

Simultaneous Extraction of High-Quality RNA and DNA from Small Tissue Samples

DEBORAH A. TRIANT AND ANDREW WHITEHEAD

From the Department of Biological Sciences, Louisiana State University, Baton Rouge, LA 70803.

Address correspondence to A. Whitehead at the address above, or e-mail: andreww@lsu.edu.

Purification of high-quality DNA and RNA from a single sample is becoming increasingly important for studies seeking both genomic and transcriptomic data. We compare different methods for isolating DNA and RNA from fish embryos (Gulf killifish; *Fundulus grandis*) and describe an optimal technique to extract high-quality DNA and RNA from a single embryo. The optimal method utilizes a chaotropic buffer and spin column technology. From embryos weighing ~4 mg, we were able to isolate an average of 6.1 μ g of DNA and 1.1 μ g of RNA per sample. Relative amounts of DNA and RNA can be adjusted as needed per study. Although these extraction trials were conducted on fish embryos, they can be potentially applied to small samples that typically do not yield high concentrations of nucleic acids.

Key words: fish embryo, *fundulus*, microarray, nucleic acid, TRIzol

Techniques that allow for the extraction of high-quality DNA and RNA from the same biological sample are essential for molecular studies involving genotyping and messenger RNA (mRNA) expression analyses. These methods are especially critical for samples that typically yield small amounts of nucleic acids. The use of microarrays for gene expression profiling has highlighted the necessity to isolate high-quality RNA (Gibson 2002; Stoughton 2005). However, methods that isolate DNA after an RNA extraction can yield DNA of low quality (Chevallard 1993; Chomczynski 1993). Various techniques have been described for the simultaneous extraction of DNA and RNA from the same sample, and commercial kits are available that utilize spin column or magnetic bead technologies to extract total nucleic acids (Hummon et al. 2007; Tolosa et al. 2007). Although these methods can extract high-quality DNA or RNA, high yields or quality of one extracted nucleic acid is often achieved at the expense of the other. With small samples that afford limited extraction opportunities, it is crucial to maximize yields.

Fish eggs and embryos present challenges for the isolation of nucleic acids because many are small in size

and few techniques have been reported for the extraction of DNA and RNA from embryos (Cary 1996; Aranishi 2006). Here, we compare several different methods for the simultaneous extraction of DNA and RNA from *Fundulus grandis* (gulf killifish) embryos and recommend an optimal approach that allows for the extraction of high-quality DNA and RNA from a single embryo. The optimal method uses organic reagents and spin column technology and enables the researcher to partition the sample prior to extraction to dictate how much of the sample is allocated for either DNA or RNA extraction. Additionally, we conduct time trials to evaluate RNA degradation that might occur during the process and amplify antisense RNA (aRNA also known as cRNA) to demonstrate that the method can produce enough high-quality RNA to be further used for microarray gene expression analyses.

Materials and Methods

RNA or DNA Extracted Separately

Total genomic DNA or RNA was first isolated from embryos before attempting extraction of both nucleic acids from single samples. This was done to provide a baseline measure of maximum yields and quality expected per nucleic acid. Ten-day-old *F. grandis* embryos each weighing ~4.0 mg were individually frozen in centrifuge tubes at -80°C . Genomic DNA was extracted using 2 methods: 1) proteinase K/phenol–chloroform–isoamyl protocol (Sambrook and Russel 2001) and 2) Qiagen DNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's protocols. Total RNA was extracted from embryos using 2 methods: 1) TRIzol reagent extraction (Invitrogen, Carlsbad, CA) and 2) chaotropic "Chaos" buffer extraction (4.5 M guanidine thiocyanate; 2% N-lauroyl sarcosine; 50 mM ethylenediaminetetraacetic acid [EDTA], pH 8; 0.1 M 2-mercaptoethanol; 0.2% antifoam-A). Embryos were homogenized in either 400 μ l TRIzol or Chaos buffer. For TRIzol extractions, 200 μ l chloroform was added to the homogenate. Samples were incubated at room temperature (RT)

for 15 min and then centrifuged at 13 200 rpms for 15 min at 4°C. An equal volume of 70% ethanol was added to the upper phase, and the mixture was then transferred to a Qiagen RNeasy spin column. For extractions with Chaos buffer, after homogenization, 40 µl 2 M NaOAc, 400 µl acidic phenol, and 200 µl chloroform/isoamyl-ethanol were added to each sample. Samples were incubated on ice for 10 min and then centrifuged at 13 200 rpms for 15 min at 4°C. An equal volume of 100% isopropanol was added to the upper phase, and the mixture was then transferred to a Qiagen RNeasy spin column. All samples were washed according to the Qiagen's protocols and were eluted in a final volume of 35 µl RNase-free water. DNA and RNA were quantified with a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE). RNA quality was assessed with an Experion standard sensitivity RNA chip (Bio-Rad, Hercules, CA), and DNA was visualized on 1% agarose gels.

