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RNA-seq in the tetraploid *Xenopus laevis* enables genome-wide insight in a classic developmental biology model organism

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Abstract

Advances in sequencing technology have significantly advanced the landscape of developmental biology research. The dissection of genetic networks in model and nonmodel organisms has been greatly enhanced with high-throughput sequencing technologies. RNA-seq has revolutionized the ability to perform developmental biology research in organisms without a published genome sequence. Here, we describe a protocol for developmental biologists to perform RNA-seq on dissected tissue or whole embryos. We start with the isolation of RNA and generation of sequencing libraries. We further show how to interpret and analyze the large amount of sequencing data that is generated in RNA-seq. We explore the abilities to examine differential expression, gene duplication, transcript assembly, alternative splicing and SNP discovery. For the purposes of this article, we use *Xenopus laevis* as the model organism to discuss uses of RNA-seq in an organism without a fully annotated genome sequence.

Keywords

RNA-seq; differential expression; *Xenopus*

1. Introduction

1.1 RNA-seq as a powerful tool in developmental biology

Over the past few decades, several advances have been made in dissecting the molecular pathways that govern basic developmental biology. Forward genetic screens in model organisms have been useful in identifying components of signaling pathways and transcription factors involved in processes ranging from the early patterning of embryos to

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the proper specification and differentiation of organs that are crucial to various stages of development [1–4]. These screens have been extremely valuable, not only in identifying the functional requirements of genes, but also in showing that no prior knowledge of a genes' molecular function is needed to order genes in a genetic pathway [5]. These phenotype-based screens have provided a directed method to identify genes and assign them functions. This information can then be better utilized in addressing how these genes organize into networks during development.

Gene network dissection has been accelerated with many collaborative efforts undertaken to sequence the genomes and transcriptomes not only of humans, but also of the model organisms, their subspecies, and additional emerging non-model organisms used in developmental biology research [6–12]. Annotation of these genomes has allowed researchers to take reverse genetic approaches to specifically knock down genes of interest and assay for phenotypes to determine gene functions using directed mutagenesis, RNA interference (RNAi), or morpholino (MO) knockdown [13–15]. These approaches have been successfully implemented to further refine our ability to order genes with conserved function in molecular networks.

The molecular basis of genetic networks was first addressed at a genome-wide level with the use of microarrays [16–19]. Microarrays allowed researchers to assay for differential gene expression during development or as a result of genetic manipulation. A disadvantage of microarrays is that they require prior knowledge of cDNA library sequences and use genome annotation data to refine their models. For this reason, microarray platform design requires constant refinement as gene models improve. This includes updating previous versions of microarrays and therefore unnecessary expense. Furthermore, although microarrays have been used extensively for some model organisms in developmental biology research, the percentage of total genes represented on microarrays for organisms with poorly annotated genomes can be low. This is especially true in light of the recent advances in the number of emerging model organisms that are being exploited to study developmental biology and evolution [20]. Moreover, arrays are limited by cDNAs or genes that have been previously described and cannot capture differences in gene models that arise. They are further limited in that they sometimes do not provide insight into small RNAs, alternative splicing, differential 3' untranslated region (UTR) patterns, or differences in expression of transcripts due to SNPs. As these new model organisms come to the forefront of developmental biology research, alternative methods have been developed to study global gene expression.

Massive parallel high-throughput sequencing has largely superseded microarray technology for most applications, but especially for transcriptomics, as it can be used to address all the limitations highlighted here. High-throughput sequencing of RNA, or RNA-seq, has become a popular method for identifying and quantifying the full set of transcripts in cells or tissues and for differential expression analysis [21–23]. This method does not require prior knowledge of the genome sequence or lists of known genes. For genomes that have been partly or fully sequenced, RNA-seq reads can be aligned with genomic sequences to perform differential expression analysis. The added advantage of RNA-seq in these organisms is that, as genome annotation improves, RNA-seq reads can be re-analyzed with the updated gene

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annotations, an option that is not available with microarrays. Moreover, data obtained from RNA-seq can help in refining existing gene and alternative splicing models.

For emerging model organisms with incomplete or no genome sequence, RNA-seq provides two major advantages to microarrays. First, the workflow of RNA-seq can be universally applied to these organisms, whereas microarrays would need to be designed specifically for each species, a potentially costly endeavor. Second, RNA-seq data can be used to generate *de novo* gene lists, which can subsequently be used to analyze differential expression [24]. This requires little to no information regarding the genome prior to analysis and avoids the pitfalls of microarrays for poorly annotated genomes, thereby providing more avenues to studies in model organisms, an example being *Xenopus*.

The western African clawed frog, *Xenopus*, has been used extensively as a model organism in vertebrate developmental biology. *Xenopus* species develop externally, allowing for various manipulations to study fertilization, induction, and cell movements. *Xenopus* are nearly transparent at tadpole stages, allowing for dynamic visualization of tissue and organ development in live animals. Tissues, such as the developing heart, can be easily dissected from the embryo and allowed to develop in culture. Antisense oligonucleotides, or morpholinos (MOs, Genetools, LLC), have been used successfully to disrupt gene function in *Xenopus*. These properties have made *Xenopus* an excellent model organism for the study of cardiac development [25].

Two *Xenopus* species, *X. laevis* and *X. tropicalis*, are widely utilized in cellular and developmental biology. The genome of the diploid *X. tropicalis* was recently published [26], making it an ideal model organism for genetic studies in the frog. *X. laevis* embryos and frogs are larger than those of *X. tropicalis* and are, therefore, preferentially exploited for experimental biology techniques. The *X. laevis* genome underwent tetraploidization 40 million years ago, and thus, classical genetic analysis was less feasible in this species [27]. Despite this hurdle, an *X. laevis* genome sequencing project is underway, and the availability of extensive expressed sequence tag (EST) sequences on Xenbase [28] allow for functional analysis of genetic pathways. Moreover, targeted mutations can be generated in *Xenopus* using TALE nuclease (TALENs) or zinc finger nucleases (ZFNs) [29–31]. These advances, combined with their suitability for experimental biology, make *Xenopus* a tractable model to dissect gene function in organ development. Here, we describe some examples of the many uses of RNA-seq technology, which we have applied in advancing our understanding of *X. laevis* cardiac development. Furthermore, these studies provide a basis to study the evolution of gene function as a result of gene duplication and polymorphisms.

The methods outlined here should provide a framework for those working not only with *X. laevis*, but with any organism, particularly those that lack a published genome sequence.

2. Preparation of total RNA

In this review, we describe the use of *Xenopus* embryos for RNA-seq experiments, including the isolation of RNA from whole embryos and dissected tissues. These approaches should be transferrable to the organism/tissue of interest. Caution should be taken to avoid

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introducing RNases into the RNA preparation protocol. RNase-free tips and tubes should be used along with gloves to minimize RNases in materials and handling.

2.1 *In vitro* fertilization of *X. laevis* embryos

To obtain sufficient numbers of developmental stage-matched embryos we fertilize embryos *in vitro*. Here we describe the fertilization method used to obtain *X. laevis* embryos. This method can be coupled with the injection of MOs at the 1-cell stage to deplete gene function in the entire embryo or in individual blastomeres fated to give rise to specific tissue or cell types [32]. In addition, microinjection of mRNA into early-stage embryos can provide gene overexpression or dominant-negative effects [33, 34]. These methods are well established in *Xenopus* and have been useful alternatives in the absence of gene knockout tools to study gene function. Recent advances in *Xenopus* mutagenesis, through forward genetic screening and the use of ZFNs or TALENs to mutate genes of interest, will further aid in functional analysis of genes in *Xenopus*. Careful planning should be used to determine when embryos will reach proper stage of development for expression studies. Embryos can also be collected at different stages for differential expression studies.

