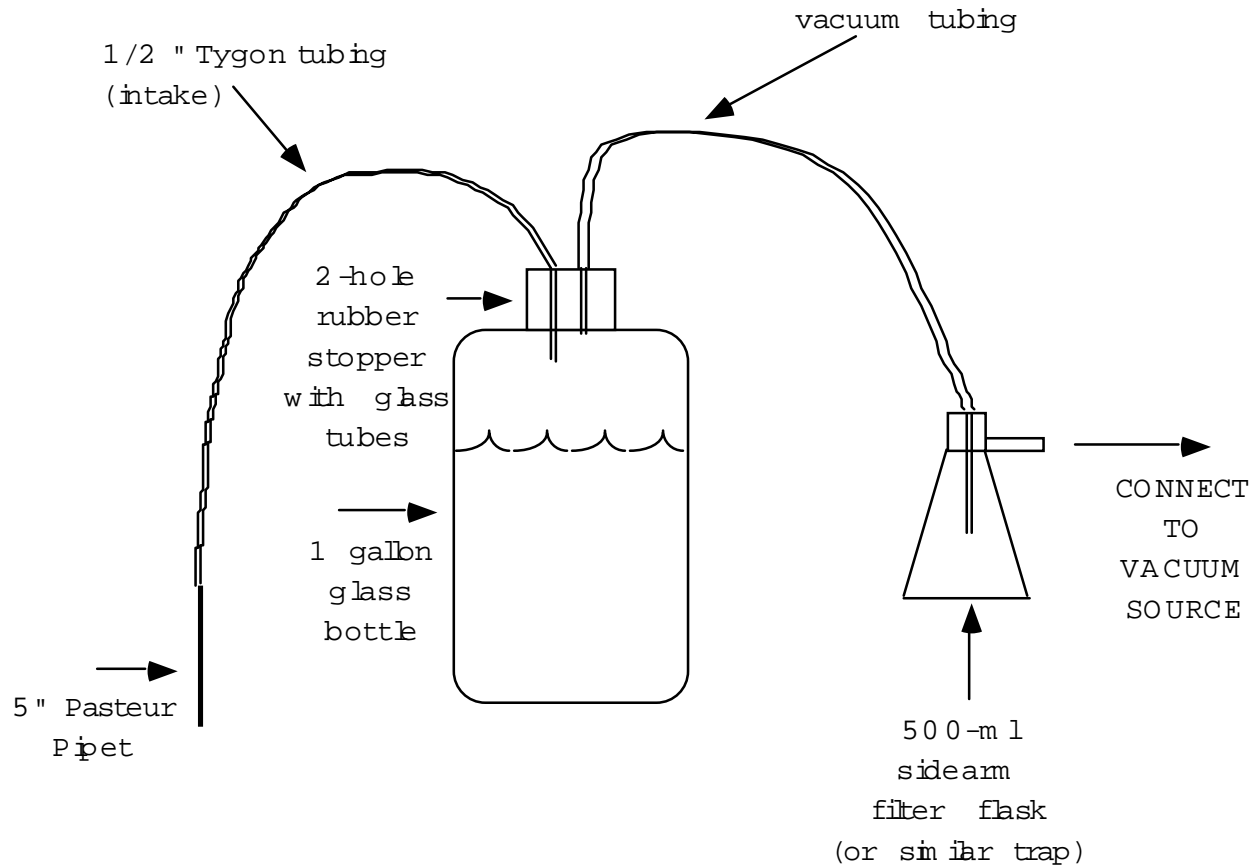


Figure 5. Vacuum apparatus for collecting waste from microplates and reagent troughs. Collection flask is a 1-gallon (or 4-liter) bottle with narrow mouth, such as a brown reagent bottle. Drill 2 holes, about 3/8" wide, into a stopper which fits the bottle. Cut 2 pieces of 1/2" diameter glass tubing: one piece about 2" long, one 4", and flame-smooth the cut surfaces. Insert the glass tubing through the stopper making sure that the intake tube enters the bottle at least 2" below the vacuum tube to prevent liquid from being drawn into the vacuum. Place the stopper into the bottle and attach intake and vacuum tubing. **Make sure there is a trap between the collection flask and vacuum source.**

e. Calculations

For samples treated with **RNase** only:

1. DNA fluorescence values = **2nd Fluorescence Reading** (from step #10)
2. RNA fluorescence values = **1st Fluorescence Reading** (from step #8) – **2nd Fluorescence Reading**
3. Calculate a DNA standard curve ($y=mx+b$ where m is the slope and b is the intercept) with the DNA fluorescence values. Do not subtract the blank value from each well; use it as the zero value in your standard curve.

