5

Constructing and Screening a cDNA Library

Methods for Identification and Characterization of Novel Genes Expressed Under Conditions of Environmental Stress

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Summary

Many organisms provide excellent models for studying disease states or for exploring the molecular adaptations that allow cells and organisms to cope with or survive different stresses. The construction of a cDNA library and subsequent screening for genes of interest allows researchers to select for genes that are likely to play key roles in the regulation or response to the condition or stress of interest, those that may not be expressed (or exist) in other systems. Determination of the open reading frame(s) of novel genes, and extensive analysis of the proteins they encode, can open up new avenues of research and promote intelligent design of downstream projects.

Key Words: Bioinformatics; cDNA library; differential screen; expression analysis; functional genomics; gene characterization; novel genes; stress induction; up-regulated.

1. Introduction

A majority of coding genes that make up an animal's genome are under selective pressure, such that there is little room for evolving function due to the importance of the wild type gene. However, in some cases, genes that are dormant (in selected regions of heterochromatin) or genes that may have arisen as a result of whole or partial gene duplication (with switching of selected exons and/or introns), may confer a selective advantage to organisms living in a certain environment or exposed to certain conditions. Extra copies of

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a gene or "spliced copies" may become activated (or mutated) to code for new proteins, in effect supplying "novel" genes to the existing suite of genes present in an organism. Although a number of genome projects are complete and numerous others are ongoing, a wide variety of fascinating organisms exist for which only selected genes have been identified ***(1,2)***. By examining the response and regulation of gene expression in systems that not only survive, but, in many cases thrive under adverse conditions, insight can be gained into the prevention and potential treatment of these types of systemic stresses. The end goal is to determine the function of such genes. Advances in the field of functional genomics is helping to address this goal and advances in bioinformatics, combined with the availability of large data sets for well-annotated genes, has provided a basis for the generation of software and analysis programs that allow researchers to examine nucleotide and protein sequences using established "rules" and algorithms. Much information can be gleaned from the nucleotide sequence itself, as well as the primary amino acid sequence of the translated gene, such that informed hypotheses can be constructed and tested prior to initiating larger downstream projects. Such preliminary analysis sets the stage for further experiments, such as the cloning and expression of open reading frames, examination of the upstream promoter, construction of mutated constructs that affect gene expression, and possibly even the creation of a knock-out animal to examine the *in vivo* effects of the loss of select genes.

The first step in this process is the identification of differentially expressed genes for the condition of interest. This chapter outlines the basic construction of a cDNA library from a select population of mRNA, including methods for mRNA/cDNA preparation and cloning, subsequent screening for differentially expressed genes, and downstream confirmation via Northern blotting. Methods for analysis of select sequences are also outlined using the previously unreported novel gene, *sarp-2*, which is included as an example. *Sarp-2* is one of several novel clones ***(3,4)*** isolated from a cDNA library constructed with mRNA prepared from hepatopancreas of the marine snail, *Littorina littorea*; the gene is induced by anoxic exposure. Identification and characterization of this novel gene was performed as outlined in this chapter and the results are reported in the accompanying figures.

2. Materials

All chemicals used are of molecular biology grade or their equivalent and of the highest purity. All plastic and glassware, including bottles and pipet tips, are autoclaved and gloves must be worn at all times during operations involving nucleic acid manipulation.

2.1. Total RNA Isolation

1. RNase-free water. Add 1 mL of diethyl pyrocarbonate (DEPC) (Sigma-Aldrich) to 1 L of water (0.1% v/v), stir overnight (>12 h), autoclave. This destroys any RNases present and this water will be used to make up solutions in this section and to dissolve RNA samples.
2. Trizol reagent (Invitrogen).
3. Chloroform (Fisher Scientific).
4. Isopropyl alcohol (Fisher Scientific).
5. 70% Ethanol. Add 30 mL of DEPC-treated water to 70 mL of ethyl alcohol (200 proof; Pharmco).
6. Oligo(dT) cellulose (New England BioLabs). Dry oligo(dT) cellulose is combined with 0.1 *M* NaOH to create a slurry and poured into a sterile column or 1-mL syringe plugged with sterile cotton or glass wool. The column is equilibrated with loading buffer prior to adding sample.
7. Loading buffer: 1 *M* NaCl, 2 m*M* phosphate buffer, pH 7.2.
8. Middle wash buffer: loading buffer + 0.3 *M* NaCl.
9. Elution buffer: 10 m*M* Tris-HCl pH 7.2–7.4, 1 m*M* EDTA.
10. 3 *M* sodium acetate, pH 5.2.
11. Ethanol (200 proof; Pharmco).

2.2. cDNA Library Synthesis

1. Cloning vector for cDNA library synthesis. Many vectors exist, often designed for a specific application. Researchers should examine the features of those available and decide on a vector appropriate for their application. Some common vectors used in cDNA library synthesis include: Uni-ZAP XR (Stratagene), plTriplEx2 (Clontech), pSPORT1 (Invitrogen), lSCREEN-1 (Novagen/Merck). The Uni-ZAP XR cloning vector will be used for illustrative purposes in this chapter.
2. 1 µg of column purified mRNA.
3. Oligonucleotide linker-primer containing a poly (dT) region. (ex. 5′-NNNNN NNNCTCGAGdT(15)-3′).
4. RNasin (20 U/µL; Promega).
5. AMV reverse transcriptase (10 U/µL) with 10× reverse transcriptase buffer (Promega).
6. Nucleotides (dATP, dGTP, dTTP; 10 m*M* each dNTP)
7. 5-Methyl cytosine analog (*dCTP; 5 m*M*).
8. Ribonuclease (RNase) H (5 U/µL; New England BioLabs).
9. DNA polymerase I (*E. coli*, 10 U/µL) and 10× DNA polymerase I buffer (New England BioLabs).
10. *Eco* RI adapters. An *Eco*RI adaptor can be purchased commercially or constructed using two primers of different lengths, which can be hybridized to form a duplex with a 5′ phosphate on the blunt end. Include an overhang of 5′-AATTCNNNN-3′ on one primer to create an *Eco*RI restriction site. The opposite end of the duplex (the blunt end of the adaptor) can be ligated to the ends of any cDNA containing

a 5′ phosphate group. The adapters should be diluted in sterile ddH_2O to a final concentration of 5 µg/µL.
11. 50× TAE buffer: 2 *M* Tris-acetate (Tris + acetic acid) pH 8.5, 100 m*M* EDTA.
12. 1% TAE agarose gel: 1× TAE buffer, 1% agarose (1 g/100 mL), ethidium bromide (1 µg/mL).
13. 100 m*M* EDTA.
14. T_4 DNA ligase (400 U/µL) and 10× T_4 DNA ligase buffer (New England BioLabs).
15. T_4 polynucleotide kinase (10 U/µL) and 10× T_4 polynucleotide kinase buffer (New England BioLabs).
16. TSM buffer: 50 m*M* Tris-HCl, pH 7.5, 100 m*M* NaCl, 8 m*M* $MgSO_4$, 0.01% (w/v) gelatin.
17. XL1-Blue (Stratagene).
18. LB-ampicillin broth: 10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, pH 7.5. Dilute up to 1 L with ddH_2O and autoclave. Broth is supplemented with 0.2% (w/v) maltose and ampicillin (100 µg/mL final concentration) after broth has cooled to room temperature.
19. NZY agar: 5 g of NaCl, 2 g of $MgSO_4 7H_2O$, 5 g of yeast extract, 10 g of peptone, 15 g of agar, pH 7.5. Dilute to 1 L with ddH_2O and autoclave. Pour plates when agar has cooled to approx. 50°C and store at 4°C until used. The agar can also be stored at 4°C and remelted in a microwave just before use.
20. NZY top agar**:** 5 g of NaCl, 2 g of $MgSO_4 7H_2O$, 5 g of yeast extract, 1 g of peptone/L, 0.7% w/v agarose, pH 7.5. Dilute up to 1 L with ddH_2O and autoclave. The top agar is stored at 4°C and remelted in a microwave just before use. The agar must be cooled to <45°C before addition of bacteria.
21. *Pfu* polymerase (2.5 U/µL) (Stratagene).

2.3. Amplification of the cDNA Library

1. NZY plates (24 × 24 cm), LB-ampicillin broth, XL1-Blue cells, NZY agar, NZY top agar (see **Subheading 2.2., items 19** and **20**).
2. TSM buffer (see **Subheading 2.2., item 16**).
3. Chloroform (Fisher Scientific).
4. Dimethyl sulfoxide (DMSO; Sigma-Aldrich).

2.4. Differential Screening of the cDNA Library

1. NZY plates (24 × 24 cm; *see* **Subheading 2.2., items 19** and **20**).
2. Hybond-N 0.45-µm nylon membranes (GE Healthcare).
3. 20× SSC: 3 *M* NaCl, 0.3 *M* Na-citrate (for 1 L: 175 g of NaCl, 88 g of sodium citrate); dilute as necessary with ddH_2O.
4. Denaturation buffer: 1.5 *M* NaCl, 0.5 *M* NaOH.
5. Neutralization buffer: 1.5 *M* NaCl, 0.5 *M* Tris-HCl, pH 8.0.
6. Rinse buffer: 0.2 *M* Tris-HCl, pH 8.0, 2× SSC.
7. Whatman filter paper or chromatography paper (Fisher Scientific).
8. dNTPs: dATP/dTTP/dGTP (10 m*M* each dNTP).