2.1.1 *In vitro* fertilization materials and reagents—

1. Human Chorionic Gonadotropin, hCG (Chorulon), 1000U/ml stock in PBS, stored at 4°C
2. 1X Marc's Modified Ringer's solution (MMR) (0.1 M NaCl, 2.0 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 5 mM HEPES (pH 7.4), 0.1 mM EDTA)
3. Testes Buffer - Leibovitz's L-15 medium (Invitrogen) supplemented with 10% fetal calf serum and 50 μg/ml gentamycin
4. Forceps
5. 0.1X Magnesium-buffered saline (MBS) (8.8 mM NaCl, 0.1 mM KCl, 0.1 mM $MgSO_4$, 0.5 mM HEPES (pH 7.8), 0.25 mM $NaHCO_3$, 0.7 mM $CaCl_2$)
6. 2% L-cysteine hydrochloride monohydrate (Sigma), pH 8.0 made fresh daily in ddH_2O

2.1.2 *In vitro* fertilization procedure—

1. Inject adult *X. laevis* females with 500U hCG 17–18 hours prior to egg collection. Allow adults to recover in frog system water at 16°C after priming injection. We prime at least two females to increase the chances of obtaining high quality eggs.
2. Transfer females to 1 × MMR and allow to lay eggs undisturbed.
3. While females are laying eggs, euthanize an *X. laevis* male. Dissect both testes and store in Testes Buffer. Testes can be stored in this solution for up to one week.
4. Collect 500–1000 eggs on a petri dish and remove excess buffer with transfer pipette.

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5. Coarsely macerate one-third of a testis. Collect pieces of testis with forceps and paint surface of eggs in petri dish.
6. After 5 minutes, flood eggs on plate with 0.1X MBS. Allow to recover for 20 minutes.
7. Remove excess 0.1X MBS and flood fertilized eggs with cysteine solution to remove jelly coats from eggs. Incubate until embryos pack together (approximately 3–5 minutes), then wash 4–5 times with 0.1X MBS.
8. Culture embryos in 0.1X MBS at desired temperature. At this point, embryos can be injected with morpholinos or mRNA as described elsewhere [32, 35–37].

2.2 Total RNA isolation

Once embryos have reached the desired stage of development, they must be processed to collect total RNA. A minimum of 1 μg total RNA is recommended to obtain enough material for RNA-seq using this protocol. When performing RNA-seq on dissected tissue, the number of explants required to reach the minimal RNA desired will need to be determined. For example, we have found that 100 dissected hearts are required for stage 37 *X. laevis* embryos to obtain 1 μg of total RNA, while a minimum of 50 hearts are required for stage 45 embryos. It is also critical from this point forward to avoid introduction of RNases. Sterile RNase-free water or DEPC-treated water should be used to make solutions needed for RNA extraction and subsequent procedures.

2.2.1 RNA isolation materials and reagents—

1. 0.5X MBS supplemented with 50 μg/ml BSA
2. Trizol reagent (Invitrogen)
3. Chloroform
4. Isopropyl alcohol
5. Ethanol (70%)
6. Forceps (for dissection)
7. RNase-free tubes and pipette tips
8. RNase-free ddH$_2$O
9. RQ1 DNase and 10X RQ1 DNase buffer (Promega)
10. Bench-top centrifuge
11. RNeasy Mini Kit (Qiagen)
12. Qubit fluorometer and RNA Assay Kit (Invitrogen)

2.2.2 RNA isolation procedure—

1. Collect 10 embryos in 1 ml Trizol. Alternatively, if collecting RNA from tissue, dissect embryos in 0.5X MBS with 50 μg/ml BSA supplement to aid dissection and

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separation of tissues. Collect dissected tissue in Trizol (25 explants per 500 μl Trizol). Flash freeze embryos/dissected tissue in Trizol in liquid nitrogen and store at −80°C. This step will enhance the homogenization process during Trizol extraction.

2. Thaw tubes at room temperature. Homogenize embryos/tissue with pipet, vortex briefly and incubate at room temperature for 5 minutes.
3. Centrifuge lysate at 22,000 × *g* for 1 minute to precipitate insoluble material. Transfer supernatant to a new tube.
4. Add 200 μl chloroform per ml initial Trizol volume. Vortex and incubate at room temperature for 3 minutes followed by centrifugation at 12,000 × *g* for 15 minutes at 4°C.
5. Transfer aqueous layer to a new tube and add 500 μl isopropyl alcohol per ml of Trizol used.
6. Incubate 10 minutes at room temperature. Precipitate RNA by centrifugation at 12,000 × *g* for 10 minutes at 4°C.
7. Wash pellet twice with 70% ethanol and centrifuge at 8,000 × *g* for 5 minutes between washes.
8. Air-dry pellet and resuspend in 25 μl DEPC-treated water. Add 2 μl RQ1 DNase and 3 μl 10X DNase buffer. Incubate at 37°C for 30 minutes.
9. Purify total RNA using the RNeasy Mini Kit, according to manufacturer's instructions.
10. Quantify purified product using a Qubit fluorometer and RNA Assay Kit according to manufacturer's instructions (Invitrogen). Store at −80°C or proceed immediately to mRNA enrichment (section 3.1). Note: This protocol is optimized for a minimum of 1 μg total RNA. If RNA yield is too low, you may consider RNA amplification protocols before proceeding [38, 39]. This may lead to amplification biases that should be taken into account downstream when analyzing RNA-seq data [40, 41].

3. RNA-seq

Once total RNA is purified from tissues or embryos of choice, poly-adenylated mRNA is purified and fragmented before library preparation. RNA-seq kits can be purchased from a number of suppliers (i.e., Illumina, NEB, Clontech and Ambion); however, prior to library preparation it is important to ensure that the provided adaptor sequences are compatible with the high-throughput sequencing machines being used. Here we describe a protocol to generate sequencing libraries for the Illumina Genome Analyzer II (GAII), using the Illumina mRNA Sequencing Sample Preparation Guide, September 2009. This protocol can be used to generate libraries from a minimum of 1 μg total RNA obtained from dissected tissue.

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3.1 mRNA purification

mRNA comprises a very small percentage of the total RNA population and is therefore difficult to quantify in a total RNA preparation. Total RNA purified must be of high quality. This can be assessed before proceeding with mRNA enrichment. For example, a small amount of total RNA can be analyzed by running on a denaturing gel and examining the ratio of 28S and 18S ribosomal RNA, which for high quality RNA should be approximately 2.0 [42]. This generally requires a large amount of total RNA, but if RNA yield is precious, samples can be analyzed for example using the Pico RNA kit for the RNA Bioanalyzer 2100 (Agilent), which requires a total RNA concentration of 50–5000 pg/μL in water.

The following protocol enriches for poly-adenylated mRNAs from the total RNA pool using magnetic bead capture. As with preparation of total RNA, great care should be taken to avoid introduction of RNases to the samples.

3.1.1 mRNA purification materials and reagents—

1. 1 μg total RNA (minimum)
2. RNase-free water
3. 1.5-ml pre-lubricated RNase-free tube (Costar, cat. no. 3207)
4. 65°C and 80°C dry heat block
5. Sera-Mag Oligo [dT] beads (ThermoScientific)
6. Dynal magnetic strand (Invitrogen)
7. Bead-binding buffer (BBB; 20 mM Tris-HCl, pH 7.5, 1M LiCl, 2 mM EDTA)
8. Wash Buffer (WB; 10 mM Tris-HCl, pH 7.5, 0.15 M LiCl, 1 mM EDTA)
9. 10 mM Tris-HCl
10. 200-μl thin-walled PCR tubes

3.1.2 mRNA purification procedure—

1. Dilute 1 μg of total RNA to a final volu*me* of 50 μl in RNase-free water in a 1.5-ml pre-lubricated RNase-free tube.
2. Heat at 65°C for 5 minutes, and immediately place on ice.
3. While RNA is heating, prepare Sera-Mag beads (15 μl/sample). For each wash step in this procedure, buffer is added to beads and then the beads are collected by placing on a magnetic stand for 1.5 minutes. To prepare beads, wash twice with 100 μl BBB.
4. After second wash, resuspend beads in 50 μl BBB and combine with 50 μl of ice-cold RNA. Rotate end-over-end at room temperature for 5 minutes.
5. Wash beads twice with 200 μl WB and resuspend in 50 μl 10 mM Tris-HCl, pH 7.5. Heat for 80°C for 2 minutes.

