Laboratory Experiments for the

*Integrative Molecular Neuroscience Module*

*Version 1.0*

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Neural Systems & Behavior Course

Marine Biological Laboratory

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Instructor Information

Module Leader: **Dr. Suzy C.P. Renn** is in the Biology Department at Reed College in Portland Oregon. She aims to understand the genomic basis of behavior from an evolutionary perspective through the synthesis of comparative genomics with functional studies in behavior, physiology, and ecology. By understanding the molecular basis of species-specific, and context-specific behaviors, she aims to determine if similar behaviors result from conserved, or converged processes of evolution. She previously worked with Hans Hofmann to pioneer the field of behavioral genomics and continues to pioneer genomic approaches to address questions that cross species boundaries.

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Module Faculty: **Dr. Hans A. Hofmann** is in the department Integrative Biology at The University of Texas at Austin and a Fellow at UT’s Institute for Cellular and Molecular Biology. He completed his doctoral thesis at the Max Planck Institute in Seewiesen under the guidance of Franz Huber. He was a postdoctoral fellow at Stanford University, a Grass Fellow at the Marine Biological Laboratory in Woods Hole, and a Bauer Genome Fellow at Harvard University, where he pioneered the new field of behavioral genomics. Hofmann was the co-initiator of the cichlid genomic consortium, which successfully completed the sequencing of five cichlid genomes. He has also been honored with the 2009 College of Natural Sciences Teaching Award for his commitment to undergraduate education at UT Austin. In March 2012 he was appointed as the Director of UT's Center for Computational Biology and Bioinformatics. In 2013, Hofmann was selected as new Co-Director of the Neural Systems & Behavior summer course at the Marine Biological Laboratory in Woods Hole (MA) where he is enhancing and extending graduate and post-doctoral training in systems neuroscience and Neuroethology.

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Teaching Assistant and Course Developer: **Rayna M. Harris** received her B.S in Biochemistry from The University of Texas at Austin and is now working towards a PhD in Cellular and Molecular Biology. Rayna is a student in the laboratory of Hans Hofmann where she is examining the neurogenomic mechanisms that regulate behavioral plasticity. Her research aims to understand how social behaviors are modulated by social networks, neural networks, and molecular networks. Ever since Hans was appointed Co-Director of NS&B, Rayna has been working to develop and teach molecular exercises that enhance and extend the study of neurophysiology and behavior in the various vertebrate and invertebrate model systems offered in the NS&B course.

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Teaching Assistant: **Chelsea A. Weitekamp** is a PhD student in Ecology, Evolution & Behavior in the laboratory of Hans Hofmann. Her interests are in understanding the neural, hormonal, and molecular mechanisms underlying cooperative behavior across species.

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Module Objective

Across animals, there is remarkable diversity of behavior both within and between species. Modern molecular and genomic approaches have made it possible to identify the neuromolecular underpinnings of such complex behavioral phenotypes. By examining species with plastic behavioral phenotypes we have begun to understand the dynamic and flexible nature of neural transcriptomes and have identified gene modules associated with variation in social and reproductive behaviors. These same techniques can be applied across diverse species to identify conserved molecular mechanisms that regulate behavior and their evolution.

The overall objective of the *Integrative Molecular Neuroethology* Module is to equip students with a comprehensive understanding of the relationships between molecular activity in the nervous system and complex behavior. A series of lectures will introduce students to the theory and practice of “neuro-molecular-ethology” as well as current research focused on social behavior in the Renn and Hofmann labs.

We will teach molecular techniques to complement the electrophysiological characterization of identified cells in the leech, stomatogastric ganglion, and electric fish. The techniques include RNA isolation, cDNA synthesis, quantitative real time PCR, bioinformatic approaches, brightfield & fluorescent immunohistochemistry, stereology, and statistical analyses using R. Knowledge and technical skills gained in this modules will be widely applicable to research in other model and non-model organisms allowing students to ask questions on multiple levels of biological organization.

Chapter 1

Gene Identification

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The molecular dogma is that **DNA** is transcribed into **RNA** which is translated into **protein**. The codon table describes which triplet nucleotides are translated in which amino acids. The coding region of RNA begins with an AUG and ends with a UAA, UAG, or GUA. RNA is highly unstable, so molecular biologists often use reverse transcription to make **complementary DNA** (cDNA), which is much more stable and thus easier to work with than RNA. The National Center for Biotechnology Information (NCBI) providing free access to biomedical and genomic information. (Ensemble, UCSD Genome Browsers, and various species specific resources provide genomic resources as well.) Let's look at one particular gene.