4. Using the DNA fluorescence values, the DNA standard curve, and the dilution factor, calculate the DNA concentration in the standard homogenate and samples.
Total μg of DNA per fish = $[(\text{DNA fluorescence value}-b)/m]*\text{dilution factor}*1.5$
5. Calculate an RNA standard curve ($y=mx+b$) with the RNA fluorescence values. Do not subtract the blank value from each well; just use it as the zero value in your standard curve.
6. Using the RNA fluorescence values, the RNA standard curve, and the dilution factor, calculate the RNA concentration in the standard homogenate and samples.
Total μg of RNA per fish = $[(\text{RNA fluorescence value}-b)/m]*\text{dilution factor}*1.5$
7. Calculate a coefficient of variation (CV) for replicate samples
 $\text{CV} = [(\text{standard deviation}/\text{mean})*100]$

For RNased and DNased samples

1. DNA fluorescence values = **2nd Fluorescence Reading – 3rd Fluorescence Reading**
(from step #16)
2. RNA fluorescence values = **1st Fluorescence Reading - 2nd Fluorescence Reading**
3. Follow steps 3-7 from the ‘samples treated with RNase only’ instructions above.

E. Reagents

Preparation

Sarcosil Tris-EDTA buffer (STEB), 2%, 1%, 0.1%

- **N-lauroylsarcosine (sarcosil)** is a very fine, light powder that dissociates proteins. Wear a mask while weighing it out and be very careful about drafts. After weighing, wipe the work area with a wet paper towel to clean up any stray particles.
- To prepare a 2% solution, dissolve 2.0 g sarcosil in 100 ml **Tris-EDTA buffer** (to make the buffer, see below). Use a small stirring bar and gentle agitation to prevent foaming.
- To prepare a 1% solution, add 80 ml 2% sarcosil to 80 ml Tris-EDTA buffer
- To prepare a 0.1% solution, add 60 ml 1% sarcosil to 540 ml Tris-EDTA buffer
If most of your fish are small and do not require diluting, you can make a smaller volume.
- Store at room temperature
- Prepare new solutions every two weeks

Tris-EDTA buffer, 5 mM Tris-HCl, 0.5 mM EDTA, pH 7.5

- In a 1-L graduated cylinder mix 50 ml of **0.2 M Tris**, 80 ml of **0.1N HCl**, and 4 ml of **0.25M EDTA** solution. (To make these reagents, see below.)
- Bring volume up to one liter with deionized distilled water (ddH₂O)
- Transfer to a capped 2-L Erlenmeyer flask
- Add one more liter of ddH₂O and stir well
- Adjust pH to 7.5 with either dilute HCl or NaOH
- Filter through a 0.45µm filter to remove any particulates (e.g. stray dog hair).
- Store at room temperature

Tris, 0.2M

- Dissolve 12.1 g **Trizma base** in ddH₂O.
- Adjust final volume (in a graduated cylinder) to 500 ml with ddH₂O
- Stir well
- Store at room temperature in a capped flask

HCl, 0.1N

- With a disposable pipette and a pipette bulb, add 4.13 ml of concentrated **HCl** to 450 ml ddH₂O
- Adjust final volume (in a graduated cylinder) to 500 ml with ddH₂O
- Stir well
- Store at room temperature in a capped flask

EDTA, 0.25M

- Dissolve 4.65 g of **Na₂EDTA** in 35 ml ddH₂O (it takes a long time to go into solution)
- Adjust final volume (in a graduated cylinder) to 50 ml with ddH₂O
- Stir well
- Store at room temperature in a capped flask

Control homogenate

- Can be made with any surplus fish in good condition. Be sure to include two control homogenate wells on each microplate to serve as a daily quality control measure and to facilitate comparison of experimental results obtained with different batches of standards.
- Combine fish and a small amount of ddH₂O in a test tube (e.g. our control homogenate is made from ~50 yolk sac cod larvae in 3.5 ml water; this makes a homogenate with a total fluorescence roughly in the middle of our working range)
- Place tube in an ice water bath and homogenize larvae **VERY** thoroughly
- With the tube still on ice, transfer 75µl aliquots to 1.5ml microcentrifuge vials, vortexing the test tube every few minutes to make sure nothing settles out - you want your aliquots to contain absolutely identical amounts of fish tissue

- Store vials at -80°C

DNA stock (~25 $\mu\text{g/ml}$)