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6. Collect beads on magnet, and transfer supernatant, containing mRNA, to a clean tube.
7. Add 50 μl BBB to the mRNA solution to bring total volume to 100 μl and heat for 5 minutes at 65°C. Immediately place on ice at end of 5 minute incubation.
8. While RNA is heating, wash beads twice with 200 μI WB.
9. After second wash, resuspend beads with 100 μl ice-cold mRNA and rotate at room temperature for 5 minutes.
10. Wash beads twice with 200 μl WB.
11. After second wash, resuspend beads in 17 μl 10 mM Tris-HCl, and heat at 80°C for 2 minutes, and immediately place on magnet.
12. Elute mRNA (16 μl) and transfer to a 200-μl thin-walled PCR tube.

3.2 mRNA fragmentation

Once mRNA has been enriched from total RNA pools, it must be fragmented. This ensures a uniform size of fragments when preparing the sequencing library and reduces the risk of fragment size-based amplification bias. We use a chemical fragmentation protocol to quickly and efficiently fragment the mRNA. This step needs to be optimized depending on the tissue source, as mRNA obtained from different species may require shorter or longer fragmentation times. Alternatively, this process can be skipped and cDNA fragments can be digested by DNAseI after generating double-stranded cDNA (section 3.4.2) as described elsewhere [43]. However, this may lead to biases of overrepresented dinucleotide sequences [41]. Optimal size of fragments ranges from 200 to 400 base pairs.

3.2.1 mRNA fragmentation materials and reagents—

1. RNase-free water
2. 10X Fragmentation Buffer (Ambion, AM8740)
3. Stop Solution (200 mM EDTA pH 8.0)
4. 70°C heat block
5. 3 M NaOAc (pH 5.2)
6. Glycogen (5 μg/μl)
7. 70% Ethanol
8. RNase-free 1.5 ml centrifuge tubes
9. Bench-top centrifuge

3.2.2 mRNA fragmentation procedure—

1. Bring mRNA solution to 18 μl with RNase-free water.

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2. Fragment by adding 2 μl 10× Fragmentation Buffer and heating at 70°C for exactly 4 minutes (The incubation time in this step is dependent on tissue. We have found 4 minutes works well for *X. laevis*).
3. Add 2 μl Stop Solution and place sample on ice.
4. Precipitate mRNA by adding 2 μl 3 M NaOAc (pH 5.2), 2 μl glycogen (5 μg/μl), and 60 μl 100% ethanol and storing at −80°C overnight.
5. Centrifuge tubes 22,000 *g* for 25 minutes at 4°C and remove supernatant.
6. Wash pellet with 300 μl 70% ethanol and air-dry.
7. Resuspend pellet in 11.1 μl RNase-free water.
8. Optional: RNA can be run on an agarose gel to check for average fragment size, concentrated at 200–400 bp. If RNA is not fragmented enough, increase reaction time in step 2. If RNA is too fragmented, reduce the time in step 2.

3.3 First strand cDNA synthesis

Once mRNA is purified and fragmented from total RNA pool, cDNA is transcribed from mRNA, followed by second strand cDNA synthesis. The ends of the generated cDNA are repaired and adaptors are ligated to these ends to prepare sequencing libraries.

3.3.1 *First strand cDNA synthesis materials and reagents—*

1. Random Primers (3 μg/μl Invitrogen)
2. 65°C heat block
3. 5× First Strand Buffer (Invitrogen)
4. 100 mM DTT
5. 25 mM dNTPs
6. RNase Inhibitor (Enzymatics)
7. SuperScript II (Invitrogen)
8. Thermal cycler

3.3.2 First strand cDNA synthesis procedure—

1. Add 1 μl of Random Primers (3 μg/μl) to mRNA. Incubate sample at 65°C for 5 minutes and immediately place on ice.
2. Make master mix containing 4 μl 5× First Strand Buffer, 2 μl 100 mM DTT, 0.4 μl 25 mM dNTP, and 0.5 μl RNase inhibitor. Add 6.5 μl freshly prepared master mix to the sample and incubate at 25°C for 2 minutes.
3. Add 1 μl SuperScript II to sample and incubate at 25°C for 10 minutes, 42°C for 50 minutes, 70°C for 15 minutes, and then maintain at 4°C.

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3.4 Second strand cDNA synthesis

3.4.1 Second strand cDNA synthesis materials and reagents—

1. 5× Second Strand Buffer (Invitrogen)
2. 25 mM dNTP
3. 2 U/µl RNase H (Enzymatics)
4. 5 U/µl DNA Pol I (Enzymatics)
5. QIAquick PCR Purification Kit (Qiagen)
6. EB buffer; 10 mM Tris-Cl, pH 8.5 (Qiagen)

3.4.2 Second strand cDNA synthesis procedure—

1. Make master mix of 52.8 µl water, 20 µl 5× Second Strand Buffer (Invitrogen), and 1.2 µI 25 mM dNTP.
2. Add 74 µl freshly prepared master mix to first strand cDNA samples. Incubate on ice for 5 minutes.
3. Add 1 µl of 2 U/µl RNase H and 5 µl of 5 U/µl DNA Pol I. Incubate at 16°C for 2.5 hours.
4. Purify cDNA using PCR purification kit according to manufacturer’s instructions. Elute in 50 µl elution buffer (EB; 10 mM Tris-Cl, pH 8.5).

3 5 Ligation of Adapters

3.5.1 Ligation of Adapters materials and reagents—

1. 10× T4 Ligation Buffer (including ATP; New England Biolabs, NEB)
2. 10 mM dNTP mixture
3. Polynucleotide Kinase (10U/µI; NEB)
4. Klenow (1U/µI; NEB)
5. T4 DNA Polymerase (3U/µI; NEB)
6. PCR Purification Kit and Mini-Elute PCR Purification Columns(Qiagen)
7. Klenow 3’-5’ Exo- (5U/µI; NEB)
8. 10 mM dATP
9. 10× NEB Buffer 2 (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1mM DTT, pH 7.9)
10. 2× Quick Ligation Buffer (NEB)
11. Genomic Adapter Oligo Mix (Illumina cat. no. 10000531)

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   a. Pair-end adapters, these can be used for single-end sequencing as well: 5' P- GATCGGAAGAGCGGTTCAGCAGGAATGCCGAG 5' ACACTCTTTCCCTACACGACGCTCTTCCGATCT

12. Quick Ligase (NEB, approximately 500 U/μl)
13. AMPure-XP (SPRI) Beads (Agencourt)
14. EB Buffer; 10 mM Tris-Cl, pH 8.5
15. 70% and 80% Ethanol