9. Anchored Oligo(dT) primer: (5′-dT(15)(A/G/C)-3′). Can be purchased commercially (Invitrogen; New England BioLabs).
10. RNasin (20 U/μL; Promega).
11. Dithiothreitol (DTT) (Sigma-Aldrich). Make up a 0.1 *M* stock with sterile ddH_2O.
12. AMV reverse transcriptase (10 U/μL) with 10× reverse transcriptase buffer (Promega).
13. [α-^{32}P]dCTP (3000 Ci/mol; GE Healthcare).
14. RNase A (60 mg/mL in ddH_2O; Sigma-Aldrich).
15. Quick Spin columns (TE; Sephadex G-25, Fine) for radiolabeled DNA purification (Roche).
16. 1× $T_{10}E1$ buffer: 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA.
17. Hybridization buffer. Modified Church's Buffer: 0.25 *M* Na_2HPO_4, 0.25 *M* NaH_2PO_4 (pH 7.5), 1 m*M* EDTA, 7% sodium dodecyl sulfate (SDS; w/v); or buffer can be purchased commercially: Ultrahybe (Ambion).
18. Membrane washing buffer: 0.1× SSC, 0.1% SDS (w/v).
19. Membrane rinsing buffer: 0.1× SSC.
20. X-ray film (Kodak).
21. TSM buffer (*see* **Subheading 2.2, item 16**).
22. Chloroform (Fisher Scientific).
23. XL1-Blue culture.
24. NZY top agar (*see* **Subheading 2.2, items 19** and **20**).

2.5. In Vivo Excision of Clones

1. XL1-Blue cells.
2. 10 m*M* $MgSO_4$.
3. ExAssist helper phage.
4. Uni-ZAP XR phage stock.
5. LB-ampicillin broth (*see* **Subheading 2.2, item 18**).
6. SOLR cells.
7. LB-ampicillin agar plates: Agar (15 g) is added to LB broth (1 L) and autoclaved. Cool to <50°C, add ampicillin (100 μg/mL), and pour into 10-cm plates at desired thickness.

2.6. Miniprep Plasmid Isolation

1. LB-ampicillin broth (*see* **Subheading 2.2, item 18**).
2. Prelysis buffer: 50 m*M* glucose, 25 m*M* Tris-HCl pH 8.0, 10 m*M* EDTA.
3. RNase A (*see* **Subheading 2.4, item 14**).
4. Alkaline lysis buffer: 0.2 *N* NaOH, 1% SDS. This buffer must be made fresh just prior to use.
5. Neutralization buffer: 5 *M* potassium acetate, 30% acetic acid, 50 m*M* glucose, 25 m*M* Tris-HCl, pH 8, 10 m*M* EDTA.
6. Isopropanol (Fisher Scientific).
7. 70% Ethanol (*see* **Subheading 2.1, item 5**).

2.7. Insert Isolation

1. Restriction enzymes: *Eco*RI and *Xho*I (New England Biolabs); these may be adjusted depending on the adaptors ligated.
2. 10× restriction enzyme buffer. Buffer will depend on restriction enzymes being used and is supplied with the enzyme.
3. DNA loading dye: 0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol FF, 50% v/v glycerol.
4. 1% TAE agarose gel and 50× TAE buffer (*see* **Subheading 2.2, item 11 and 12).**
5. DNA ladder (Invitrogen). A ladder should be chosen based on the size of the clones expected (ranging from 100 bp up to several kilobases).
6. Filtered pipette tips (1 mL).
7. Sterile cotton or glass wool.

2.8. Synthesis of Labeled Probe

1. dNTPs without dCTP (dATP/dTTP/dGTP; 5 m*M* each).
2. Random primer (100 m*M* $d(N)_6$; New England BioLabs).
3. 10× random primer buffer: 0.5 *M* Tris-HCl, pH 7.6, 20 m*M* DTT, 50 m*M* $MgCl_2$, 0.4 *M* KCl.
4. Klenow fragment of DNA polymerase I (5 U/μL; New England BioLabs).
5. [α-^{32}P]dCTP (3000 Ci/mol; GE Healthcare).
6. Quick Spin columns (TE; Sephadex G-25, Fine) for radiolabeled DNA purification (Roche).

2.9. Northern Blotting

1. 10× MOPS buffer: 200 m*M* 3-(*N*-Morpholino)propanesulfonic acid (MOPS), 50 m*M* sodium acetate, 10 m*M* EDTA, pH 7.
2. 1.25% Agarose formaldehyde denaturing gel. Melt 3.75 g of agarose in 217 mL of ddH_2O containing ethidium bromide (1 μg/mL) in a sterile flask. Place solution in an incubator set to 60°C. Into a separate sterile flask, add 30 mL of MOPS 10× buffer and 53 mL of formaldehyde 37% (v/v) and place this solution at 60°C. Once both solutions have equilibrated to 60°C, combine the contents of both flasks together, gently swirl without introducing bubbles, and pour into large gel tray to desired thickness.
3. RNA sample buffer: 1× MOPS buffer, 2.2 *M* formaldehyde, 50% (v/v) formamide.
4. RNA loading buffer 6× stock: 1× MOPS buffer, 50% (v/v) formamide, 40% (v/v) glycerol. Add a few flakes of bromophenol blue and xylene cyanol as tracking dyes.
5. Hybond 0.45-μm nylon membrane (GE Healthcare).
6. 20× SSC (*see* **Subheading 2.4, item 3**). Dilute as necessary with ddH_2O.
7. Whatman filter paper or chromatography paper (Fisher Scientific).
8. Paper towels.
9. Flat piece of Plexiglas or sequencing gel plate.
10. Solid weight.

11. Glass or plastic pipet (5- or 10-mL) for smoothing the gel/membrane/filter paper and removing air bubbles from transfer set-up.
12. Plastic wrap.
13. Northern blot fixing solution: 0.05 *N* NaOH.

2.10. Probing Northern Blots

1. Hybridization solution: Ultrahybe Hybridization buffer (Ambion) or modified Church's buffer (*see* **Subheading 2.4. item 17**).
2. Radiolabeled probe (prepared in **Subheading 2.8**).
3. Northern blot washing solution: 0.1× SSC, 0.1% SDS in sterile ddH_2O.
4. X-ray film or phosphoimager screen.
5. Northern blot stripping solution: boil a solution of ddH_2O and 0.5% SDS and immerse blots to be stripped. Repeat as necessary until radioactive label (cpm) detected using a handheld Geiger counter is at or below background levels.

3. Methods

Commercial products have been designed for specific types of experiments (high vs. low copy number genes, etc.) that examine and identify differentially expressed genes. Although the technology changes rapidly, cDNA library construction using mRNA isolated from a tissue/organ/organism of interest remains the standard method for identifying new and novel genes and proteins. Synthesis of such a library can be carried out using a wide variety of available products; a common standard method for library construction, screening and positive clone analysis is outlined in this chapter. These instructions assume the use of the Uni-Zap-cDNA synthesis kit (Stratagene; *see* **ref. *5***), but can be adjusted for the use of other vectors. Some common phagemid cloning vectors available include, but are not limited to: plTriplEx2 (Clontech), pSPORT1 (Invitrogen), and lSCREEN-1 (Novagen).

Following construction of the library, differential screening will identify selected clones that are induced (or repressed) in the condition being tested. In the example provided here, we follow the steps used to find and characterize the novel gene *sarp-2*, which is induced in the hepatopancreas of *Littorina littorea* in response to anoxia exposure. This up-regulation is confirmed with Northern blotting. Following sequencing of the *sarp-2* clone, sequence analysis can be performed using the applications outlined in **Subheading 3.11**.

3.1. Total RNA Isolation

1. A number of kits are available today for total and mRNA isolation (Ambion, Roche, Qiagen, Invitrogen, Novagen, etc.). These kits often provide high quality, pure RNA, but are relatively expensive. A traditional method for RNA isolation is outlined in this section.