- Supplied by the manufacturer as 5 units (250 μg) of DNA in solid form. To dilute to ~25 $\mu\text{g/ml}$ add 10 ml of Tris-EDTA buffer and mix gently but thoroughly
- Check DNA concentration on a UV spectrophotometer:
Dilute 100 μl of the DNA stock with 400 μl of ddH₂O (i.e. dilution factor of 5)
DNA stock concentration in $\mu\text{g/ml} = (A_{260}) * (\text{extinction coefficient}) * (\text{dilution factor})$.
The extinction coefficient supplied by the manufacturer for this DNA stock is 1 A_{260} unit/ml = 50 $\mu\text{g/ml}$
- Transfer 160 μl aliquots (enough for >3 microplates) to individual microcentrifuge vials.
- Store at -80°C (for long-term storage)
- Use the concentration determined from the A_{260} as the DNA stock concentration

RNA stock (~25 $\mu\text{g/ml}$)

- Supplied by the manufacturer as 500 μg of rRNA in 250 μl of solution. To dilute to ~25 $\mu\text{g/ml}$ add 19.75 ml of Tris-EDTA buffer and mix gently but thoroughly.
- Check rRNA concentration on a UV spectrophotometer:
Dilute 300 μl of the RNA solution with 300 μl of ddH₂O (i.e. dilution factor of 2)
rRNA stock concentration in $\mu\text{g/ml} = (A_{260}) * (\text{extinction coefficient}) * (\text{dilution factor})$.
The extinction coefficient supplied by the manufacturer for this RNA stock is 1 A_{260} unit/ml = 40 $\mu\text{g/ml}$.
- Transfer 400 μl aliquots (enough for 3 microplates) to individual microcentrifuge vials
- Store at -80°C (for long-term storage).
- Use the concentration determined from the A_{260} as the RNA stock concentration

Ethidium Bromide (EB) Stock (1 mg/ml)

- ***EB is a mutagen, and extreme caution should be used when handling it.*** Handle in a confined area lined with disposable lab bench paper. Always wear nitrile gloves when handling EB; they provide far better protection than latex gloves.
- Supplied by the manufacturer as 10 mg/ml in liquid form. To dilute to 1 mg/ml, add 300 μl EB to 2.7 ml ddH₂O.
- Store in a foil-covered bottle in the dark at room temperature.

RNase working solution (20 U/ml)

- Handle RNase in a confined area lined with disposable lab bench paper. Use disposable labware only. RNase is extremely difficult to remove and inactivate.
- Supplied by the manufacturer as 50 mg protein in solid form. The activity of the enzyme (measured in Kunitz units (U) per mg protein) varies depending upon the lot. Dissolve one bottle of RNase in the appropriate amount of Tris-EDTA buffer to yield 20 U/ml.
- Mix well

- Using a disposable plastic pipette and a pipette bulb, transfer 3ml aliquots into plastic test tubes (enough for 3 microplates).
- Cap tightly and store at -20°C in a clearly marked container.

MgCl₂ (0.1 M)

- Dissolve 0.203 g of MgCl₂ in 10 ml of ddH₂O
- Store capped in the refrigerator

CaCl₂ (0.08 M)

- Dissolve 0.118 g of CaCl₂ in 10 ml of ddH₂O
- Store capped in the refrigerator

DNase (1U/ μl)

- Supplied by manufacturer as 2000 Kunitz units (U) of DNase in powdered form. To dilute to 1 U/ μl , add 2 ml ddH₂O and mix gently but thoroughly.
- Transfer 480 μl aliquots (enough for 2 microplates) to individual microcentrifuge vials
- Store at -80°C (for long term storage)

Sources

Chemicals were obtained from the following sources:

1. Liquid ethidium bromide (10mg/ml) from ISC Bioexpress (product no. EB-5515-10; phone 800-999-2901).
2. Sigma Chemical Company (phone 800-521-8956 or www.sigma-aldrich.com)

<u>Chemical</u>	<u>Prod. #</u>
Trizma base	T 6066
Na ₂ EDTA	E 5134
HCl (12.1N)	H 1758
Sarcosil (n-lauroylsarcosine, sodium salt)	L 9150
rRNA from calf liver, 18S and 28S	R 0889
DNA from calf thymus, genomic, ultrapure, 5 units	D 4764
RNase (A from bovine pancreas)	R 6513
DNase	D 4263
MgCl ₂	M 0250
CaCl ₂	C 3881

F. Technical Notes

Microplate Fluorometer Information

1. Microplates

There are three microplate varieties: clear, white, and black. Our Bio-Tek FL500 fluorometer reads microplates from the bottom; thus it will only function with clear plates. You need to determine which type of microplate works best with your fluorometer. If your blank fluorescence values are off-scale, try using a different type of microplate.