3.5.2 Ligation of Adapters procedure—

1. Blunt cDNA by incubating 16 μl cDNA with 20 μl 10× T4 Ligation Buffer (including ATP), 2 μl 10 mM dNTPs, 1 μl polynucleotide kinase (NEB), 1 μl Klenow (NEB), and 1.2 μl T4 DNA polymerase (NEB) for 30 minutes at 20°C.
2. Purify blunted fragments on a Qiagen PCR Purification Column and elute in 42 μl EB.
3. To purified fragments, add 3 μI Klenow 3’–5’ Exo- (NEB), 1 μl dATP, and 5 μl 10× NEB Buffer 2 (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9) for a total volume of 50 μl for 1 hour at 37°C to generate “A” overhangs.
4. Purify fragments with Qiagen Mini-Elute PCR Purification Columns and elute with 17 μl EB.
5. Ligate adapters to DNA in a reaction mixture consisting of 16 μl DNA, 20 μl 2× Quick Ligation Buffer, 2 μl 1:10 Genomic Adapter Oligo Mix, and 2 μl Quick Ligase at 23°C for 25 minutes.
6. Unused adapter oligonucleotides and dimers are removed using AMPure Beads. Add 10 μl EB to reaction sample, bringing the total volume to 50 μl.
7. Add 45 μl AMpure XP Beads to samples and mix by pipetting up and down. Incubate at room temperature for 10 minutes.
8. Place tubes in magnetic stand for 5 minutes and carefully remove and discard the supernatant without disturbing the beads.
9. Rinse the bead pellet twice with 200 μl 80% ethanol. Keep the tubes on the magnet and try not to disturb the beads.
10. After removing second ethanol wash, briefly spin down tubes and return to magnetic stand. Remove remaining liquid with a small bore pipet.
11. With lid open, incubate tubes at 37°C, 2 minutes or until cracks begin to appear on the pellet surface.
12. Add 50 μl EB directly to the pellet and resuspend well, pipetting up and down 20 times. Incubate at room temperature for 2 minutes.
13. Place on magnet for 2 minutes and save the library-containing supernatant to a new tube.

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14. Repeat steps 7–13, (using 70% ethanol in place of 80% ethanol) to remove adapters and dimers a total of two times.

3.6 PCR Amplification of cDNA libraries

Once adapter oligonucleotides are ligated onto cDNA fragments, PCR is used to amplify these fragments to generate the libraries that will be used for high throughput sequencing. PCR reactions are carried out in duplicate for each library to avoid jackpot effects in which some transcripts may become overrepresented by amplification bias. The number of PCR cycles is dependent on the initial amount of cDNA present in the sample. For example, when starting with larger initial amounts (>100 ng), fewer cycles are required, as less amplification will be needed to reach the desired amount of library. Moreover, increasing the number of PCR cycles can result in log phase amplification of genes very highly expressed in the sample and therefore leading to a bias of these samples and generating additional variation from one sample to the next. This can be observed by increased numbers of duplicated sequences in RNA-seq data sets [44], therefore potentially masking other transcripts that are present in a sample at much lower levels. Verify the DNA amount/ concentration required for a specific machine or sequencing platform to minimize amplification cycles needed. Note: we use the AMPure beads for selection of amplified products; however, if cDNA fragment size is not optimal (~200–400 bp on average), or for cost-reduction, gel extraction of cDNA fragments of the correct size range can be used [43] .

3.6.1 PCR Amplification of cDNA libraries materials and procedure—

1. DNase-free water
2. 5× Phusion Buffer HF (NEB)
3. 10 mM dNTP
4. 25 µM primer Solexa PE.1 (5'AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGC TCTTCCGA TCT)
5. 25 µM primer Solexa PE.2 (5'CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAAC CGCTCTT CCGATCT)
6. Phusion Polymerase (NEB)
7. AMPure-XP (SPRI) Beads (Agencourt)
8. EB buffer; 10 mM Tris-Cl, pH 8.5
9. 70% ethanol
10. Qubit Fluorometer and dsDNA HS Assay Kit (Invitrogen)
11. Bioanalyzer 2100 HS DNA chip (Agilent)
12. Bioanalyzer 2100 (Agilent)

3.6.2 PCR Amplification of cDNA libraries procedure—

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1. Set up PCR reactions in duplicate for each library.
2. Make PCR master mix consisting of 24 μl water, 20 μl 5× Phusion Buffer HF (NEB), 3 μl 10 mM dNTP, 1 μl each 25 μM primers Solexa PE.1 and PE.2, and 1 μl Phusion polymerase (NEB).
3. Add master mix (50 μl) to 50 μl cDNA and divide sample equally between two tubes.
4. PCR with initial denaturation at 98°C for 30 seconds followed by 16 cycles of 98°C for 10 seconds, 65°C for 30 seconds, and 72°C for 30 seconds, followed by final extension at 72°C for 5 minutes. PCR cycles may be altered to account for differences in starting RNA amounts. Fewer cycles of PCR are recommended to minimize amplification bias of highly expressed genes.
5. Add 40 μl AMpure XP Beads to each 50 μl PCR sample. Mix by pipetting up and down and incubate at room temperature for 10 minutes.
6. Place tubes in magnetic stand for 5 minutes and carefully remove and discard the supernatant without disturbing the beads.
7. Rinse the bead pellet twice with 200 μl 70% ethanol. Keep the tubes on the magnet and try not to disturb the beads.
8. After removing second ethanol wash, briefly spin down tubes and return to magnetic stand. Remove remaining liquid with a small bore pipet.
9. With lid open, incubate tubes at 37°C, 2 minutes or until cracks begin to appear on the pellet surface.
10. Add 15 μl EB directly to the pellet and resuspend well, pipetting up and down 20 times. Incubate at room temperature for 2 minutes.
11. Place on magnet for 2 minutes and save the library-containing supernatant to a new tube. For each sample, pool the two 15 μl elutions into one 30 μl sample
12. Quantify purified product using a Qubit Fluorometer and dsDNA HS Assay according to manufacturer's instructions (Invitrogen). Check specific sequencing machine or platform to determine minimal DNA amount and concentration for submission.
13. Prior to sequencing, it is usually necessary (consult your high throughput sequencing facility) to check quality of your RNA-seq library by running 2 ng of the sample on a Bioanalyzer 2100 (Agilent) to determine fragment size distribution at a mean length of 300–400 bp. Figure 1 shows an example of an RNA-seq library made from *X. laevis* stage 37 heart explants. The library contains primarily DNA averaging approximately 400 bp in size. This is consistent with the fragmentation size of RNA described in section 3.2, combined with the size of sequencing adapters. The absence of a peak at 50–100 bp indicates that purification of PCR products and removal of primers and primer-dimers was successful.

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4. Sequencing results and data analysis

In this section, we explore the output of high-throughput sequencing, initial quality assessment, and briefly summarize methodology that can be used by biologists to interpret the millions of sequence reads obtained per sample. It should be noted that this knowledge is important to have before undertaking an RNA-seq experiment, as the end-point analysis can largely depend on the methodology used to prepare and run the sequencing libraries. For example, if the goal of an RNA-seq experiment is transcriptome discovery, external RNA controls of known quantity, or spike-ins, may be crucial to determine the sensitivity of RNA-seq to identify rare transcripts in a sample [45]. Alternatively, if analyzing global differential expression among samples, it is necessary to have biological replicates to determine the variability that exists between samples. It may be beneficial to prepare and index libraries from biological replicates so they can be sequenced in bulk on a single lane of a flow cell. Securing the help of a bioinformatics specialist can be quite useful, especially prior to designing the experiment, to outline the goals of the RNA-seq experiment and plan accordingly.

4.1 Sequencing platforms

The protocol outlined here is designed for RNA-seq experiments using the Genome Analyzer II platform from Illumina. We typically use 76 cycles, or 76 base pairs (bp) per read, and single-end sequencing for differential expression studies. Note that as technologies for high-throughput sequencing advance, machines available at sequencing cores may differ greatly. Alternative platforms include the HiSeq2000 and MiSeq (Illumina), Ion Torrent (Invitrogen), and 454 (Roche). These platforms are capable of different size reads (36-, 76-, 50-, and 100-bp, etc. as well as single- or paired-end). Consult with the manufacturers to determine minimum library amount and proper adapter sequences. The choice of sequencing platform and ultimately, the size of reads needed may be dictated by the type of experiment performed, the cost to user, and platform availability.