2. Homogenize tissue samples in Trizol reagent (1 mL per 100 mg of tissue) and add chloroform (0.2 mL/mL of reagent). Invert samples repeatedly (15 s), incubate at room temperature (2–3 min), and centrifuge at 12,000*g* at 4°C for 15 min.
3. Remove the aqueous layer and transfer to a sterile Eppendorf tube. Add an equal volume of isopropanol to precipitate the RNA. Incubate samples at room temperature for 10 min.
4. Pellet the RNA by centrifuging the samples at maximum speed in a microcentrifuge for 10 min at 4°C. Aspirate the supernatant and wash the pellet with 70% ethanol (250 µL). Recentrifuge the RNA at maximum speed for 5 min. Aspirate the ethanol and air-dry the pellet for 10 min on the lab bench. Dissolve the RNA in an appropriate amount of DEPC-treated water (assuming you get 10 µg for every 10 mg of tissue) and store at 4°C (if it will be processed within a week) or at –20°C (long-term storage).
5. Determine RNA concentration and purity spectrophotometrically by measuring absorbance at 260 and 280 nm. For RNA of acceptable quality, the A_{260}/A_{280} ratio should fall between 1.6 and 2.0. (*See* **Note 1.**)
6. Purify the poly(A)$^+$ mRNA from total RNA samples using oligo(dT) cellulose. chromatography. Heat total RNA to 65°C, mix with loading buffer (1:1 v/v), and load onto an oligo(dT) cellulose column.
7. Wash the column with loading buffer (1 bed volume) and collect the eluate in a sterile Eppendorf tube. Load the recovered eluate onto the column, collect it as it elutes, then reload onto the column (repeat twice).
8. Wash the column with loading buffer (1 bed volume), followed by middle wash buffer (1 bed volume), and finally with elution buffer (1.5 bed volume). This last step elutes the poly(A)$^+$ mRNA, to which 0.1 volumes of 3 *M* sodium acetate, pH 5.2, and 2.5 volumes of absolute ethanol are added. The poly(A)$^+$ mRNA is placed at –20°C and left to precipitate overnight. The protocol can be stopped here.
9. The following day, centrifuge the tubes at 13,000*g* for 45 min at 4°C. Discard the supernatant and wash the pellet with 70% ethanol (250 µL) and centrifuge at the same speed for 10 min. The resulting pellet consists of mRNA, which is air-dried and resuspended in DEPC-ddH_2O (*see* **Note 2**). If the mRNA is not going to be used immediately, store at –80°C until further use.

3.2. cDNA Library Synthesis

1. Combine mRNA (5 µg; <30 µL) with the oligonucleotide linker-primer (3 µg) and RNasin (5 µL) in an RNase-free Eppendorf tube. Synthesize a complementary first strand of cDNA, using the mRNA as template, with the addition of dNTPs (3 µL), 5-methyl-dCTP (3 µL), 10× reverse transcriptase buffer (5 µL), and adjusting the total volume up to 45 µL. Mix the contents gently and allow primer and template to anneal at room temperature for 10 min.
2. Add reverse transcriptase to the reaction tube (5 µL; 50 U) to bring the final volume up to 50 µL. Gently mix the contents of the tube, briefly centrifuge to collect contents at the bottom of the tube, and then place at 42°C for 1 h. (*See* **Note 3.**)

3. Second-strand cDNA synthesis (complementary to the first strand) is carried out in the same tube. The following components are added to the first strand reaction: dNTPs (6 µL), 10× DNA polymerase I buffer (20 µL), [α-^{32}P]dCTP (1 µL), RNase H (1 µL; 5 U), DNA polymerase I (10 µL; 100 U). Adjust the final reaction volume to 200 µL with sterile ddH_2O and incubate for 2 h at 16°C.
4. Place the newly double-stranded cDNA reaction on ice and blunt-end the termini by adding 22.5 µL of dNTPs (A/G/C/T) and *Pfu* DNA polymerase (2.5 µL) into the tube and incubate at 72°C for 30 min.
5. Add an equal volume of phenol–chloroform (1:1 v:v, pH 7.4) and vortex-mix the tube briefly. Centrifuge the sample at maximum speed in a desktop centrifuge for 10 min at room temperature. Transfer the aqueous layer to a fresh Eppendorf tube and add an equal volume of chloroform. Vortex-mix the tube briefly and centrifuge as above for 5 min. Transfer the aqueous layer to a fresh tube.
6. Add an equal volume of isopropanol to the sample, followed by addition of sodium acetate (to a final concentration of 0.1 *M*). Place the tube at –20°C overnight to allow the cDNA to precipitate. The protocol can be stopped at this step.
7. The following day, centrifuge the precipitated cDNA for 1 h at maximum speed in a microcentrifuge at 4°C. Transfer the supernatant into a new tube and store until cDNA precipitation has been confirmed. Using a Geiger counter, confirm radiolabel incorporation into the precipitated cDNA pellet. Check the supernatant to confirm that most of the radioactivity is in the pellet.
8. Wash the pellet by adding 70% ethanol (250 µL), followed by gentle agitation. Centrifuge the tube at maximum speed in a microcentrifuge for 10 min. Carefully remove and discard the ethanol, taking care not to disturb the pellet, and air dry for 10 min at room temperature. The location of the pellet should be marked on the outside of the tube with a labelling marker because it will "disappear" once it is dried.
9. Resuspend the cDNA in sterile ddH_2O (10 µL). Gently flick the sample and briefly centrifuge to collect contents at the bottom of the tube. The tube is kept on the bench for 10 min at room temperature (or at 4°C for 30 min) to allow the cDNA to fully dissolve. Transfer the cDNA to a new tube and check the old tube with a Geiger counter to confirm cDNA was completely resuspended. (*See* **Note 4.**)
10. Ligate *Eco*RI adapters (1.5 µL) to the blunt-ended termini by adding T_4 DNA ligase (5 U) and 10× T_4 DNA ligase buffer (1.5 µL). Final reaction volume is 15 µL (make up the final volume with sterile ddH_2O if required). Incubate the reaction for 16 h at 10°C.
11. Heat inactivate the ligase by placing the tube at 75°C for 15 min, followed by brief centrifugation to collect the contents at the bottom of the tube. Place the sample on ice and add the following components: T_4 polynucleotide kinase (10 U), 10× T_4 polynucleotide kinase buffer (0.5 µL). Adjust the final reaction volume in the tube to 20 µL. Incubate the reaction at 37°C for 30 min, followed by heat inactivation of the kinase at 75°C for 15 min. Centrifuge the tube briefly to collect the contents at the bottom and place tube on ice.

12. Digest the linker primer's *Xho*I site by adding 4 µL of the appropriate 10× restriction enzyme buffer, 10 µL of sterile ddH_2O, and 6 µL of *Xho*I enzyme to the cDNA (to a final volume of 40 µL). Mix the reaction by flicking, centrifuge briefly to collect contents at the bottom of the tube, and place at 37°C for 2 h. Add an equal volume of isopropanol and place tube at –20°C overnight to precipitate the digest. (*See* **Note 5.**)
13. Centrifuge the precipitated cDNA at maximum speed in a microcentrifuge for 1 h at 4°C. Aspirate and discard the supernatant, taking care not to disturb the pellet.
14. Wash the pellet with 70% ethanol (250 µL) and centrifuge for 10 min at maximum speed at 4°C. Carefully aspirate the supernatant and air-dry the pellet on the benchtop for 10 min (*see* **Note 6**). Resuspend the pellet in the appropriate volume of ddH_2O (in this case we will use 50 µL) and store at 4°C.
15. Prepare 10 mL of 1% TAE agarose gel, pour into a 10-cm plate and allow the gel to polymerize. Create a grid on the bottom of the plate with 10 squares.
16. Using a 100 m*M* EDTA solution, make serial dilutions (from 10 ng/µL up to 250 ng/µL) of a DNA standard with a known concentration (such as the DNA ladder in **Subheading 2.7.5.**).
17. Label a square for each of the serial dilutions (0, 5, 10, 25, 50, 75, 100, 150, 200, 250 ng/µL).
18. Carefully pipet 1 µL of each dilution onto the surface of the plate.
19. Below each dilution, spot 1 µL of the cDNA sample and allow all the liquid to absorb into the plate for approx. 20 min.
20. View the plate using ultraviolet (UV) light and estimate the concentration of the cDNA sample by comparing with the standards.
21. Combine an aliquot of the resuspended cDNA (~100 ng) with the vector arms (1 µg), 10X DNA ligase buffer (1 µL), T_4 DNA ligase (2.5 U), and sterile ddH_2O up to 10 µL. Incubate the reaction for 16 h (overnight) at 10°C.
22. The ligation is packaged into phage using a commercially available packaging kit, compatible with the phagemid vector being used, according to the manufacturer's instructions (in this example, GigapackIII XL from Stratagene would be used). Experimental DNA is combined with the packaging extract, mixed and incubated for a set amount of time, chloroform separated, and then the phage is ready to be titered.

3.3. Titration and Amplification of the cDNA Library

1. From a stock vial, streak XL1-Blue cells onto an LB-ampicillin agar plate and incubate overnight at 37°C. The following day, prepare 25 mL of LB-ampicillin broth culture by inoculating a single colony into a flask and incubating on a shaker at 37°C until the OD_{600} = 1.
2. Centrifuge the XL1-Blue culture at 1000*g* for 10 min at 4°C. Resuspend the cell pellet in sterile 10 m*M* $MgSO_4$ and adjust the volume until the OD_{600} is 0.5.
3. Prepare aliquots (100 µL each) of serial library dilutions (1, 1:10, 1:100, 1:1000, 1:10,000) in TSM buffer, mix each with an equal volume of freshly cultured XL1-Blue cells, and incubate at 37°C for 20 min.