2. Wavelengths

For the EB fluorophore, some researchers use an excitation wavelength of 360-365 nm while others use 525 nm (e.g. Le Pecq and Paoletti 1966, Morgan et al. 1979 Part I). You need to determine which excitation wavelength works best with your fluorometer. If you use an excitation wavelength of 525, try to buy a filter with a narrow bandwidth to prevent overlap between the excitation and emission wavelengths.

3. Optimization

It is important to optimize your fluorometer control settings according to the manufacturer's recommendations. Each manufacturer uses different controls and different terms for the controls; thus the values of the settings will not be comparable between different instruments. The following settings are listed as an example only.

Settings for a Bio-Tek FL500 (purchased in 1995)

Excitation 530

Emission 590

Sensitivity 66 (out of a possible range of 1-99)

Each well is read 10 times, with a total duration of 0.1 second per well. The 10 readings are averaged and the mean is recorded. All microplates are read with the covers off.

4. Linear range and detection limits

The functional detection limits of your fluorometer, and the range of fluorescence values where the response is linear, must be determined.

- a. Determine the linear response range by measuring a series of dilutions of a highly concentrated standard or sample. Plot the fluorescence units (FU) vs. the nucleic acid concentration or sample size, and determine the range where the plot is linear (e.g. our fluorometer response is linear up to 45,000 FU).
- b. Determine the functional detection limits of your fluorometer by measuring a series of combined RNA and DNA standards. Make a series of five dilutions

of RNA and DNA standards, ranging from very dilute to concentrated. Prepare all 25 combinations of the standards and assay them. Calculate the recoveries - you should recover the correct concentrations of the standards. If you don't, those values will determine your upper and lower limits for functional detection of a specific nucleic acid from a sample containing both nucleic acids. Your standard curves should fall within these functional limits and all of your samples should fall within the limits of your standard curve. (e.g. We determined that the minimum functional detection limits for RNA and DNA were 4,000 and 6,000 FU respectively, thus our minimum RNA and DNA standards yield 5,500 FU. We determined the minimum functional detection limit for the total fluorescence of a sample to be ~15,000 which is the sum of our blank (~4,500), the minimum functional FU for RNA, and the minimum functional FU for DNA. The maximum functional detection limit for RNA and DNA was determined to be 24,000 and 17,000 FU respectively because combining these two values with the blank value will yield a total fluorescence near the upper limit of the linear range of the fluorometer (45,000 FU). The total fluorescence of our control homogenate value is currently ~20,500 FU, consisting of ~8,500 FU from RNA and ~12,000 FU from DNA. Autofluorescence of standards plus reagents is ~ 1,900 FU. The fluorescence of a totally empty well is ~3,000 FU). Remember that the total fluorescence values of your samples (**1st Fluorescence Reading**) should fall within the linear range of your fluorometer, while the DNA fluorescence values of your samples (**2nd Fluorescence Reading**) and the RNA fluorescence values of your samples (**1st Fluorescence Reading** minus **2nd Fluorescence Reading**) should fall within the range of your standard curves.