The NCBI Database

The National Center for Biotechnology Information (NCBI) advances science and health by providing access to biomedical and genomic information. One of the most useful features is the nucleotide database, which you can search to find published gene sequences. One example is:

*Astatotilapia burtoni* melanocortin receptor 4 (*mc4r*) cDNA sequence Accession number : NM\_001287403.1

Gene: 1-1648. Coding region: 127-1110

CGTTGTCCTCCAGATAATGAGGATGATCCAAAGCCACACACACTGAGACTTTAAAACGGACAACATAGCTGGACACTTAACAACGGAGAAATAACCCAAGGAGAAGGGAATTTAAAAGAAGCCATTATGAATGCCACAGAATACCATGGACTGATCCAAGGCTACCACAACAGAAGCCAAACCTCAGGCAATTTGCCATTTAACAAAGACTTATCAGGAGAGGAAAAAGACTCATCTTCTGGATGTTATGAGCAGCTGCTTATTTCCACTGAGGTTTTCCTGACTCTGGGCATCATCAGTTTGCTGGAGAACATCCTGGTTGTTGCTGCTATAATCAAAAACAAGAACCTTCATTCACCTATGTACTTTTTCATCTGTAGCCTTGCAGTTGCTGACATGCTCGTCAGTGTCTCTAATGCCTCAGAGACTATTGTTATAGCACTCATCAATGGAGGCAGCCTGACCATTCCTGTCACACTGATTAAAAGCATGGACAATGTGTTTGACTCTATGATCTGTAGCTCTCTGTTGGCATCCATCTGCAGTTTGCTAGCAATCGCCATCGACCGCTATATCACCATCTTCTATGCCCTGCGATACCACAACATTGTCACCCTGCGTCGTGCAATGCTGGTCATCAGCAGCATCTGGACGTGCTGCACCATTTCCGGCATCTTGTTCATCATCTACTCGGAAAGCACCACTGTACTCATCTGCCTCATCACCATGTTCTTCACCATGCTGGTGCTCATGGCATCACTCTACGTCCACATGTTCCTGTTGGCTCGCCTGCACATGAAGCGGATCGCAGCCCTACCGGGCAACGCTCCCATCCAGCAGCGTGCCAACATGAAGGGGGCCATCACCCTCACCATTCTCCTTGGGGTATTTGTGGTATGCTGGGCGCCCTTTTTCCTCCACCTCATCCTTATGATCAGCTGCCCCAGGAACCCCTACTGCACCTGCTTCATGTCCCATTTCAACATGTACCTCATCCTCATCATGTGCAACTCTGTCATTGACCCCATCATCTATGCTTTTCGCAGCCAAGAGATGAGAAAAACCTTCAAAGAGATTTTCTGCTGCTCGCATGCTCTGCTGTATGTGTGAGCTGCCTGTAAAGGGTCCGCTGTGAACCCACAGCCTGCAATTTAAATCGAAACCTGGCCAACATGGACTCTGAGAGTATGATATCTCACATGTTGCCGGTAAATGATTTATTTGGGCACCCATCTTTGGTTTCAGGTTGCTGAAGAGGTAGGTTTACAAACATCTTGTAATATAGCACATTGCTCTTCA

BLAST: Finding Homologous Genes or Proteins by Sequence Alignment

The Basic Local Alignment Search Tool (BLAST) finds regions of local **similarity between sequences**. The program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches. BLAST can be used to infer functional and evolutionary relationships between sequences as well as help identify members of gene families. There are multiple ways to use the blast program.

**Blastn**: search nucleotide database with nucleotide sequence

**Blastp**: search protein database with protein sequence

**Blastx**: search protein database with nucleotide sequence

Scan the QR Code to Blast the *A. burtoni mc4r* sequences against the nucleotide database (blastn) to identify *mc4r* genes in other species

Clustal Omega: Sequence Identification

Clustal Omega is a multiple **sequence alignment program** for proteins. It produces biologically meaningful multiple sequence alignments of divergent sequences. Evolutionary relationships can be seen by viewing cladograms or phylograms.