RNA and DNA Extractions from Single Embryos

We extracted both DNA and RNA from embryos using a variety of methods: 1) DNA extractions from the organic phase and interphase remaining after RNA extraction from

TRIzol and Chaos buffer, 2) DNA and RNA extracted from separate portions of subdivided TRIzol and Chaos buffer homogenates, 3) spin column technology, and 4) magnetic beads and bead-based sample disruption.

DNA Extraction after RNA Extraction

After RNA extraction from TRIzol and Chaos buffers, we added 250 µl back extraction buffer (4 M guanidine thiocyanate; 50 mM sodium citrate; 1 M Tris, pH 8.0) to the phenol phase and interphase and let the mixtures sit at RT for 10 min. Samples were then centrifuged at 13 200 rpms for 15 min at 4 °C. The upper phase was removed, an equal volume of 100% isopropanol was added, and the samples were incubated overnight at -80°C. After incubation, samples were centrifuged at 13 200 rpm for 15 min at 4°C. The supernatant was removed, and the pellets were washed 3 times with 70% ethanol. Samples were eluted in a final volume of 50 µl low Tris EDTA (10 mM Tris; 0.1 mM EDTA, pH 8.0). We added a polyacryl carrier (Molecular Research Center, Cincinnati, OH) to a subset of samples to test for increased yields. Additionally, we added the following reagents (during separate extractions) to the

Table 1. RNA and DNA concentrations produced during extraction trials

Extraction technique	Average (range) RNA yield (ng/µl)	Average A _{260/280}	Average (range) DNA yield (ng/µl)	Average A _{260/280}
DNA only				
Organic extraction			6.8 (5.2–11.0)	1.9
Qiagen DNeasy kit			9.9 (8.3–13.3)	1.9
RNA only				
TRIzol/Qiagen RNeasy spin columns	2.7 (1.0–5.2)	2.1		
Chaos/Qiagen RNeasy spin columns	2.5 (1.6–2.4)	1.8		
RNA and DNA				
TRIzol/Qiagen RNeasy spin columns ^a	2.7 (1.0–5.2)	2.1		
TRIzol organic phase DNA extraction				
Back buffer			3.6 (1.1–5.7)	1.7
Back buffer with polyacryl carrier			3.7 (3.3–4.0)	1.7
1× Tris EDTA			3.4 (2.8–3.7)	1.8
TNES buffer			3.6 (2.2–4.6)	1.9
SNET buffer			3.0 (2.7–3.4)	1.9
Chaos/Qiagen RNeasy spin columns ^a	2.5 (1.6– 2.4)	1.8		
Chaos organic phase extraction				
Back buffer			3.2 T(2.3–3.6)	1.8
Back buffer with polyacryl carrier			3.1 (1.9–3.5)	1.7
1× Tris EDTA			3.2 (3.0–3.3)	1.9
TNES buffer			3.9 (2.8–4.4)	1.8
SNET buffer			3.0 (2.3–3.7)	1.9
Chaos split homogenate				
DNA/RNA–50:50	1.8 (1.1–2.6)	1.9	3.9 (3.3–4.7)	1.9
DNA/RNA–75:25	1.1 (0.4–2.0)	1.9	6.1 (4.2–9.7)	1.9
Qiagen Allprep DNA/RNA Micro kit	1.5 (1.1–2.0)	2.2	1.9 (1.4–2.8)	1.8
MagMAX-96 Total RNA Isolation kit	1.8 (0.5–3.8)	2.0	2.4 (0.7–4.7)	1.9
MagMAX total Nucleic Acid Isolation kit	0.9 (0.5–1.3)	2.2	2.1 (1.9–2.6)	2.2

^a RNA concentrations are equal to those obtained during RNA only extractions.

phenol phase and interphase to test whether they affected DNA yields or quality 1) 100% ethanol, 2) chloroform/isoamyl ethanol, 3) 1× Tris EDTA, TNES buffer (10 mM Tris-HCl, pH 7.5; 125 mM NaCl; 10 mM EDTA; 1% sodium dodecyl sulfate [SDS]; 6 M Urea) (Kimura et al. 2004), and 4) SNET buffer (20 mM Tris, pH 8.0; 5 mM EDTA, pH 8.0; 400 mM NaCl; 1% SDS) (Sambrook and Russel 2001). Lastly, we used a ChargeSwitch gDNA tissue kit from Invitrogen substituting the phenol phase and interphase for the lysate as outlined in the manufacturer's protocols.