4.2 Sequencing format and quality analysis

RNA-seq analysis can be complicated if initial read qualities are poor. Prior to sequencing a library, samples are checked for quality of fragment size and concentration to ensure that equal amounts of library are loaded for each sample (see section 3.6.2). Once sequencing is completed, it is important to document a workflow of data handling/analysis, including sequence manipulation, versions of software used to map sequences, and parameters used to determine differential expression. Many of the programs discussed here are continually being updated and therefore parameters used by the programs change frequently. Alternatively, sequence analysis can be performed using Galaxy (http://galaxyproject.org/), a publicly available website preloaded with sequence analysis software. Other commercially available software, such as Partek Genomic Suite or CLC Genomics Workbench, can also be useful to those wishing to avoid using UNIX-based analyses.

4.2.1 General quality assessment—High throughput sequence reads are reported in large text files for each lane of the flow cell. Each sequence read generated by the sequencing platform is assigned a set of quality scores used to determine the reliability of

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each nucleotide per read. Each sequence read output consists of four lines of text (fastq format, Figure 2), which will vary among sequencers and sequencing facilities. However, the general organization remains the same for a fastq file. Lines 1 and 3 contain information that uniquely identifies each cluster that has been read and the machine that performed the sequencing. Line 2 contains the actual sequence of the read and line 4 contains the quality score assigned to each corresponding nucleotide in line 2. Quality scores are presented in ASCII format, with quality value identities varying depending on the sequencing platform used. FastQC is a powerful Java-based program that can be used to load fastq files generated from high-throughput sequencing and examine various aspects of read quality.

Once fastq files are uploaded to FastQC, parameters of the sequence reads will be displayed. These include: 1) per base quality score, 2) per sequence quality score, 3) sequence length distribution, 4) duplication levels, 5) k-mer profiles (a string of distinct sequences), 6) per_base n content, 7) per base sequence content, 8) per sequence GC content, and 9) per base GC content. Each attribute is taken from the sequence data and compiled into an easy-to-view graphical format (Figure 3 for examples). Careful attention should be given to the quality scores for each base, as lower quality scores may represent erroneous base calls from the sequencing platform. Generally, scores above 20 are acceptable. Also, very high or very low GC content may represent errors in sequencing – we generally see a mean of 50% of GC content per read (Figure 3C) – while duplicated sequences may represent amplification bias or presence of adaptor-dimers formed during library preparation.

4.2.2 Filtering reads—Low-quality reads can be trimmed using a number of different methods, such as the FASTX toolkit (http://hannonlab.cshl.edu/fastx_toolkit/), Btrim, Trimmomatic, and ConDeTri [46–48]. Trimming aids in removal of ambiguous reads that may be erroneously identified as SNPs or mutations. Trimming also improves mapping of reads by reducing the number of mismatches to the reference genome or gene list. Sequences commonly overrepresented are those of sequencing adapter primer-dimers remaining in the library. These reads, if present, can easily be removed using the program TagDust [49].The tradeoff of trimming or removing reads is the generation of a reduced number or shorter reads, potentially resulting in less coverage. The choice is generally left to the investigator. If trying to align more reads, reads can be left without filtering and mapping can be altered to allow for more mismatches.

4.3 Mapping sequence reads

Once reads have been checked for quality, and filtered based on preferences determined in section 4.2, these reads are then mapped to the reference genome using programs such as Bowtie, Bowtie2, BWA, and BFAST [50–53]. Because of the file sizes involved (greater than 3–4 GB), mapping analyses can be done by using UNIX commands, for example, on the UNC Research Computing Cluster. This cluster employs a Load Sharing Facility (LSF), which manages jobs on multiple processors and computing nodes, greatly shortening the time needed to perform analysis of RNA-seq data. The LSF can also have modules (programs), including the ones discussed here, pre-installed on the system available for use. Check with your institution or company for access to LSF and for a list of programs available.

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4.3.1 Genome/gene reference lists—For sequenced and annotated genomes, a reference list of all annotated genes is usually preloaded by these alignment programs and no further preparation would be required prior to mapping. For species without annotated genomes, a fasta file consisting of gene names and their sequences can be loaded and subsequently formatted by these programs. For example, although the complete *X. laevis* genome sequence is not yet available, extensive efforts are underway to obtain full-length cDNA libraries (http://xgc.nci.nih.gov/). This allows for the compilation of cDNA sequences to which sequence reads can be mapped to obtain read counts for known genes. For example, all *X. laevis* cDNA sequences can be downloaded from XenBase [28], and annotated RefSeq sequences can be selected to restrict the analysis to high-quality sequences. As a result, 8,879 cDNA sequences (on May 1, 2011) are used to generate a reference library for mapping, reducing the redundancy of genes and partial transcripts from the larger sets of cDNA sequences available, thereby simplifying the mapping of reads to unique cDNA sequences in subsequent steps. These sequences are available at http://www.marcottelab.org/index.php/Xenopus_reference. To maximize the number of unique genes for mapping, other groups have chosen to align reads to one another to generate *de novo* transcripts [24]. This method can generate additional *X. laevis* transcripts compared to the reference list used here (Taejoon Kwon, personal communication) and be more applicable to those attempting to determine the transcriptome of a sample.

The Bowtie2 alignment program can be used to map reads back to the generated reference list. It allows alignments across gaps compared to the reference list, which is useful in the case of SNPs and incomplete cDNA sequences. The program also integrates well with other software packages used to assemble transcripts, such as Cufflinks, or detect splice junctions, such as Tophat [54]. Before Bowtie2 can be used to map reads to the reference list, it must first index the list for mapping. This can be done at the command line of the LSF as follows:

```
bowtie2-build <ref_list.fa> <base_name>
```

base_name refers to name given to indexed library and ref_list is a FASTA file that contains the entire reference library

4.3.2 Obtaining gene counts—Once a reference library is indexed, Bowtie2 will map reads to this indexed library and report the reads in a Sequence Alignment/Map (.sam) file. The default Bowtie2 usage for this is:

```
bowtie2 -x index_file –U RNA-seq.fastq -S aligned.sam
```

-x refers to reference list that was indexed, -U refers to file name of sequence reads, -S instructs bowtie2 to output data in SAM format with the indicated name.

To determine the number of reads mapping to each gene, .sam files must first be converted to smaller binary mapping (.bam) files, sorted, then indexed by gene in order to be tabulated to give gene counts using the following commands in Samtools [55]:

```
samtools view -bt ref_list.txt -o aligned.bam aligned.sam

samtools sort aligned.bam aligned.sorted

samtools index aligned.sorted.bam
```

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```
samtools idxstats aligned.sorted.bam
```

The resultant output file generated after these commands are executed will be presented in tab-delimited format with gene names and number of reads mapped to each gene reported.

4.3.3. Alternative alignment parameters—Bowtie2 default parameters assign reads to a single gene in the reference. If a read maps to more than one location in the genome, and the alignment among these regions of the genome is identical, the read is randomly assigned to one of the regions. These parameters can be modified to report all alignments, rather than a single random assignment, or to report user-specified number of alignments. These options should be considered based on the application of RNA-seq experiments. For example, in a species with duplicated genomes, most reads will map to at least two genes.

The *X. laevis* genome underwent tetraploidization 40 million years ago [27], resulting in duplication of many genes. Gene duplication followed by functional divergence is one mechanism underlying evolution of novel gene function [56]. At least one example of this has been demonstrated in *X. laevis*, in that alloalleles from two unique, independent loci of *hairy2* exhibit different expression patterns and functional requirements [57]. This poses a minor challenge in mapping because duplicated *Xenopus* genes are quite similar in sequence. Known duplicated genes are present in the cDNA reference library used for mapping. Under default mapping parameters in Bowtie2, reads that map to more than one gene are randomly assigned to one of these genes. These reads are reported in the output file from mapping.

It is unclear what the general contributions of alloalleles of duplicated genes in the *Xenopus* genome are during development. In the reference list described in section 4.3.1, there are duplicated genes present, corresponding to known alloalleles previously characterized. To determine if any alloallele-specific biases occur in a sample, i.e. one alloallele is more prevalent than the other but is masked by the random mapping, uniquely mapped reads from the .sam files can be filtered out from the Bowtie2 alignment output. This allows one to determine the number of reads that are alloallele-specific in the sample by comparing to mapping of all reads that mapped to the alloallele, including reads that also map to other genes in the reference list. This can be done by selecting all reads that have an “AS” header, but not an “XS” header in the .sam file using the UNIX commands and the bowtie2 output file from section 4.3.2:

```
grep “AS:” aligned.sam | grep -v “XS:” > unique_alignments.sam
```

The new file generated here can be processed using Samtools as described in section 4.3.2 to generate gene counts for uniquely mapped reads. These can then be directly compared to the gene counts of all mapped reads from the same sample.

Here, we provide an example of selecting specific parameters to analyze RNA-seq data from stage 37 *X. laevis* wild-type hearts. The results are summarized in Table 1 and show genes known to be expressed in the heart and to have characterized alloalleles: *bmp2; gata4, gata5, gata6; nkx2–5, foxc1*, and *myl3* [28, 58–62]. We compared the relative amounts of alloalleles present in the sample using all mapped reads (initial bowtie2 output file) or uniquely mapped reads (filtered bowtie2 output file). Using these criteria, we see slight

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differences in these ratios for some genes. However, the ratios still indicate whether one alloallele is more abundant than the other in the sequencing sample (Table 1). Similar studies could further elucidate the potential divergent expression of alloalleles in different tissues, or duplicate genes in other organisms.

4.4 Differential expression analysis

The end goal of an RNA-seq for many developmental biologists is in most cases to identify and quantify differential gene expression between two or more samples. In this section, we describe the methodology used to identify differential expression and identify putative pathways or cellular functions. Although proper statistical methods can be used to identify classes of differential expression, it is important to also use alternative methods of validation. We briefly discuss such methods here.

4.4.1 Normalization methods and statistical analysis—Biological replicates allow the researcher to use correlation between experiments and conduct statistical analysis to determine the significance of differential expression [63, 64]. To properly determine differential expression between samples, normalization must be performed to account for unequal sampling (unequal number of reads among samples). There are a number of methods used for normalization of read counts between samples. These methods have been developed in the absence of truly invariant genes among these different sample types. Spike-in controls of known quantities can be used to mimic the invariant genes and properly compare two or more samples. Alternatively, if spike-in controls are not used, the trimmed mean of the M value (TMM) and an upper-quartile normalization method (RPKM) are popular methods to normalize RNA-seq reads across samples [65, 66].

A number of statistical programs have been designed for R that can be used to analyze differential expression. These include edgeR, DEGseq and DESeq [67–69].

4.4.2 Gene ontology (GO) analysis—When performing differential expression studies, it is often desirable to examine the global differences between two samples. Once a list of differentially expressed genes is determined, this list can be loaded, along with a list of all genes examined for differential expression, into gene ontology software. For example, the program GOrilla identifies GO terms overrepresented in the differentially expressed genes compared to the total number of genes tested [70]. GOrilla examines GO term enrichment in three aspects: cellular process, function and component.

4.4.3 Verification of differential expression—Proper statistical methods and additional biological replicates may limit the number of genes falsely identified as differentially expressed in an RNA-seq experiment, but additional validation is often necessary to eliminate the possibility of improper conclusions. One manner in which RNA-seq results can be validated is by performing quantitative reverse transcription-PCR (qRT-PCR) [71, 72]. PCR primers can be designed against the differential expressed genes and results can be validated on a gene-by-gene basis.

In addition to examining levels of differential gene expression, other methods of validation provide visual verification of RNA-seq results. Standard protocols for *in situ* hybridization

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and antibody staining exist in *Xenopus* that allow researchers to examine spatial-temporal expression of genes and their proteins [73, 74]. This information can be crucial to understanding differential expression. For example, organs such as the heart are comprised of different cell types. RNA-seq or qRT-PCR may identify transcripts present in the heart, but not other organs. Examining spatial expression could give further resolution as to the transcript localization to specific chambers or cell types, as shown recently with differentially expressed genes in *tcf21*-depleted embryos [75]. This information, together with quantification, will help elucidate gene networks that lead to proper specification and differentiation of the multitude of cell types generated during development.

4.5 Additional RNA-seq applications

The amount of information obtained from an RNA-seq experiment greatly exceeds the uses of the microarray. In addition to differential expression analyses and studies of gene duplication, RNA-seq data can be applied to examine transcript structure and evolution as well as building gene models.

An additional advantage of RNA-seq is the ability to directly test the efficacy of a MO. For example, we have previously shown that *casz1* morpholinos (MOs) efficiently block proper splicing of *casz1* intron 8, thereby introducing extraneous sequence, and causing a frameshift in the rest of the open reading frame and a premature stop in translation [76]. This can be seen in the results of RT-PCR performed on mRNA derived from hearts dissected from *casz1* MO embryos (Figure 4A). To examine these effects with RNAseq data, genomic sequence must be used for the reference sequence for mapping. We sequenced the entirety of *casz1* intron 8 from the RT-PCR product and included this sequence between exons 8 and 9 in a .fasta file consisting of the *casz1* coding sequence. For simplicity, this sequence was used as the reference file for mapping RNA-seq reads from *wild-type* (WT) and *casz1* MO hearts. Subsequently, after we sequenced this intron, the draft of the *X. laevis* genome was made publicly available on Xenbase. Therefore, genomic sequences containing intronic sequences can be extracted from Xenbase to generate .fasta format reference sequences for the gene(s) of interest. To determine the effects of splice-blocking by MO using RNA-seq, we use the program Tophat (version 2.0.5) [77]:

```
tophat -o /WThea xCasz1_w_intron8.fa /WT_read_file.txt

tophat -o /MOhea xCasz1_w_intron8.fa /MO_read_file.txt
```

The “-o” option generates an output folder to where the Tophat results are placed.

Tophat integrates Bowtie2 alignments of RNA-seq reads to the reference genome while also giving information of splice junctions within transcripts. In the output folder for the Tophat alignments, these junctions are represented in .bed files, which can be viewed, together with the individual reads that map to the reference sequence (.bam files that are generated by Tophat using Bowtie2 and Samtools), using IGV viewer [78] or uploaded to a genome browser (when the genome has been annotated).

As shown in Figure 4B, we were able to identify ten reads in wild-type (WT) hearts in which intron 8 had been spliced out, and only a single read that mapped to intron 8. In

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contrast, *casz1* MO hearts had no reads with intron 8 spliced out, but over 100 reads that mapped to intron 8, suggesting that proper splicing was not occurring in MO hearts. Moreover, aberrant splicing was observed downstream of intron 8 in the MO heart (Figure 4B), presumably a secondary result of the first splice-blocking events. Taken together, these results suggest that RNA-seq can be used to not only determine MO efficiency, but also the full consequences of MOs on RNA splicing.

Another option for RNA-seq users is to assemble transcripts *de novo*. This is especially useful for organisms that have neither a sequenced genome nor an extensive list of ESTs like the one used to generate the *Xenopus* reference list described here. The program Cufflinks accepts RNA-seq reads and assembles them into a parsimonious set of transcripts [79]. Moreover, Tophat and Cufflinks results can be integrated with statistical software such as CummeRbund to examine differential expression from this newly assembled set of transcripts [54].

Finally, RNA-seq is becoming a popular method in single nucleotide polymorphism (SNP) detection. The abundance of reads obtained in an RNA-seq experiment, together with the quality scores obtained for each base pair in these reads (see section 4.2) allow for the rapid detection of SNPs. Detection of SNPs in transcripts will help identify conserved and rapidly evolving portions of genes and their protein products, giving insight to the structure and function of these proteins. SNPs can be detected using the publicly available program Maq [80], or commercially available programs such as Partek Genomic Suite or CLC Genomics Workbench.

5. Concluding remarks

The advances made in sequencing technology during the last decade have revolutionized our ability to construct gene expression profiles. Here we have described methodology covering new ways to probe the transcriptomes of the model organisms of choice, regardless of genome annotation. We have provided avenues to study differential gene expression and evolution of gene families as discussed with gene duplication, splicing and SNPs. These methods will accelerate the progress being made in understanding gene networks required for proper development, without restricting researchers to organisms with sequenced genomes.

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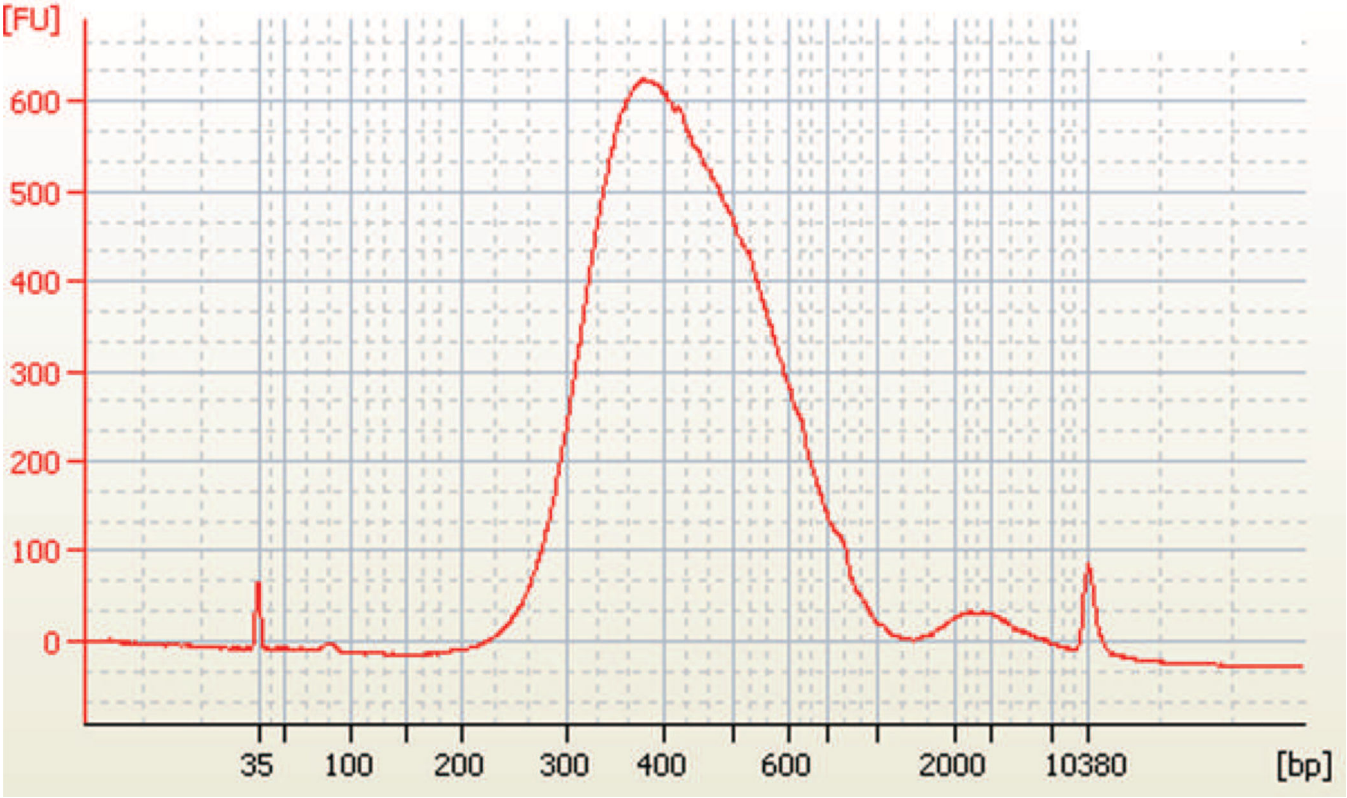

Figure 1. Fragment analysis of RNA-seq libraries

Bioanalyzer trace files for final RNA-seq libraries prepared from stage 37 *X. laevis* hearts. Graph represents DNA content with amount on *y*-axis measured by fluorescent unit (FU) and fragment size on the *x*-axis. Two peaks at 35 and 10380 base pair (bp) correspond to DNA standards used. Note the abundance of DNA in the 300–600 bp range.

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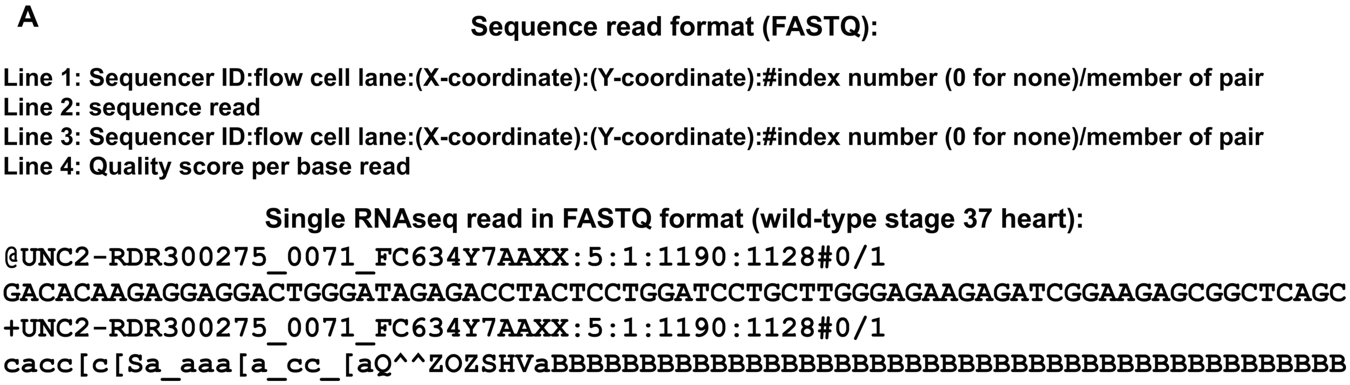

A

Sequence read format (FASTQ):

Line 1: Sequencer ID:flow cell lane:(X-coordinate):(Y-coordinate):#index number (0 for none)/member of pair
Line 2: sequence read
Line 3: Sequencer ID:flow cell lane:(X-coordinate):(Y-coordinate):#index number (0 for none)/member of pair
Line 4: Quality score per base read

Single RNAseq read in FASTQ format (wild-type stage 37 heart):