To create an alignment, paste the amino acid preceded in .fasta format (description preceded by an “>” and followed by a hard return, underscores used to separate words).

>fish\_mc4r

MNATEYHGLIQGYHNRSQTSGNLPFNKDLSGEEKDSSSGCYEQLLISTEVFLTLGIISLL

>rat\_mc4r

MNSTHHHGMYTSLHLWNRSSHGLHGNASESLGKGHSDGGCYEQLFVSPEVFVTLGVISLL

>bird\_mc4r

MNSTQPHGMHTSLHSWNRSGHGLPTNVSESPAKGYSDGGCYEQLFVSPEVFVTLGVISLL

>cow\_mc4r

MNSTQPLGMHTSLHSWNRSAHGMPTNVSESLAKGYSDGGCYEQLFVSPEVFVTLGVISLL

Example: Clustal output

Fish\_mc4r MNATEYHGLIQGYHNRSQTSGNLPFNKDLSGEEKDSSSGCYEQLLISTEVFLTLGIISLL

rat\_mc4r MNSTHHHGMYTSLHLWNRSSHGLHGNASESLGKGHSDGGCYEQLFVSPEVFVTLGVISLL

bird\_mc4r MNSTQPHGMHTSLHSWNRSGHGLPTNVSESPAKGYSDGGCYEQLFVSPEVFVTLGVISLL

cow\_mc4r MNSTQPLGMHTSLHSWNRSAHGMPTNVSESLAKGYSDGGCYEQLFVSPEVFVTLGVISLL

\*\*:\*. \*: . \* .::. : \* . \* : \*..\*\*\*\*\*\*::\* \*\*\*:\*\*\*:\*\*\*\*

\* indicates positions which have an identical residues.

: indicates conservation between groups of strongly similar properties (i.e. all acidic residues)

. indicates conservation between groups of weakly similar properties (i.e. all polar, but some more than others)

Exercise in Multiple Sequence Alignment:

For this exercise, students will learn how to search NCBI database for a sequence of interest and use Clustal Omega to create a multiple alignment to identify highly similar regions.

1. Think of a gene you are interested in.
2. Search for that gene in the NCBI “nucleotide” or “protein” database using the full name or abbreviation.
3. Click search to view the results.
4. Over on the left, click “TREE view” to view all the taxonomic groups who have a record for that gene. Click on a group to narrow your results. OR, add “AND species name” in the search bar.
5. Select five entries and copy the .fasta sequence in a text editor. (Note: it is very important to keep track of the accession number for future analyses and publication.)
6. Paste your sequences into Clustal omega and view the output.
7. Identify conserved regions of your sequence.
8. Note: since fasta sequences being with the accession number and end with the species and gene name, you may want to format the header (i.e. change “>gi|565671712|ref|NM\_001287403.1| Haplochromis burtoni melanocortin 4 receptor (mc4r), mRNA” to “>Aburtoni\_mc4r.”

Chapter 2

Tissue Processing

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Recording Sample Collection Information

In this module, we will be working with biological samples collected during the previous cycles. Essentially, this is collaborative research because you will likely be working with samples collected by your peers rather than by yourself. Therefore, students in the previous cycles were instructed to keep detailed notes regarding sample collection and storage.

Paper data sheets such as this one were used to record sample information and have now been transferred to an electronic file on the server in Y:\NSB\_2014\3\_Int Mol Neuro\Sample Information.

Refer to these data sheets to identify the tissues to be processed.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Please use this sheet to record **sample information**. Handle tubes with gloves. Obtain tube from 4C fridge and keep tube on ice while working. Record sample information on the side of the tube and on this sheet. Store sample in the appropriate location and record the location. | | | | |
| **Tube #** | **Description** | **Collector(s)** | **Date** | **Location** |
|  |  |  |  |  |
|  |  |  |  |  |
|  |  |  |  |  |
|  |  |  |  |  |

Record the following information:

1. Using a sharpie, **label the side of a collection tube** with the following:
   1. description of the neuron or ganglion
   2. your name(s) or initials
   3. today’s date Transfer the isolated tissue to a tube.
2. **In your notebook**, record the following:
   1. tube name
   2. description of the sample
   3. today’s date
3. **On the data sheet** on the freezer, record the following:
   1. description of the tissue
   2. your name(s) or initials(s)
   3. today’s date

Storage of Single Neurons and Ganglia for qPCR

Single neurons and ganglia isolated for gene expression profiling have been stored in 200 μL homogenization buffer from the Maxwell® 16 Tissue LEV Total RNA Purification Kit and stored in a -80°C freezer. When ready to process, these tissues should be placed on ice for use.