DNA and RNA Extraction from Split Portions of Homogenate

To conduct simultaneous DNA and RNA extractions with TRIzol and Chaos buffers, we divided the homogenate immediately after homogenization and allocated a portion of it for DNA extraction with a Qiagen DNeasy kit and the other for RNA extraction. It is important to note that the tissue homogenate was split and allocated to different protocols rather than the tissue itself. This is because, like many tissue types, fish embryos encompass a heterogeneously distributed population of cell types. Dissecting the tissue prior to nucleic acid extraction could lead to inconsistencies in mRNA profiling among samples because of inconsistent splitting of tissue types. This can be avoided by splitting the tissue homogenate instead, which should result in a more randomly distributed sample of tissue types. We split the homogenate 50:50 but found that the TRIzol buffer was too viscous for the Qiagen spin column filters (see Results and Discussion). We also split the homogenate 25:75 (RNA/DNA) but only proceeded with the extractions for tissues homogenized in Chaos buffer because of viscosity problems with TRIzol. RNA extractions were conducted as described above, and DNA extractions were conducted according to the Qiagen instructions with 1.25 µg RNase added to the spin column during the first washing step. If the fraction allocated for DNA is extracted before the fraction allocated for RNA, the RNA might suffer degradation while sitting in the homogenate at RT. Thus, we conducted time trials extracting RNA immediately after homogenization, after 1 h at RT, and after 2 h at RT.

DNA and RNA Extractions from Commercial Kits

We tested 3 commercially available kits that utilized different technologies to simultaneously extract RNA and DNA from the same sample. The first was the Qiagen AllPrep DNA/RNA Micro kit, which we used according to the manufacturer's instructions. For homogenization, we used the ATL buffer provided with the kit and conducted separate trials substituting TRIzol for ATL. We also extracted RNA and DNA using the magnetic bead technology of the MagMAX-96 Total RNA Isolation kit (Applied Biosystems/Ambion, Austin, TX) and with the bead-based sample disruption of the MagMAX Total Nucleic Acid Isolation kit (Applied Biosystems/Ambion). We followed the instructions provided with the kits but divided the samples during the final washing steps and applied either DNase or RNase to obtain estimates of total RNA and DNA yields, respectively.

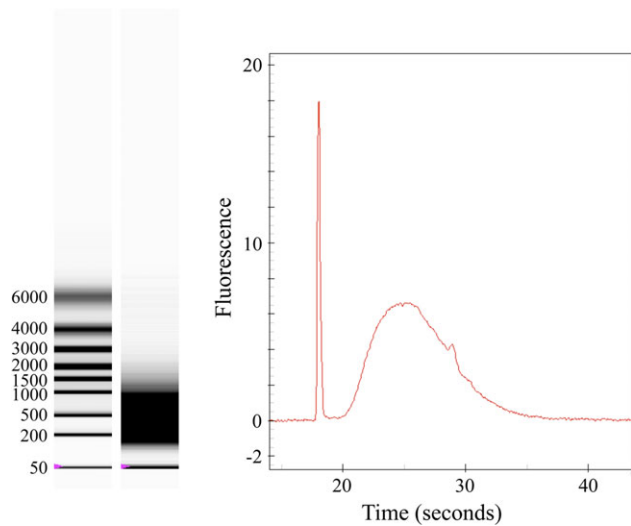


Figure 1. Gel image and electropherogram from Bio-Rad's Experion system depicting aRNA amplified with Ambion's Amino Allyl Message Amp aRNA Amplification kit.

Assessing Sample Quality

Samples from all trials were quantified with a NanoDrop 1000 spectrophotometer. RNA quality was assessed with an Experion standard sensitivity RNA chip, and genomic DNA was visualized on 1% agarose gels. Polymerase chain reactions (PCRs) were performed on DNA obtained from all methods using the primer pair FhATG-18 (Adams et al. 2005) in a final volume of 21 µl as an additional test of DNA quality. PCRs included 1× buffer (Applied Biosystems), 1.2 mM MgCl₂ (Applied Biosystems), 0.2 mM deoxynucleoside triphosphates (Fisher Scientific, Pittsburg, PA), 0.25 µM each primer, and 1.2 U *Taq* DNA polymerase. The thermal profile consisted of initial denaturation at 95 °C for 5 min; 30 cycles of 95 °C for 45 s, 56 °C for 45 s, 72 °C for 45 s, and a final elongation at 64 °C for 45 min. PCR products were visually inspected on 1% agarose gels.