4. Mix the contents of each tube with NZY top agar (~3 mL for each dilution; cooled to <50°C) and immediately spread on 10-cm plates. Allow plates to cool (until top agar has solidified), then invert and incubate at 37°C until plaques are observed to form. Count the number of plaques on each plate and determine library titer using the following formula: pfu/mL = (number of plaques × dilution factor)/volume of extract plated (*see* **Note 7**).
5. To amplify the cDNA library, prepare 10 NZY plates (24 × 24 cm) and an overnight LB-ampicillin broth culture of XL1-Blue cells (as described in steps 1 and 2 of this subheading).
6. Pipet XL-1 Blue cells (100 µL) into 10 sterile 1.5-mL tubes, add an aliquot of packaged phage mixture (containing ~50,000 phage particles) to each tube, and incubate for 15 min at 37°C.
7. Combine the contents of each tube with 7 mL of molten NZY top agar and spread evenly onto each of the NZY plates.
8. Incubate the plates at 37°C overnight to allow plaque formation on the bacterial lawn. Following plaque formation, spread TSM buffer (10 mL) over the surface of each plate, followed by incubation at 4°C overnight with gentle shaking to release the phage from the solid media and into solution.
9. Pool the TSM media containing bacteriophage from the 10 plates and add chloroform to 5% v/v. Incubate this mixture at room temperature for 15 min, centrifuge at 500*g* for 10 min, then transfer the supernatant to a new sterile container.
10. Add DMSO to the supernatant (7% v/v final) and aliquot the amplified library into sterile 1.5-mL tubes and store at –80°C. The titer of each amplified library should be determined as described above.

3.4. Differential Screening of the cDNA Library

1. Prepare NZY plates (10 plates; 24 × 24 cm) as described in **Subheadings 3.3.1–3.3.4**. Adjust the amount of phage library used to infect the bacteria using the calculated titer, so each plate contains approx. 20,000–50,000 pfu.
2. Plaques will develop over the course of approx. 12 h and should be viewed regularly until they are round in shape and measure approx. 1 mm across.
3. Place a Hybond-N nylon membrane onto each plate containing the newly developed plaques for one min (adsorption time). Orientation marks should be made on the membrane and on the plate so that the correct plaques can be chosen when going back to select positive clones.
4. Soak each membrane in denaturation buffer for 1 min, neutralization buffer for 5 min, and rinse buffer for 0.5 min.
5. Allow membranes to air dry on filter paper and UV cross-link the phage DNA to membranes using an ultraviolet (UV) crosslinker.
6. Repeat steps 3–5 using a second membrane on each plate, with the adsorption time lengthened to 2 min.
7. Bake the plaque lifts in a gel dryer for 1 h at 80°C between two fresh pieces of filter paper.

8. Screening requires the synthesis of two sets of probe. One set is synthesized using control mRNA as a template and a second set using mRNA from the stress or condition being examined.
9. Synthesize probe using a protocol similar to that used for first strand cDNA synthesis (**Subheading 3.2.**; materials listed in **Subheading 2.2.**). Aliquot poly(A)$^+$ mRNA template (about 1 µg) into an autoclaved, DEPC-treated, 1.5-mL microfuge tube and heat at 65°C for 5 min.
10. Cold-snap the mRNA on ice, with subsequent addition of 10× reverse transcriptase buffer (5 µL), dNTPs (3 µL, without dCTP), oligo(dT) primer (3 µg), RNasin (5 µL) into each tube. Adjust the total volume up to 40 µL. Flick the tubes gently to mix, centrifuge briefly to collect contents at the bottom of the tube, and allow the primers to anneal to the RNA at room temperature for 10 min.
11. Add 5 µL of reverse transcriptase and 5 µL of [α-^{32}P]dCTP (3000 Ci/mol) to each tube and incubate at 42°C for 1 h. Transfer the tubes to a water bath preset to 16°C.
12. To degrade the RNA, add RNase A (5 µL) and incubate at 37°C for 30 min. This will leave a radiolabeled first strand of cDNA, which is loaded onto a quick spin column and centrifuged at 400*g* for 3 min. The eluant (labeled cDNA) is used to probe membranes.
13. Plaque lift membranes are pre-hybridized in hybridization solution (enough to cover the membrane) for 30 min in a rotating hybridization tube at 55°C.
14. Boil the radiolabeled probe for 5 min (*see* **Note 8**) and place immediately on ice. Add probe directly to the existing hybridization solution (*see* **Note 9**) to a concentration of 1×10^6 cpm/mL. To allow for direction comparison, add identical levels of radioactivity (cpm) to paired blots (control vs. experimental). Hybridize blots overnight (~16 h) with rotation at 45°C.
15. After hybridization, wash the plaque-lift membranes in 0.2× SSC, 0.1% w/v SDS at 55°C for 10 min. Repeat this wash step 3 additional times to reduce background radioactivity. If background levels remain high (as detected using a handheld Geiger counter), washes can be repeated as desired.
16. Place washed membranes radioactive side down on plastic wrap while still damp and seal tightly so that liquid does not leak out. Wrap the membranes a second time using a plastic wrap and smooth the surface using a paper towel (to remove wrinkles or air bubbles). Expose the blots to X-ray film (or a phosphoimager screen) for an appropriate length of time. Exposure time depends on the cpm of the specific probe bound to each membrane and should be adjusted accordingly.
17. Develop the X-ray film (or phosphoimager screen) and analyze the resulting pattern of spots on the autoradiograms. An example of a primary screen autoradiogram is shown in **Fig. 1A**. Each spot represents a plaque where probe has hybridized to cDNA.
18. Using the orientation markers, trace differentially expressed clones back to their corresponding phage plaques on the NZY plates and excise. A sterile Pasteur pipet is used to excise each section of agar, which is then placed into a 1.5-mL Eppendorf tube containing 500 µL of TSM buffer. If it is not possible to isolate a single clone (especially common during the primary screen) putatively positive "regions" can be excised.

19. Add chloroform to each tube (to 5% w/v), vortex-mix for 30 s, and leave at room temperature for 30 min, allowing the phage to desorb from the agar into the buffer.
20. Centrifuge samples for 5 min at 3000*g* at 4°C and transfer the supernatants (containing isolated phage) to fresh Eppendorf tubes and store at 4°C.
21. Perform a spot titer for each putative clone or "region" to determine the approx. pfu/mL (as described in **Subheading 3.3.**).
22. Re-plate each putative positive clone or "region" on a separate circular (10 cm diameter) NZY plate at 50–100 pfu/plate.
23. Construct plaque lifts for this secondary screen using small, circular nylon membranes and follow the same steps outlined above for a primary screen (starting at step 3). Repeat differential screening in an identical manner (starting at step 8.). Select positive clones representing genes of interest (differentially expressed) by carefully examining the resulting autoradiograms. An example of a secondary screen autoradiogram is shown in **Fig. 1B**.

3.5. In vivo Excision of Clones

1. The procedure followed in this section depends upon the cDNA library vector used. Components must be purchased commercially (generally in the form of a kit from the same manufacturer that created the vector). A brief overview of the procedure used with the Uni-Zap cDNA libraries (ExAssist Helper Phage with SOLR cells; Stratagene, see **ref. *6***) is provided.
2. Prepare a stock of XL1-Blue cells as outlined in **Subheading 3.3.1.** Prepare a stock of SOLR cells using the same protocol as that for XL1-Blue cells. Cells are grown at 37°C in LB-ampicillin broth overnight (~16 h) and are centrifuged at 3000*g* for 5 min.
3. Discard the supernatant and resuspend each bacterial cell pellet in 10 m*M* $MgSO_4$ to an OD_{600} = 1.0. Store the SOLR cell pellet at 4°C until step 8.
4. Aliquot 200 µL of each phage stock into a separate Eppendorf tube, add an equal volume of XL1-Blue Cells, and 1 µL of helper phage.
5. Incubate the tubes at 37°C for 15 min. Prepare an equal number of 15-mL tubes containing 3–5 mL of LB-ampicillin broth. Following the incubation, transfer the contents of each Eppendorf tube into labeled 15-mL tubes. Incubate these tubes at 37°C with gentle shaking for approx. 3 h.
6. Centrifuge the tubes for 15 min at 400*g* to pellet cells and debris and transfer the supernatant, containing the intermediate filamentous phage, to a fresh tube and incubate at 70°C for 15 min.
7. Centrifuge the new tubes for 15 min at 3000*g* to pellet any remaining cells, and then transfer the supernatant containing the filamentous phage stock to fresh 15-mL tubes and store at 4°C.
8. Add filamentous phage stock (100 µL) representing each selected phagemid into labeled tubes. Resuspend SOLR cells with brief mixing and aliquot 200 µL into each tube and incubate at 37°C for 15 min.