Storage of Electric Fish Brains for qPCR

We will use a technique called “tissue punching” to micro-dissect specific brain regions of interest from weekly electric fish. Brains dissected for this purpose were frozen in O.C.T. mounting medium and stored at -80°C. If these brains have been sectioned, they will be stored in slide boxes in the -80°C.

Storage of Electric Fish Brains for Immunohistochemistry

Brains processed for immunohistochemistry were fixed, cryoprotected, embedded, and stored at

-80°C**.** If these brains have been sectioned, they will be stored in slide boxes in the -80°C. See Chapter 7 for details on sample processing.

Storage of Electric Fish Electric Organs for qPCR

Electric organs were dissected and placed in RNALater® (Life Technologies) solution and stored at 4°C overnight than transferred to -20°C for long-term storage.

Storage of Mouse Brain Slices for qPCR

The mouse brain was dissected and slices on a vibratome at 350 μM. Sagittal slices were placed in

RNALater® (Life Technologies) solution and stored at 4°C overnight than transferred to -20°C for long-term storage.

Storage of Mouse Brain Slices for qPCR

The mouse brain was dissected and sliced on a vibratome at 350 μM. The sagittal slices were then transferred to a cutting mat and placed under a dissecting scope. Brain regions of interest were punched and placed in 200 μL homogenization buffer from the Maxwell® 16 Tissue LEV Total RNA Purification Kit. The tissue was homogenized with a pestle and stored at -80°C.

Sectioning

**Set-up**

* 1. Make sure cryostat is on and cold (~ –20°C). Place tissue to be sectioned in chamber to bring it to temperature (tissue stored at –80°C should be in the chamber for at least 30 minutes prior to sectioning). Also place specimen disk (chuck), brushes, forceps, slide cases, etc. in the chamber. If you are not using a disposable blade, also put the microtome knife in place. Leave slides at room temperature (at least until you have mounted and dried all of the desired sections on them).
  2. Mount your tissue. First, place the specimen disk on the platform inside the chamber and apply a generous circle of O.C.T. compound (or other freezing mounting medium). Place tissue specimen in medium and freeze completely. You may add additional mounting medium around tissue for added security. Wait at least 15 minutes for O.C.T to completely freeze.
  3. Using a pencil, clearly label your slides. It is useful to mark your starting location on the slide.
  4. Once specimen is securely embedded in frozen medium, place the specimen disk in the specimen head, orient your tissue as desired, and tighten the knob to secure. To adjust the plane of your specimen, release the lever and use the knobs behind the head to make the needed adjustments. **Note:** For your safety, keep the microtome knife guarded at all times while you are working in the chamber!

**Trimming**

1. Begin by putting the specimen head in “home” position (all the way back). Use the stage forward button to bring your specimen very close to the knife.
2. When you are ready to begin trimming your tissue, press the trim/section button or change thickness to 60 um.
3. Use any of the motorized sectioning buttons and footswitch, or the manual hand wheel to trim your tissue until you near the place where you will begin to collect tissue sections. As you are trimming, you may need to make further adjustments to the plane of your tissue.
4. When you are near the place you will collect tissue sections, turn the trim function off by pressing the trim function button once and/or change setting to your desired thickness.
5. Before collecting tissue sections, put the anti-roll plate in place and practice a few sections so that you can make the proper adjustments for smooth tissue sections. If sections are curling, turn the knob of the anti-roll plate clockwise. If sections are shredding, turn the knob counter-clockwise. Usually, only minute adjustments to the anti-roll plate are necessary to make a difference in tissue section quality.

**Collecting** **Tissue** **Specimens**

When you have made all of the necessary adjustments and are at the place where you wish to begin collecting tissue sections: slice a section, carefully lift the anti-roll plate away, use a brush to position your section, and carefully collect the section on a room temperature slide within the region that will be covered by the coverslip. **For immunohistochemistry**, be sure to allow all sections to dry at room temperature before storing your slides in the freezer. **For tissue punching,** proceed immediately.