After determining which method provided the highest quality DNA and RNA (see Results and Discussion), we tested whether that method would provide sufficient template for microarray analysis. Thus, we chose an RNA sample with the lowest yield (0.42 µg) and amplified 0.3 µg of the sample with the Amino Allyl Message Amp aRNA Amplification kit (Ambion) following the product instructions. This kit yields labeled aRNA copies of mRNA that can be used with most microarray gene expression platforms and typically requires at least 5 µg of amino allyl-modified aRNA for the labeling process. We visualized aRNA quality on the Experion system.

Results and Discussion

RNA and DNA yields for all trials are summarized in Table 1. All methods resulted in successful isolation of RNA.

Extractions using TRIzol, Chaos Buffer, and Qiagen AllPrep DNA/RNA Micro kit consistently produced high-quality RNA as indicated with the Experion system. Ambion MagMax kits also produced high-quality RNA though not as consistently. DNA was successfully isolated with all methods with the exception of the Invitrogen ChargeSwitch extraction of phenol phases and interphases. ChargeSwitch uses a magnetic bead-based technology that is dependent on the pH of the surrounding buffer, which was likely disrupted by the organic phases resulting from initial extraction steps. High-quality DNA, as visualized on agarose gels, was produced with the Chaos/spin column method and with the Qiagen AllPrep DNA/RNA Micro kit. The remaining methods generated low-quality DNA that either did not appear on agarose gels or was not viewed as a crisp band without smearing. Despite the apparent low quality, genomic DNA could be PCR amplified successfully. All methods, with the exception of Invitrogen ChargeSwitch protocol, produced PCR products ~160 bp in length, the expected

size range within *Fundulus* for this primer pair (Adams et al. 2005).

In addition to extracting both high-quality DNA and RNA, we sought to maximize the yield of each nucleic acid isolated from a single sample. Total RNA and DNA were initially isolated independently of each other to determine the maximum amount of nucleic acids that we could expect to extract from a single embryo. The TRIzol/spin column extraction method produced the highest yield of RNA (average = 2.7 μg , range = 1.0–5.2 μg), and the Qiagen DNeasy kit produced the highest yield of DNA (average = 9.9 μg , range = 8.3–13.3 μg). The Qiagen AllPrep DNA/RNA Micro kit generated high-quality RNA and DNA but at low concentrations (RNA: average = 1.5 μg , range = 1.1–2.0 μg ; DNA: average = 1.9 μg , range = 1.4–2.8 μg). Ambion MagMax kits also produced RNA and DNA at low concentrations but not of consistently high quality (Table 1).

The DNA extractions conducted after RNA isolation using TRIzol or Chaos buffers generated low-quality DNA in