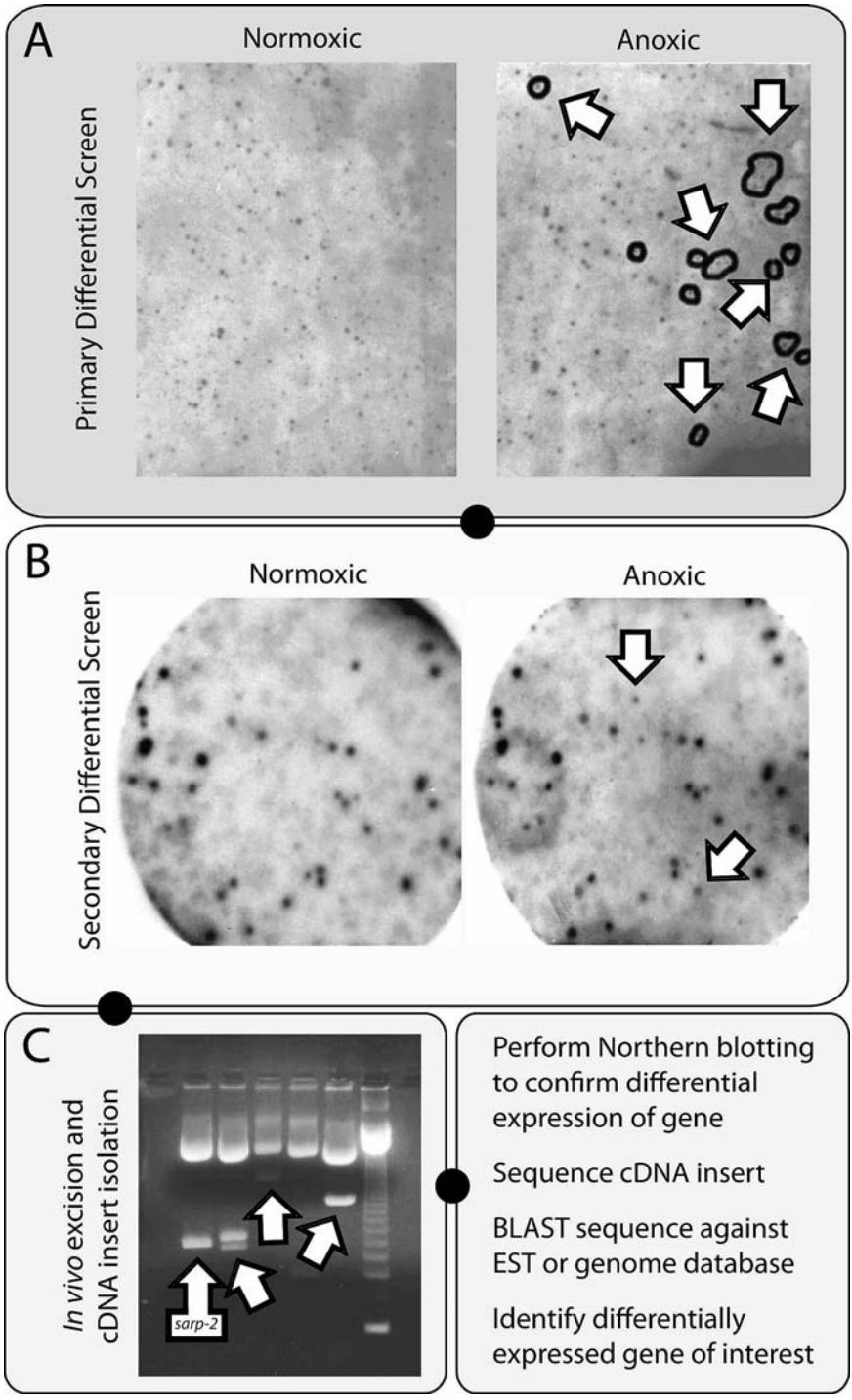

Fig. 1. (Continued)

9. Pipette an aliquot of cells (100–200 μL) from each tube onto an LB-ampicillin plate and incubate at 37°C overnight.
10. Streak a single colony from each plate onto a fresh LB-ampicillin plate to create a master plate of SOLR cells containing the pBluescript plasmid of interest. Incubate these plates overnight at 37°C.
11. Inoculate a single colony from a master plate into 3 mL of LB-ampicillin broth. When the OD_{600} = 1.0, a glycerol stock is created by combining an aliquot of the log-phase bacterial culture with sterile glycerol (15% v/v final concentration). Each tube is mixed and kept at –4°C for 30 min, then stored at –80°C.

3.6. Miniprep Plasmid Isolation

1. A variety of kits are commercially available that provide a quick and clean plasmid isolation. Such kits are available from a number of companies including, but not limited to Nucleospin (BD Biosciences), Qiagen, and Invitrogen. A standard "non-kit" protocol is outlined here.
2. Select a single colony from a master plate and inoculate 3 mL of LB-ampicillin broth in a 15-mL tube. Incubate overnight (~16 h) at 37°C with shaking.
3. Pipet 1.5 mL of the overnight culture into an Eppendorf tube and centrifuge at 3000*g* at 4°C for 10 min. Aspirate and discard the supernatant.
4. Resuspend the pellet in 400 μL of pre-lysis buffer, transfer to a sterile Eppendorf tube, add RNase A (5 μL) and vortex-mix.
5. Add an equal volume of freshly diluted alkaline lysis buffer and keep tube at room temperature for 5 min.
6. Add an equal volume of neutralization buffer and vortex-mix the tube until the white precipitate (protein, genomic DNA, and cell debris) is broken up. Keep on ice for 5 min and then centrifuge in a microcentrifuge at maximum speed for 10 min at 4°C.

Fig. 1. Differential screening of a cDNA library. (**A**) Primary differential screen of duplicate plaque lift membranes using [^{32}P]dCTP labelled cDNA synthesized from mRNA isolated from the hepatopancreas of the marine snail, *Littorina littorea*. Normoxic: mRNA isolated from animals kept at 4°C under normal aerated conditions. Anoxic: mRNA isolated from animals kept under a nitrogen gas atmosphere for 1 h, 12 h, and 24 h (equal amounts of mRNA from each time-point combined). (**B**) Secondary differential screen of duplicate plaque lift membranes using the same probe as in **(A)** to confirm putative anoxia-responsive clones. (**C**) In vivo excision of plasmids from phage stock, with subsequent plasmid purification from bacteria, allowed insert isolation from putative anoxia-induced clones. Lane 1 shows a particular clone of interest, encoding snail anoxia-responsive protein 2 (*sarp-2*); various inserts of other nonspecific genes are in the subsequent lanes. Positive clones should be confirmed as differentially expressed with Northern blots and then further characterized as outlined in **Fig. 2**.

7. Transfer the supernatant to a fresh 1.5-mL Eppendorf tube and add an equivalent volume of isopropanol. Invert tube repeatedly to mix and incubate for 10 min at room temperature.
8. Centrifuge the tube at maximum speed in a microcentrifuge for 10 min at room temperature. Aspirate and discard the supernatant and wash the pellet (DNA plasmid) with 70% ethanol. Recentrifuge as described previously for 5 min, Aspirate ethanol and allow the pellet to air-dry for 10 min at room temperature.
9. Resuspend the plasmid DNA in an appropriate volume of sterile ddH_2O (generally start with 20 µL) and quantify using spectrophotometry (1 OD = 50 µg/mL of double- stranded DNA). Plasmid is stored at 4°C (short term) or –20°C (long term) until further use.

3.7. Insert Isolation

1. Digest the plasmid DNA with appropriate restriction enzymes (in this case, *Eco*RI and *Xho*I).
2. Combine an aliquot of plasmid DNA (5 µL) with 0.5 µL of each enzyme, 1 µL of 10× reaction buffer, and adjust the final volume up to 10 µL with sterile ddH_2O. Incubate digest at 37°C for at least 1 h.
3. Stop the restriction digest by heating at 94°C for 5 min and then adding 0.1 volumes of DNA loading dye.
4. Load samples onto a 1% TAE agarose gel and separate the restriction fragments by running the gel at 90 V in 1× TAE buffer. An appropriately sized DNA ladder should be used as a molecular weight standard, allowing the size of the insert to be estimated. An example of digested inserts separated using agarose gel electrophoresis is shown in **Fig. 1C**.
5. Carefully slice each insert band out of the gel using a sterile razor blade and place each into a labeled Eppendorf tube. To isolate the DNA from the gel agarose, a number of options are available. Commercial kits using filtered columns or glass milk are available, however a quick and easy protocol is outlined here.
6. Place the gel slices at –80°C until frozen (~15 min). The collar of a 1-mL filtered pipet tip is removed using a razor blade and the bottom half of the tip is fixed inside it. This apparatus is then placed firmly into the top of a labeled Eppendorf tube. Each frozen gel slice is transferred quickly into the opening of the tip on top of the filter and the tube is placed into a microcentrifuge set to spin at 10,000*g* for 3 min. Remove the tubes from the centrifuge and discard the filter apparatus. Insert DNA is present in the eluant and can be stored at 4°C (short term) or –20°C (long term).