Tissue Punching

1. Rapidly dissect the brain.
2. Embed the brain.
   1. Obtain a tube or cryomold of appropriate size for your tissue.
   2. Fill tubes ¾ of the way with O.C.T. compound. Using the spinal cord to grip the brain, place it into the O.C.T. compound. Straighten out as much as possible. Mark the dorsal midline with a marker.
   3. Place on dry ice until frozen then store upright in the -80˚C.
3. Prepare the cryostat
   1. RNase Zap interior of cryostat, blade, punch tool, tweezers, and anything else that may come in contact with the frozen tissue.
   2. Acclimate the brains in the cryostat at -20°C for 30 min.
4. Remove brain from tube and mount on chuck with O.C.T. compound. Add an additional line of O.C.T compound to the side of the frozen block of O.C.T, compound, so that the orientation of the slices will be clear after cutting.
5. Slices will be cut at 300 µm. Since our cryostat only cuts up to 60 µm, do 4 partial turns then cut on the 5th turn. A partial turn is done by turning the handle clockwise until the bottom of the tissue is at the blade, then a counterclockwise turn to restart.
6. Arrange sections in order on the stage in the cryostat.
7. Chill the end of the punch tool on dry ice before using and between each section, which prevents the tissue from thawing and sticking to the punch.
8. Press firmly with the punch tool over the desired brain region. Record punch tool size.
9. Very quickly deposit the punch in your buffer of choice for DNA or RNA isolation. Wipe the punch tool with RNase Zap before collecting the next punch.

Chapter 3

RNA Isolation

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Gene expression is the most fundamental level at which the genotype gives rise to the phenotype. Obtaining high-quality RNA is the first, and often the most critical, step in performing many molecular techniques such as reverse transcription followed by quantitative real-time PCR, transcriptome analysis using next-generation sequencing, and microarray analysis. To generate the most sensitive and biologically relevant results, the RNA isolation procedure must include some important steps before, during, and after the actual RNA purification. RNA is less stable than DNA and proteins and is subject RNA-ases, enzymes that degrade RNA. For short-term storage, RNase-free H2O (with 0.1 mM EDTA) or TE buffer (10 mM Tris, 1mM EDTA) may be used. RNA is generally stable at -80°C for up to a year without degradation. With good technique, RNA is not difficult to work with. Surfaces tools, tubes and equipment must be kept RNase free, reagents kept on ice, clean gloves warn at all times and barrier filter tips are often used for extra precaution.

COMPARISON OF METHODS

**COLUMN-BASED RNA EXTRACTION**

The **ReliaPrep™ RNA Tissue Miniprep System** provides a fast and simple technique for preparing purified and intact total RNA from animal tissue in as little as 30 minutes. The system incorporates a DNase treatment step to reduce genomic DNA contamination. Column based approaches such as the ReliaPrep™ RNA Tissue Miniprep Systems are affordable and require only a centrifuge for use. For the complete ReliaPrep™ RNA Tissue Miniprep System protocol, scan the QR code to the right. On the following pages you will find an abbreviated version that you can print and paste in your notebook each time you do an isolation.

**AUTOMATED RNA EXTRACTION**

The Maxwell® 16 systems allows rapid isolation of RNA with very few pipetting steps, but this requires investment in the instrument. The **Maxwell® 16 Tissue LEV Total RNA Purification Kit** is a medium throughput automated technique for RNA extraction. For the complete Maxwell® 16 Tissue LEV Total RNA Purification Kit protocol, scan the QR code to the right. You will find an abbreviated version in the following pages.

**TRIZOL**

TRIzol is a chemical solution used in RNA/DNA/protein extraction (hence the “tri” in the name). This method uses a guanidinium thiocyanate-phenol-chloroform extraction, and is similar to the method of DNA extraction taught in most introductory biology labs.

In this module, you will perform exercises with the column based approach, but you will use the Maxwell system for your independent projects.