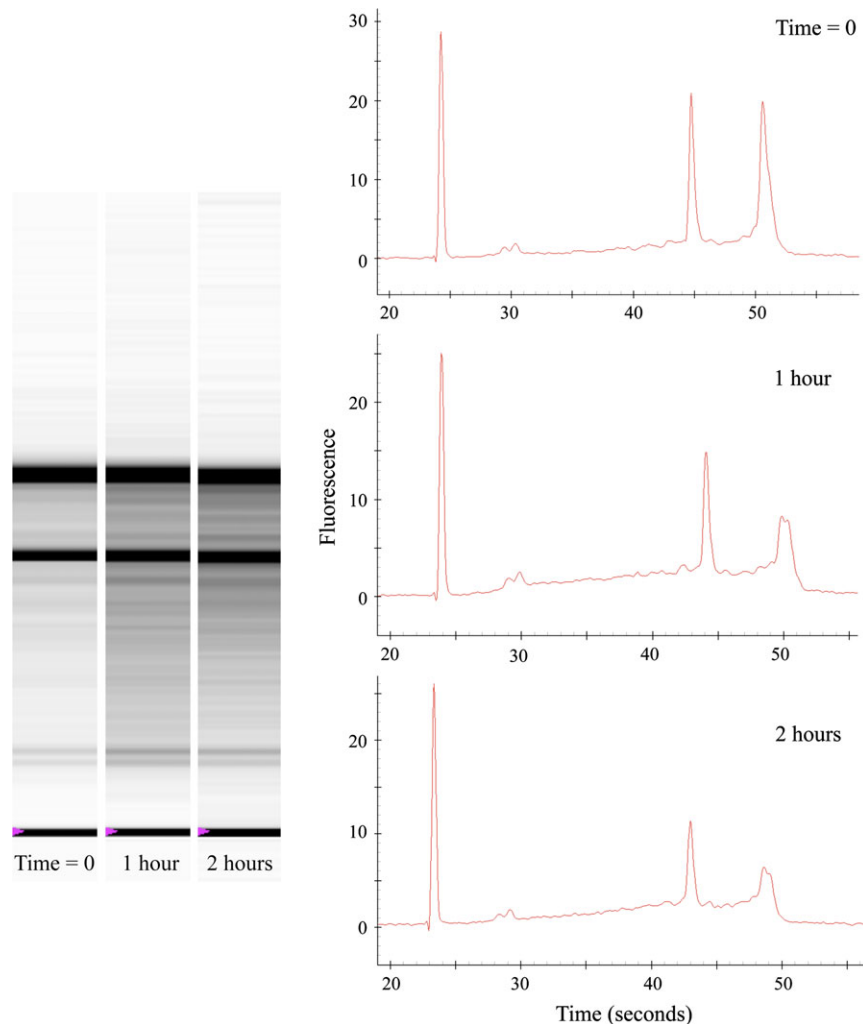


Figure 2. Gel images and electropherograms from Bio-Rad's Experion system depicting RNA quality and degradation over time. Images depict RNA extracted immediately after homogenization (time = 0), after 1 h at RT, and after 2 h at RT.

almost all cases. The use of different buffers or a polyacryl carrier during the extraction did not significantly affect the yield (Table 1). However, when we divided the Chaos buffer homogenate prior to nucleic acid purification, then separately extracted DNA and RNA from each portion, we consistently obtained high-quality DNA and RNA at high concentrations. TRIzol homogenate was not compatible with this method because the buffer appeared to be too viscous for the spin column filters. DNA yields from TRIzol homogenates were higher than those obtained from the Chaos buffer homogenates but did not appear as clean or as robust when samples were electrophoresed on an agarose gel, which led us to conclude that the sample was contaminated by TRIzol that passed through the filter. This also occurred when we substituted TRIzol for the ATL buffer as provided in the Qiagen AllPrep DNA/RNA Micro kit.

Dividing the homogenate prior to extraction not only generates high-quality product but also permits researchers to allocate the sample according to their experimental needs. We required at least 5 µg of DNA and 0.5 µg of RNA for genetic mapping and microarray analyses, respectively. Thus, we divided the homogenate with 75% of the sample for DNA isolation and 25% for RNA isolation. Nucleic acid quality and yields obtained from a single embryo for both DNA (average = 6.1 µg, range = 4.2–9.7 µg) and RNA (average = 1.1 µg, range = 0.4–2.0 µg) were more than sufficient for our studies. None of the other methods provided enough high-quality nucleic acid. The aRNA generated with the Amino Allyl Message Amp aRNA Amplification kit was of high quality (Figure 1) and given a final concentration of 5.5 µg was enough template for the labeling procedure prior to microarray hybridization.

Although the optimal extraction method, Chaos buffer/spin columns, has the advantages of high sample quality and preferential isolation of either nucleic acid type, it does require that both extractions be carried out simultaneously. If RNA is extracted first, the sample designated for DNA extraction will sit as homogenate during the process, but we did not notice any decline in DNA quality over time. Because RNA is more prone to degradation, we conducted time trials and recorded RNA degradation after 1 h and after 2 h (Figure 2). There was noticeable degradation after 1 h, but there did not seem to be any additional degradation after the second hour. There were no differences in yield across these time periods. Therefore, if the split samples cannot be extracted simultaneously for RNA and DNA because of lack of personnel or equipment, we suggest that the RNA portion be purified before DNA.

Conclusions

We tested a number of ways by which nucleic acids can be extracted from a single sample and found that the Chaos buffer/spin column method maximizes the amount of high-quality RNA and DNA isolated. Importantly, splitting the homogenate rather than splitting the whole tissue minimizes

potential inconsistencies in mRNA profiling that could arise from inconsistent surgical splitting of a tissue with heterogeneous distribution of cell types. DNA and RNA can be extracted from a single sample in proportions that can be tailored to individual investigator's needs. It is a simple procedure and because it uses spin column technology, it can be easily adapted to a 96-well plate format. Although we used fish embryos, this method can be particularly useful for anyone working with small samples that are not amenable to multiple independent extractions.

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