3.8. Synthesis of Labeled Probe

1. Transfer an aliquot of the insert DNA (12 µg) into a sterile 1.5-mL Eppendorf tube and adjust the final volume of 8 µL. Denature the DNA by placing at 94°C for 3 min, centrifuge briefly to collect contents at the bottom of the tube and place immediately on ice. Add the following to the tube on ice: dNTPs (ATP, TTP, GTP; final concentration = 1 m*M* each), 1 µL random primer (100 m*M* $d(N)_6$;

NEB), 2 µL of 10× random primer buffer, 2.5 U of Klenow fragment of DNA polymerase I (NEB), 2.5 µL of [α-^{32}P]dCTP (3000 Ci/mol; Amersham).
2. Gently mix the tube and place in a heating block at 37°C for 45 min. Load the labeled reaction onto a quick spin column and centrifuge at 400*g* for 3 min. The eluted radiolabeled probe is used for Northern blotting.

3.9. Northern Blotting

1. Prepare a 1.25% agarose formaldehyde denaturing gel and submerse it in enough 1× MOPS buffer to cover the wells. Pre-run the gel for 15 min (as RNA samples are being prepared).
2. Aliquot the appropriate volume of total RNA, containing between 10 and 20 µg, into labeled tubes on ice. Add an equal volume of RNA sample buffer into each tube.
3. Incubate samples at 55°C for 10 min and place immediately on ice. Add the appropriate amount of RNA loading buffer (final concentration = 1×) to each sample.
4. Mix the RNA samples gently and briefly centrifuge tubes to collect the entire sample at the bottom of each Eppendorf tube.
5. Load the entire contents of each tube into the wells of the gel and record sample order for reference.
6. Perform gel electrophoresis at 100 V until the loading dye front reaches the end of the gel.
7. Place the gel on plastic wrap and visualize the RNA using a UV light. A photograph (digital or Polaroid) should be used to maintain a photographic record. The ribosomal RNA (rRNA) bands are used as an indicator of degradation and as a rough guide to ensure equal loading of the wells. **Fig. 2A (lower panel)** provides an example of ethidium bromide stained ribosomal RNA visualized with UV light.
8. The separated RNA will be transferred from the gel onto a 0.2-µm nylon membrane by the neutral transfer method. Soak the gel in 10× SSC for 10–15 min to remove excess formaldehyde and ethidium bromide.
9. While the gel is soaking, set up the transfer apparatus. Fold a large square of plastic wrap over onto itself (2 ft × 3 ft) and place on the benchtop.
10. Center a square piece of Plexiglas or glass sequencing plate (larger than the gel being transferred) on the plastic wrap. The wrap should extend 3–4 in. beyond the edges of the plate.
11. Cut two pieces of filter paper (used as a wick) such that they are approx. 1–2 inches longer than the plate on all sides.
12. Prewet the plate with 10× SSC and place each filter paper sequentially over the plate. Remove wrinkles or air bubbles by smoothing each layer with a sterile pipet.
13. Orient the gel face down on the second piece of filter paper, directly in the center.
14. Cut a membrane to the exact size of the gel. Briefly rinse the membrane in sterile water, then rinse in 10× SSC, and place it over the entire gel. Use a pipet to smooth the membrane over the gel, removing any wrinkles or air bubbles.
15. Cut two pieces of filter paper (the same size as the gel and membrane) and place each sequentially on top of the membrane. Prewet the filter paper in 10× SSC and roll smooth using the pipet.

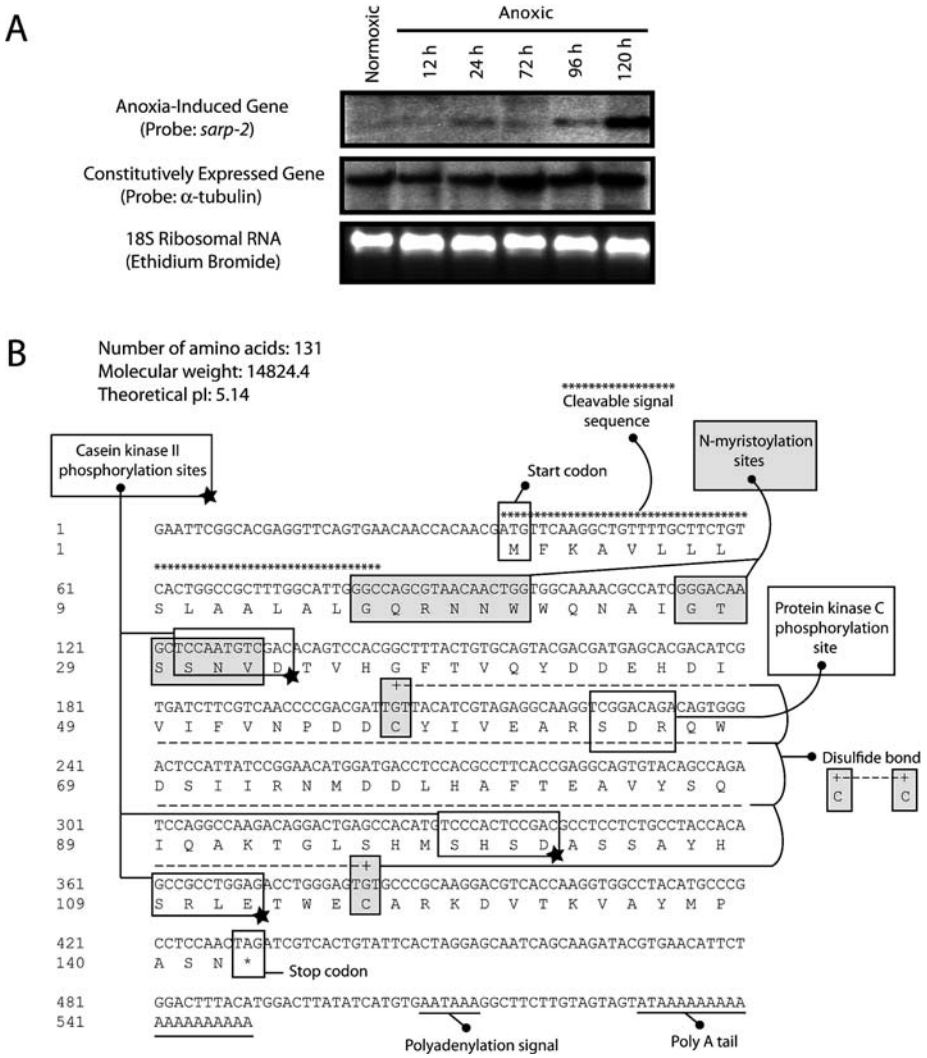

Fig. 2. Characterization of the novel gene from *L. littorea*, *sarp-2*. **(A)** Following insert isolation from a clone designated *sarp-2* (as described in **Fig. 1**), expression of the *sarp-2* transcript was monitored over a time course of anoxia exposure by Northern blotting. The expression of *sarp-2* was normalized by examining the expression of a control gene (α-tubulin) on the same blot, as well as by examining the intensities of the ribosomal RNA band on the gel before transfer. **(B)** Sequencing of the insert *sarp-2* showed a 550 nucleotide sequence containing the full open reading frame for a 131 amino acid protein. Multiple common protein domains and motifs were identified in the *sarp-2* mRNA and deduced SARP-2 protein sequences using the applications outlined in **Subheading 3.11.** and selected results are illustrated here.

16. Place a piece of plastic wrap (the same size as that on the benchtop) over the entire transfer apparatus. Using a sharp razor blade, cut a "window" around the gel/filter paper. Remove this square of plastic wrap and place a stack of paper towels (5 in. high) on top of the filter paper. Add 10× SSC (10 mL) into each end of the transfer apparatus (between the inner and outer layers) and fold over the plastic wrap to seal. Place a weight (250–500 g) on top of the paper towels and allow the transfer to occur overnight (*see* **Note 10**).
17. Following transfer, cross-link the RNA to the membrane following the same protocol described in **Subheading 3.4.5.**, or the membrane can be soaked for 5 min in 0.05 *N* NaOH and then rinsed in sterile ddH_2O.

3.10. Probing Northern blots

1. Probing Northern blots is similar to probing plaque lift membranes. Prehybridize Northern blots at 55°C using hybridization solution (enough to cover the surface of the blot) in a hybridization oven for a minimum of 30 min.
2. Heat radiolabeled cDNA probe at 94°C for 5 min, cold-snap on ice, then add directly to the hybridization solution.
3. Incubate Northern blots with probe at 45°C for at least 16 h (overnight). Following hybridization, wash blots with Northern blot washing solution at 55°C for 10 min. Repeat a total of 4 times.
4. If radioactive background appears high when scanned with a handheld Geiger counter, wash steps can be repeated as desired.
5. Wrap Northern blots in plastic wrap, expose to X-ray film or a phosphoimager screen for the appropriate length of time, and develop.
6. Perform densitometric analysis on the resulting autoradiogram using appropriate imaging software (Example, Imagequant (GE Healthcare) or QuantityOne (Bio-Rad)). An example of a Northern blot is shown in **Fig. 2A (upper panel)**.
7. As needed, probe can be removed from a blot by boiling a solution of ddH_2O and 0.5% SDS and pouring it on top of the blots in a wash container. Repeat until radioactive counts reach (or are below) background levels. Rinse stripped blots in sterile water (to remove residual SDS) and re-probe as desired. Reprobing for a constitutively expressed or "housekeeping" gene is used to normalize the relative levels of your differentially expressed gene against a gene whose expression does not change under the experimental condition being examined. The Northern blot in **Fig. 2A** was stripped and reprobed with α-tubulin to control for loading, as illustrated in the **Fig. 2A (middle panel)**.
8. Additional experimental protocols can and should be performed to further characterize a novel gene. The promoter of the gene can be examined to determine if stress or condition responsive elements are present which may play a role in gene expression. Transcriptional analysis can be examined with nuclear runoff assays to determine if a gene is transcriptionally regulated or if the mRNA is stabilized. The gene can be expressed as a protein and basic biochemistry can be carried out to determine a possible role or function *in vitro*. The protein can also be used as an antigen for producing an antibody that can be used for Western

blotting and subcellular localization experiments. A knockdown (using siRNA) or knockout (using genetic manipulation) of a gene can be performed to determine the effects and/or phenotype resulting from loss of the novel gene. These methods and techniques are outside the scope of this chapter and they represent downstream experiments from the "starting point" outlined here. Refined protocols can be found in other volumes of the *Methods in Molecular Biology* series, as well as in classic laboratory manuals such as *Molecular Cloning: A Laboratory Manual* ***(7)***. **Figure 3** provides a basic view of experiments used to examine the novel gene, *kvn*, discovered and characterized by the authors.