```
@UNC2-RDR300275_0071_FC634Y7AAXX:5:1:1190:1128#0/1
GACACAAGAGGAGGACTGGGATAGAGACCTACTCCTGGATCCTGCTTGGGAGAAGAGATCGGAAGAGCGGCTCAGC
+UNC2-RDR300275_0071_FC634Y7AAXX:5:1:1190:1128#0/1
cacc[c[Sa_aaa[a_cc_[aQ^^ZOZSHVaBBBBBBBBBBBBBBBBBBBBBBBBBBBBBBBBBBBBBBBBBBBBB
```

Figure 2. RNA-seq data interpretation and quality analysis

Overview of fastq file format and example of a single read output for wild type heart sample.

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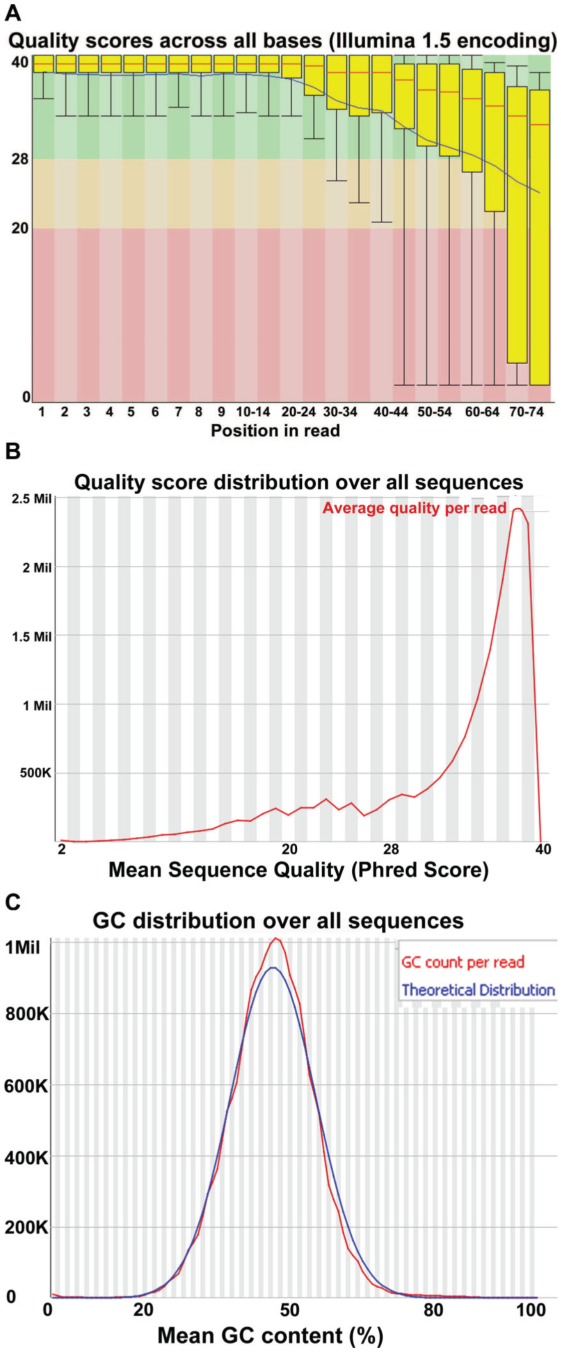

Figure 3. Output from fastqc software for RNA-seq data quality analysis

(A) Quality score per base position for reads. *x*-axis represents base pair position and *y*-axis represents interquartile range of quality value (from 0 to 40). Note the overall reduction in quality of scores toward the end of the 76 bp reads. (B) Graphical summary of quality scores shown in (A). (C) G/C distribution over all sequence reads represented on *x*-axis, with total reads corresponding on *y*-axis. Overall distribution (red line) versus the theoretical distribution in sample (blue line).

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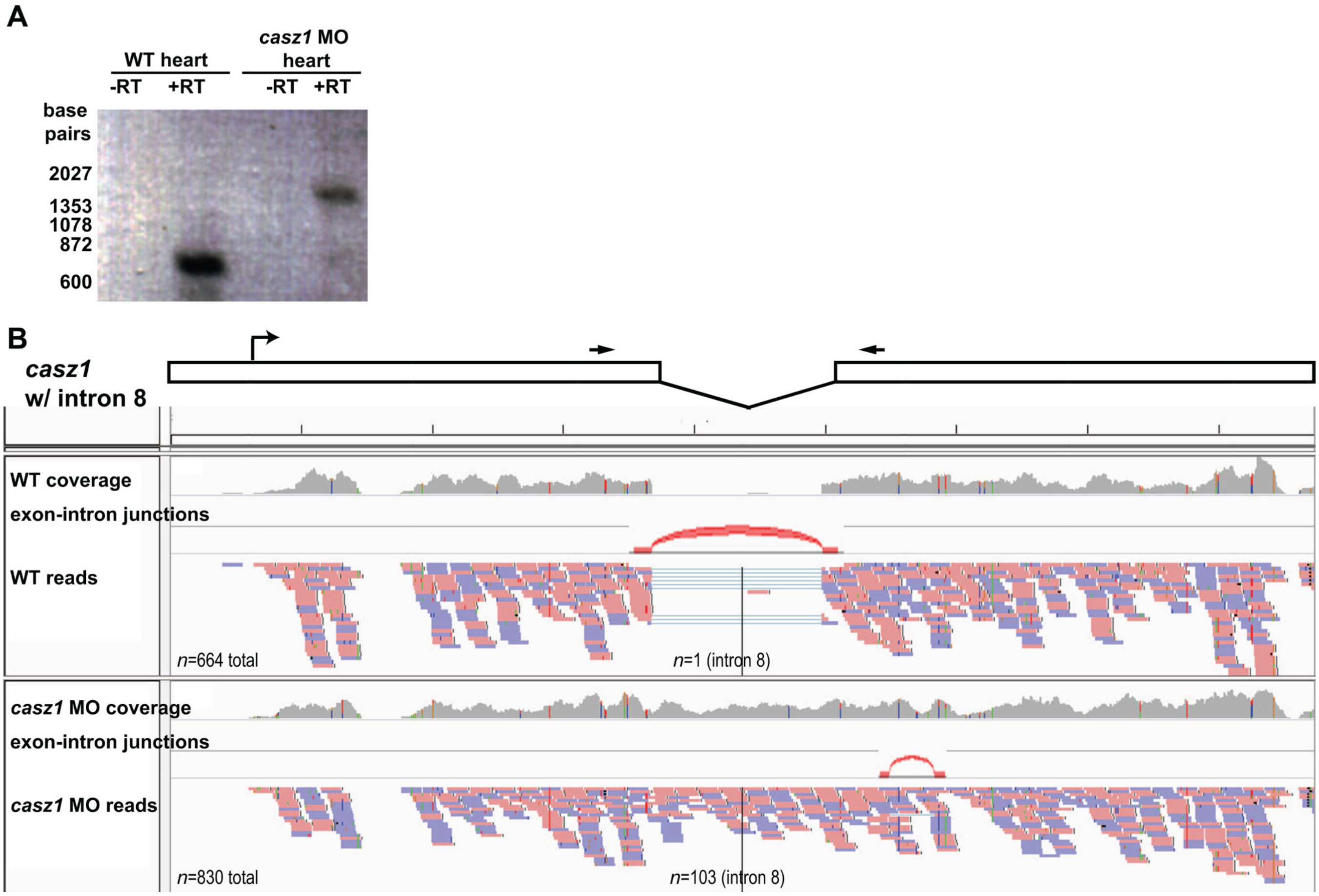

Figure 4. Determining morpholino (MO) efficacy by RNA-seq

(A) RT-PCR of cDNA generated from wild type and *casz1* MO hearts shows aberrant splicing of *casz1* intron 8 in MO-treated embryos. (B) Modified IGV viewer screenshot with schematic of *casz1* mRNA (boxes) with inclusion of intron 8. Arrows indicate primers used for PCR shown in (A). Overall read coverage and individual reads mapping to *casz1* mRNA in wild type and *casz1* MO hearts are shown below schematic. Exon-intron boundaries predicted by the program Tophat based on read coverage are displayed on genome browser.

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Table 1

Read counts and ratios of alloallele-specific reads from RNAseq of *X. laevis* hearts.

"A" alloallele	gene length (bp)	total reads mapping	unique reads mapping	"B" alloallele	gene length	total reads mapping	unique reads mapping	A/B ratio (total)	A/B ratio (unique)
bmp2|NM_001085884	2630	1281	740	bmp2|NM_001101666	1992	159	68	8.06	10.88
foxc1|NM_001088214	2400	992	376	foxc1|NM_001096377	2378	736	279	1.35	1.35
gata4|NM_001090629	1626	1219	568	gata4|NM_001091886	1639	1770	942	0.69	0.60
gata5|NM_001086362	2095	2764	1912	gata5|NM_001088493	2050	3823	2677	0.72	0.71
gata6|NM_001087983	4183	4555	3447	gata6|NM_001090256	3720	4821	3237	0.94	1.06
myl3|NM_001089148	1189	3383	1756	myl3|NM_001089150	855	15106	7763	0.22	0.23
nkx2–5|NM_001086721	2506	6175	4568	nkx2–5|NM_001172192	903	1115	280	5.54	16.31