ReliaPrepTM RNA Tissue Miniprep System

1. Determine the number of samples to be processed. **N=\_\_\_\_\_\_\_\_\_**
2. Place sample in RNA later on ice
3. Thaw DNase 1 on ice.
4. Obtain the following: € two 1.5 tube, € one minicolumn, € two collection tubes, € one elution tube, and € one 0.5 μL Axygen tube.
5. Label the top and side of each tube with sample name (or code for each sample). Additionally, label the elution tube with today’s date and your initials
6. Prepare **DNase1 Incubation Mix** by combining the following in the order listed. DO NOT VORTEX rather mix by gently pipetting up and down 3-4 times.

|  |  |  |
| --- | --- | --- |
| DNase 1 Incubation Mix | | |
| Solution | Vol (μL) | Vol \* N |
| Yellow Core Buffer | 24 |  |
| MnCl2, 0.09M | 3 |  |
| DNase 1 | 3 |  |

1. Verify that 1-Thioglycerol has been add to the LBA buffer (stored at 4°C)
2. Add 250 μL LBA with 1-Thioglyercol to a 1.5 tube (one tube per sample)
3. Using forceps, transfer a mouse hippocampal slice to tube with buffer.
4. Homogenize with a pestle. Then pipette up and down 7-10 times with P200 to shear DNA.
5. Centrifuge 3 minutes at 14,000g. Then transfer the supernatant to a new tube.
6. Add 85 μL100% Isopropanol to each sample. Vortex 5 seconds.
7. Transfer sample to a minicolumn in a collection tube. Centrifuge at 12-14,000g for 1 min. Discard the liquid in the collection tube by first removing the Minicolumn. Replace column.
8. Add 500 μL RNA wash solution to the minicolumn. Centrifuge at 12-14,000g for 1 min. Discard the liquid in the collection tube by first removing the Minicolumn. Replace column.
9. Apply **30 μL DNase 1 Incubation Mix** to minicolumn. Incubate 15 min at room temp.
10. Add **200 μL Column Wash Solution** to column. Centrifuge at 12-14,000g for 15 sec.
11. Add **500 μL RNA Wash Solution** to column. Centrifuge at 12-14,000g for 30 sec. Discard the liquid in the collection tube by first removing the Minicolumn.
12. Transfer minicolumn to a new collection tube. Add **300 μL RNA Wash Solution** to column. Centrifuge at 14,000g for 2 min.
13. Transfer minicolumn to an elution tube. Add **15 μL Nuclease Free Water**. Place the tube in the centrifuge with the elution tube cap facing to the outside. Centrifuge at 12-14,000g for 1 min.
14. Discard Minicolumn tube.
15. Transfer 2 μL RNA to an Axygen tube for quantitation with the Quantus Fluorometer.
16. Store RNA at -80°C.

Maxwell® 16 LEV simplyRNA Tissue Kit Protocol

**Setup**

1. Remove samples from -80°C. Thaw cells in 200μLof homogenization buffer on ice to avoid RNA degradation.
2. Thaw blue DNase 1 solution on ice. Do not vortex. Gently mix once thawed.
3. To maintain an RNase-free environment during processing, change gloves before handling cartridges, LEV Plungers and Elution Tubes.
4. Label 0.5 mL tubes from the kit with sample names and date.
5. Add 30-50 μl of Nuclease-Free Water to the bottom of each Elution Tube. Record volume\_\_\_\_\_
6. Carefully peel back the seal on the cartridge.
7. Add 5µl of blue DNase 1 to well 4 (yellow reagent). Well 4 appears green after addition.
8. Place the LEV plunger in well #8 of the cartridge (the well closest to the Elution Tube).
9. Add 200µl of Lysis Buffer to each sample.
10. Vortex vigorously for 15 sec then place on ice.
11. Pipette the 400 μL of lysates into well #1 of the cartridge.
12. Place the cartridges in the Maxwell® 16 LEV Cartridge Rack with the **label side facing away** from where the Elution Tubes go. Press down on the cartridge to snap it into position.
13. Place 0.5ml Elution Tubes in the front of the Maxwell® 16 LEV Cartridge Rack. Leave the cap open. Turn the cap out or in, but not sideways