3.11. Clone Analysis

Differentially expressed clones should be bidirectionally sequenced using standard methods (either in the laboratory or by a DNA/Genomics facility). Subsequent sequence analysis is performed using commercially available computer software and a wide array of analysis tools available online. An index of some excellent bioinformatics programs and applications available to the scientific public for analysis of cDNA and putative amino acid sequences is listed in this subheading.

As a first step, DNA sequences should be copied and pasted into NCBI's BLAST server for comparison to existing and annotated sequences. Genes sharing sequence identity or similarity to existing expressed sequence tags (ESTs) will be identified with an E-value. The E-value represents the probability of each alignment occurring due to chance, where a value $<e^{-5}$ signifies that an alignment is not due to error. Novel or unknown genes will often come back with no determinable match, although some conserved domains and/or motifs may be identified and provide a short sequence match.

3.11.1. National Center for Biotechnology Information (NCBI)

http://www.ncbi.nlm.nih.gov/

1. **Basic Local Alignment Search Tool (BLAST):** http://www.ncbi.nlm.nih.gov/BLAST/ BLAST first lists four letter "words" of residues in one sequence; then the words are expanded to segments (profile based). The programs used for nucleotides are blastx (compares a nucleotide query sequence translated in all reading frames against a protein sequence database) and tblastx (compares the six-frame translations of a nucleotide query sequence against the six-frame translations of a nucleotide sequence database), whereas blastp (compares an amino acid query sequence against a protein sequence database) is used for protein analysis.
2. **ORF (open reading frame) finder:** http://www.ncbi.nlm.nih.gov/gorf/gorf.html ORF finder allows identification of potential coding regions in all 6 reading frames of a nucleotide sequence. Selected ORFs can then be transferred directly into

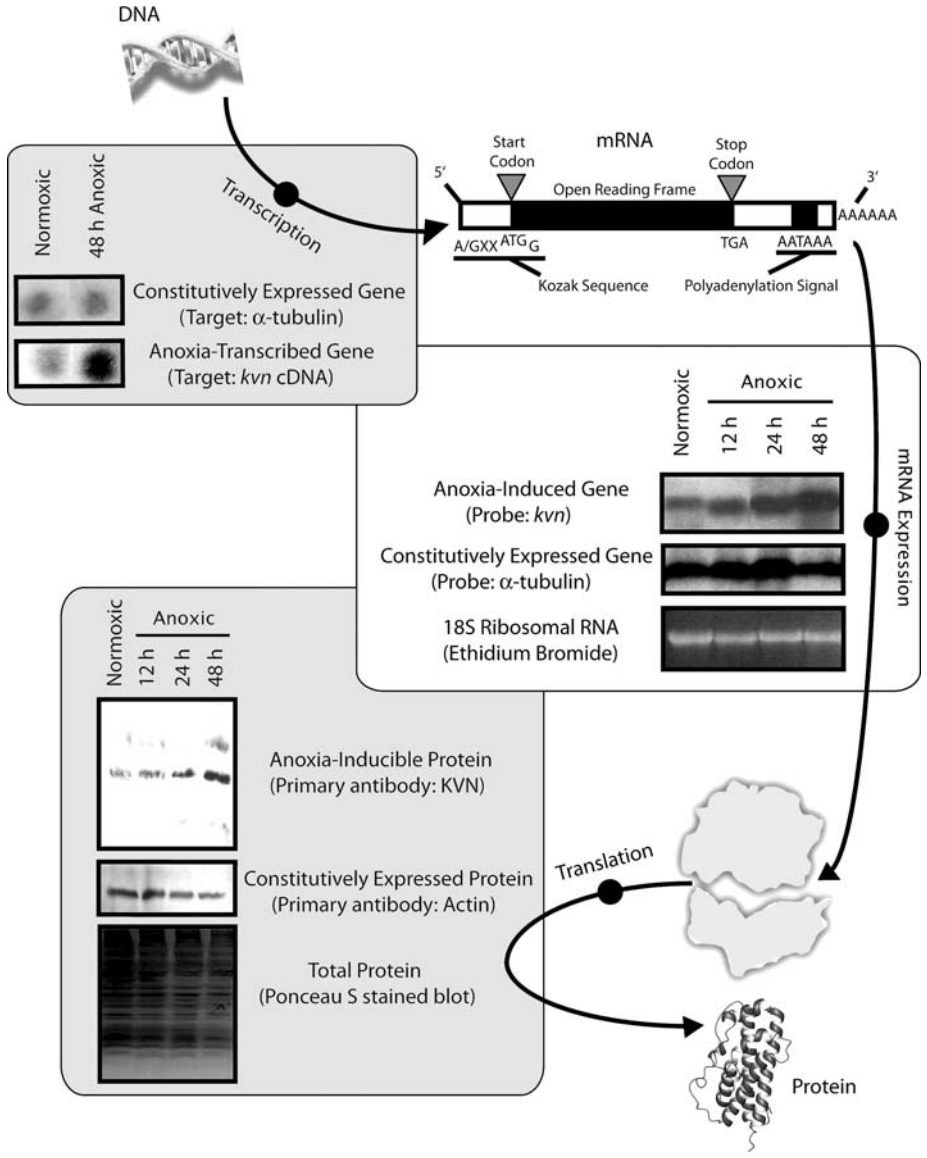

Fig. 3. Flowchart outlining the characterization of a novel gene. Starting with genomic DNA, the transcription of a gene can be measured using a nuclear run-off assay (top left panel). The gene examined here (*kvn*) is actively transcribed in nuclei isolated from the hepatopancreas of *L. littorea* kept under anoxic conditions for 48 h. This gene has been characterized previously ***(4)*** and contains all of the features common to eukaryotic genes that are identifiable in mRNA.

BLAST to search for conserved domains and similar sequences using blastp (for proteins).

3.11.2. Gene Translation and Characterization of a Protein Sequence

Determining the function of novel genes is a challenge currently facing many of the large genome projects. An mRNA sequence contains much information, but it is traditionally the functioning protein that performs the important task. In order to anticipate the function of a particular gene, its sequence is usually translated into a predicted protein, which is then analyzed for structural homology and conserved regions (known as domains or motifs) that have been identified in proteins having known functions. Many times a cDNA clone will represent a known homologue. The genes/proteins represented by these clones can be assimilated into an existing scheme, since they have often been characterized in other systems, organs or tissues (as well as during various stresses or conditions) and perform a known role or function. However, if the clone of interest does not have any known homologues, it must be further analyzed and characterized using the tools available. Some tools for analyzing a novel gene/protein include the following:

PredictProtein:
http://www.embl-heidelberg.de/predictprotein/predictprotein.html
The translated protein sequence of interest is queried against characterized sequences using the PredictProtein server, which retrieves similar sequences in the database and predicts aspects of protein structure.

Protein Sequence Analysis (PSA):
http://bmerc-www.bu.edu/psa/request.htm
PSA predicts protein secondary and tertiary structure based on amino acid sequence.

SOSUI:
http://sosui.proteome.bio.tuat.ac.jp/sosui_submit.html
This tool predicts secondary structure of membrane proteins from an input protein sequence by examining the physicochemical properties (such as hydrophobicity and charges) of amino acid sequences.

Fig. 3. *(Continued)* Expression of *kvn* was monitored over a time course of anoxia exposure as shown by Northern blotting (middle panel). Northern blots were normalized by examining the expression of a control gene (α-tubulin) on the same blot, as well as the intensity of ribosomal RNA on the gel before transfer. Expression of the KVN protein was measured over a time course of anoxia exposure as shown by Western blots (bottom left panel). Western blots were standardized by re-probing the blot with an antibody for a control protein (β-actin), as well as comparing the protein levels in each sample lane after the membrane is stained with a reversible Ponceau S stain.

PROSITE:
http://www.expasy.ch/prosite/
PROSITE is a database of conserved protein motifs. The query output provides matches for biologically significant patterns and profiles based on the primary sequence of the protein.