Comments and Answers to FAQ

1. ***Ethidium bromide (EB) liquid waste and contaminated labware should be handled and disposed of in accordance with local hazardous waste regulations.***
2. Calibrate pipettes on a regular basis (at least 4X per year) and after each time they have been accidentally dropped.
3. Fluorometer bulbs should be changed after ~600 hours of use or according to manufacturer's directions. The fan filter should be cleaned every six months, more often if the lab is dusty.
4. The microplate fluorometric assay can be scaled-up for use with a cuvette fluorescence reader - however, be aware that RNase binds to plastic and can remain active for days/weeks. If a flow-through fluorometer or autosampler is being used, check to be sure that the RNase is not binding to the sipper or sample chamber by thoroughly cleaning the system, and then measuring a set of RNA standards before and after running a set of RNased samples.
5. If a cuvette fluorescence reader is being used, we recommend using disposable cuvettes to insure against RNase carry-over from one sample to the next. Check to be sure that the cuvettes are suitable for fluorescence assays.
6. Fluorescence assays are sensitive to changes in temperature (Chen 1967, Caldarone and Buckley 1991, Karsten and Wollenberger 1977). A one-degree change in temperature can result in a 1% change in fluorescence yield. If your fluorometer is temperature-controlled and it is set to a temperature that differs from your room temperature, you will need to equilibrate your microplates in the fluorometer for a sufficient amount of time to reach a consistent stable temperature before you read the microplates. For cuvette fluorometers, if it is impossible to control the room temperature, standard curves must be run more frequently to compensate for any changes in fluorescence yield.
7. Be aware that certain types of microcentrifuge tubes will adsorb small amounts of nucleic acids. Since this microplate assay measures relatively large quantities of nucleic acids this is usually not a problem, but if your samples will also be used for future analysis of very small amounts of nucleic acids (e.g. a single mRNA), then proper selection of tubes is critical.
8. The ratio of the slope of the DNA standards to the slope of the RNA standards should be calculated for every run. The ratio should not vary widely from day to day.
9. Plan the size of your batches of RNA stock, DNA stock and control homogenate so that only one standard (RNA, DNA, or control homogenate) is changed at a time. The new configuration should be run at least 10 times before another standard is changed. This functions as a double-check that your UV-derived nucleic acid standard concentrations are accurate and that your assays are working properly.
10. The RNase must be certified to be free of any DNase activity.

11. The more uniform your control homogenate is, the more reproducible your control homogenate values will be from run to run, and the more confident you can be about your techniques and standards.
12. If the control homogenate values for a run are outside of the normal range, the sample values for that run are likely to be spurious.
13. Tissues and larvae can be stored frozen until time of analysis. Samples may be freeze-dried and then analyzed without any loss in nucleic acids. Thawing, refreezing, and then analyzing samples does not appear to be an option - the DNA seems to degrade (Caldarone, unpub., Ferguson and Draushchak 1989).
14. Different parts of a fish have different R/D values; therefore, the R/D from a subsample of a fish cannot be directly compared to the R/D of the whole fish.
15. If a detergent such as N-lauroylsarcosine or SDS is used, the larva should be vigorously shaken until it disintegrates. The vigorous shaking shortens the extraction time. Adding one or two glass beads will speed up the process. If muscle tissue or very large fish are being extracted, it is probably a good idea to increase the concentration of the detergent, increase the extraction time, add beads, or sonicate (Caldarone and Buckley, 1991). If you increase the concentration of the extraction detergent, be sure to dilute down to the normal concentration (0.1-0.2% for N-lauroylsarcosine) before adding EB, or your blank fluorescence will be too high.
16. Unlike Gremare and Vétion (1994) we have not seen any differences in nucleic acid concentration from samples that were, or were not, centrifuged or were centrifuged at different speeds (with fish <10 mm live length). We centrifuge to sediment out any bones or particles that may interfere with the fluorescence of the assay.
17. Control of growth rate or the cellular rate of protein synthesis occurs primarily through regulation of the number of ribosomes per cell (Nomura et al. 1984), although ribosome activity can be important (Lied et al. 1983). Temperature affects ribosome activity. We have found the relationship of R/D to growth to be temperature-dependent. If you are comparing ratios of fish from differing temperatures (>2-3°C), a laboratory study should be conducted to quantify the effect of temperature on the R/D - growth relationship in that species (Buckley 1984 and see review in Buckley et al. 1999).
18. See Buckley et al. (1999) for a discussion of normalization of nucleic acids to larval size/developmental stage, and a discussion of R/D in juvenile stages.
19. See Buckley et al. (1999) for a discussion of diel cycles and R/D.
20. When writing journal articles, report in the methods section the source of the standards you used (e.g. genomic ultrapure calf-thymus DNA, 18S- and 28S-rRNA). Also, if you are using

RNase only, report that you empirically determined that the addition of DNase was unnecessary.

21. Observations on Gremare and Vétion (1994).

Gremare and Vétion (1994) compared seven spectrofluorometric methods for measuring RNA and DNA concentrations in a deposit-feeding bivalve. They concluded that there were problems with a commonly used purification step (involving phenol-chloroform-isoamylalcohol), that a centrifugation step was essential, and that detergent extraction was absolutely necessary. They did not however, test the effectiveness of heparin or proteinase K for dissociating proteins. They found highly significant differences in DNA concentrations, RNA concentrations, and R/D using the seven spectrofluorometric methods; however they did not check for residual fluorescence after digestion with both RNase and DNase, nor did they determine the recovery of spikes. Results of these analyses might have explained some of the discrepancies they observed between the methods. The article also did not mention the source or type of standards used in the different assays.

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