PSORT:
http://psort.nibb.ac.jp/
After a gene is translated into a protein, the amino acid sequence often contains a region that represents a "transport" or "localization" signal. This signal determines the cellular localization of the protein and may help in determining its function. PSORT compares an input sequence to known consensus sequences to determine if a sorting signal is present.

SignalP:
http://www.cbs.dtu.dk/services/SignalP/
The SignalP server predicts the presence and location of signal peptide cleavage sites in input amino acid sequences.

Submitting DNA Sequences to NCBI GenBank
http://www.ncbi.nlm.nih.gov/Genbank/submit.html
Prior to publication of research that has retrieved and characterized DNA sequences, the sequence must be annotated and submitted to a sequence database. The submission may include such information as coding regions, start and stop codons, or any information provided by the applications listed here. Using an internet browser, the new sequence is submitted to Genbank through Bank It (web based tool) or Sequin (stand alone submission tool).

4. Notes

1. Total RNA sample quality can also be assessed via denaturing agarose gels (see **Subheading 3.9.**). Both the integrity of the ribosomal RNA (rRNA) and the distribution of mRNA in each lane are indicators of degradation. The 28S rRNA band should be approximately twice as intense as the 18S rRNA (although with many invertebrates, only a single rRNA band is observed due to a cryptic nick in the 28S rRNA, resulting in a single rRNA band migrating at the approximate size of the 18S rRNA). There should be an even distribution of mRNA throughout the lane (as indicated by a light pink ethidium bromide smear when viewed using UV light).
2. The final reaction volume in the cDNA library synthesis step (**3.2.1**) will be 50 µL, so the RNA must be resuspended in the appropriate volume of ddH_2O. If the RNA is not concentrated enough, the sample can be concentrated using a SpeedVac centrifuge (preferred method) or reprecipitated using isopropanol as before (some loss will occur).
3. Remove 5 µL of the first-strand synthesis reaction and aliquot it into a new tube. Add 0.25 µL of [α-^{32}P]dCTP and incubate with the first-strand synthesis reactions.

Place the radioactive first-strand synthesis control reaction at –20°C until ready to resolve by electrophoresis on an alkaline agarose gel. (*See* **Note 4.**)

4. Remove an aliquot of the second strand cDNA synthesis reaction (1 µL) and analyze it with the aliquot saved from the first strand reaction (*see* **Note 3**) using an alkaline agarose gel. These radiolabeled "control" reactions allow determination of the size and quality of synthesized cDNA. Gel electrophoresis is performed similar to the procedure outlined in **Subheading 3.7 or 3.9.** Melt agarose (3.75 g) in ddH_2O (270 mL) containing ethidium bromide (1 µg/mL) and cool to 60°C. Add 0.1 volumes of 10× alkaline agarose electrophoresis buffer (500 m*M* NaOH, 10 m*M* EDTA, pH 8.0), swirl without introducing bubbles, pour gel, and allow it to polymerize. Submerse the gel in enough 1× alkaline agarose electrophoresis buffer to cover the wells and prerun for 5 min. To each of the samples (first- and second-strand reactions, 1 µL), add 10 µL of 1× alkaline agarose electrophoresis buffer and 2 µL of alkaline loading buffer (300 m*M* NaOH, 6 m*M* EDTA, 18% Ficoll 400, 0.15% bromocresol green, 0.25% xylene cyanol FF). If possible, electrophorese gel in a cold room (or at low voltage) to prevent overheating. Following electrophoresis, fix the gel in 7% trichloroacetic acid (w/v), dry in a gel dryer, and perform autoradiography.
5. To enhance precipitation, add NaCl to a final concentration of 25 m*M*.
6. Depending on how mRNA was originally prepared, the quality may be suitable to create a library without size fractionation. If transcripts above or below a certain size are desired, cDNA fractionation can be performed. One method for this is briefly outlined here. A Sepharose drip column (~1 mL) can be used to separate cDNA by size; longer cDNA sequences will elute off the column first followed by progressively shorter sequences. Columns can be made in the lab using Sepharose having the desired molecular weight cutoff (often S500), or can be purchased from a company such as Invitrogen). Equilibrate column with loading buffer (10 m*M* Tris-HCl, pH 7.5, 0.1 m*M* EDTA, 25 m*M* NaCl) by loading 1 mL onto the column and allowing it to drain completely. Repeat 3–5 times. Load sample cDNA onto the column (in a volume of ~100 µL of loading buffer) and allow it to enter the Sepharose. Aliquot 100 µL of loading buffer onto the column, allow it to enter the Sepharose and collect the eluant into the first tube. Continue adding 100-µL aliquots of loading buffer on top of the column (allow each to enter the Sepharose prior to adding subsequent aliquots). Collect the eluant in approx. 50 µL fractions. A few microliters of a sample dye containing glycerol (50%) and bromophenol blue (50%) can be added after the sample is loaded to track the progress of the cDNA elution. Each collected fraction can be analyzed by loading 5 µL on a 5–6% standard nondenaturing acrylamide gel, followed by autoradiography (wet or dry gel), to determine cDNA size in each. Combine the fractions containing the desired lengths of cDNA into a single tube and then process as described in **Subheading 3.2.5**–**3.2.8**. Resuspend the dried pellet in the appropriate volume of ddH_2O and proceed to **Subheading 3.2.15**.
7. The Uni-ZAP vector contains β-galactosidase within the multiple cloning site, allowing a "blue-white" color assay to be performed to determine the ratio of

recombinants (white plaques) to nonrecombinants (blue plaques) in the cDNA library. Transfer an aliquot of the final packaged reaction (1 µL) into two Eppendorf tubes. Label the first tube *1* (undiluted). Add 9 µL of ddH_2O to the second tube, then aliquot a single microliter of this 1:10 dilution into a fresh Eppendorf tube labeled *2* (1:10 dilution). Add an aliquot of XL1-Blue cells (100 µL; prepared as described in **Subheading 3.3.1**) into each of the labelled tubes and incubate at 37°C for 15 min. Combine IPTG (15 µL of a 0.5 *M* stock) and X-gal (50 µL of a 250 mg/mL stock) with 3 mL of molten NZY top agar. Cool the agar to <50°C, add the infected XL1-Blue cells, spread the mixture on top of a standard NZY plate, and allow top agar to solidify. Incubate the plate at 37°C overnight (~16 h) until colored plaques develop. Examine both plates (one of which should contain ~250–500 plaques) and count blue vs. white plaques to determine the background of the library.

8. Sheared salmon sperm DNA (Sigma-Aldrich) can also be included as a non-specific, unlabelled DNA source to reduce background binding to membranes. Add sheared salmon sperm DNA (0.2 mg DNA/mL hybridization solution) to the Eppendorf tube containing probe and follow the protocol from **Subheading 3.4.14**. This note is also applicable to **Subheading 3.10**.
9. When adding probe to the hybridization tube, take care not to "spray" the membrane. If concentrated probe contacts the membrane, the result can be a "hot spot" which may obscure important regions of the blot. Probe should be dispensed directly into hybridization solution (using longer pipet tips) or added to an aliquot of hybridization solution (~1 mL), which is carefully transferred to the tube drop by drop.
10. A layer of plastic wrap can be placed around the stack to prevent a potential "leak" (and resulting short circuit) in the transfer setup between the paper towel and the wicks. This extra layer ensures that buffer must flow through the gel and membrane to reach the paper towel, improving Northern blot transfer.

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References

1. Larade, K. and Storey, K. B. (2002) A profile of the metabolic responses to anoxia in marine invertebrates, in *Cell and Molecular Responses to Stress*, *Vol. 3: Sensing*, *Signaling and Cell Adaptation* (Storey, K. B. and Storey, J. M., eds.), Elsevier Press, Amsterdam, pp. 27–46.
2. Storey, K. B. and Storey, J. M. (2004) Physiology, biochemistry and molecular biology of vertebrate freeze tolerance: the wood frog, in *Life in the Frozen State* (Benson, E., Fuller, B., and Lane, N., eds.) CRC Press, Boca Raton, pp. 243–274.

3. Larade K. and Storey K. B. (2004) Anoxia-induced transcriptional up-regulation of *sarp-19*: cloning and characterization of a novel EF-hand containing gene expressed in hepatopancreas of *Littorina littorea*. *Biochem. Cell. Biol*. **82,** 285–293.
4. Larade K. and Storey K. B. (2002) Characterization of a novel gene up-regulated during anoxia exposure in the marine snail, *Littorina littorea*. *Gene* **283,** 145–154.
5. Stratagene Instruction Manual Revision #073013b. 2003. For: cDNA Synthesis Kit, ZAP-cDNA Synthesis Kit, and ZAP-cDNA Gigapack III Gold Cloning Kit.
6. Stratagene Instruction Manual Revision no. 073002f. 2003. For: ExAssist Interference-Resistant Helper Phage with SOLR Strain.
7. Sambrook, J., Russell, D.W. (2001) *Molecular Cloning: A Laboratory Manual*, 3rd lab edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.