**Instrument run**

1. Select “Run” on the Menu screen
2. Press the Run/Stop button to select the “Simply RNA” method and select okay.
3. Open the door when prompted. Press the Run/Stop button to extend the platform.
4. Load the cartridge with the elution tubes closest to the door.
5. Press the Run/Stop button. The platform will retract. Close the door.
6. The Maxwell® 16 Instrument will immediately begin the purification run. The screen will display the steps performed and the approximate time remaining in the run.
7. Wait 58 min.
8. Follow the on-screen instructions at the end of the method to open door. Verify that the plungers are located in well #8 of the cartridge at the end of the run. If the plungers are not removed from the magnetic plunger bar, push them down by hand to remove them.
9. Press the Run/Stop button to extend the platform out of the instrument
10. Remove the Maxwell® 16 LEV Cartridge Rack from the instrument.
11. Remove Elution Tubes containing total RNA, and close the tubes. Place on ice.
12. If paramagnetic particles are present in the elution tubes, centrifuge at 10,000 × g for 2 minutes.
13. Proceed immediately to reverse transcription or store RNA at -80°C.

Chapter 4

Nucleic Acid Quantification

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Methods for Nucleic Acid Quantification

DNA and RNA quantification is an important and necessary step prior to most downstream analysis methods. The traditional method for assessing RNA concentration and purity is UV spectroscopy. The absorbance of a diluted RNA sample is measured at 260 and 280 nm. Certain fluorescent dyes exhibit a large fluorescence enhancement when bound to either dsDNA, ssDNA, or RNA. To accurately quantitate DNA or RNA, unknowns are plotted against a standard curve produced with a sample of known concentration. The Agilent® 2100 Bioanalyzer™ instrument uses a combination of microfluidics, capillary electrophoresis, and fluorescent dye that binds to nucleic acid to evaluate both RNA concentration and integrity.

In this course, we will use the Quantus™ Fluorometer with dsDNA-, ssDNA-, and RNA- specific fluorescent dyes to quantify nucleic acids.

Quantus™ Fluorometer Protocol

Watch the Quantus™ Fluorometer Sample Quantification video for detailed instructions on how to operate the instrument. The instrument has already been calibrated, so you do not need to prepare the standard for use when measuring your samples.

PROTOCOL

1. Thaw QuantiFluor® dye at room temperature, protected from light.
2. Calculating the volume of working dye solution needed

# samples \_\_\_\_\_\_\_ x 110 μL = \_\_\_\_\_\_\_\_\_\_\_

1. Dilute the QuantiFluor® dye 1:1000 (i.e 2 μL dye + 1998 μL 1X TE buffer)
2. Add 1 μL of sample from each well (1-8) to a 0.5 mL Axygen tube.
3. Add 199 μL 1X TE buffer to each sample.
4. Add 100 μL dye working solution to each tube. Vortex. Wait 5 min.
5. Quantify each sample using the Quantus™ Fluorometer.

RNA Quantification Exercise

In this exercise, students will practice serial dilutions RNA quantification.

1. Place RNA sample provided by your TA on ice.
2. In your notebook, record information about this sample.
3. Add 25 μL water two all 8 wells of a PCR strip.
4. Add 25 μL RNA sample to well 1. Vortex.
5. Take 25 μL from well 1 and add to well 2. Vortex.
6. Repeat step 4 for wells 2-7.
7. Optional: Discard 25 μL from well 7 to give an equal volume in all tubes.
8. Well 8 is you blank (0 μg RNA).
9. Save the data in Y:\NSB\_2014\3\_Int Mol Neuro\Quantus Data. Name the file “Quantus Data. “Date”.” Initials”. “short description”
10. Calculate the standard deviation from the expected and actual DNA concentration.

RNA Degradation Exercise

This exercise will allow you to see firsthand how quickly RNA can degrade at room temperature.

1. Place one tube of RNA on ice and another on your bench top.
2. Wait 1 hour (i.e. go have lunch or dinner)
3. Run RNA sample on a gel to examine the size of RNA fragments in each sample.
4. Quantify the samples using the Quantus.

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Reverse Transcription

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Promega GoTaq 2-Step RT qPCR System 19

Overview

Transcription is the process of creating RNA from a DNA template, but reverse transcription (also known as cDNA synthesis) is to the process of creating single stranded DNA from a RNA template.

Reverse transcription reactions require a reverse transcriptase enzyme, primers, buffers, and a ribonuclease inhibitor.

The primers most often used are:

oligo(dT), which binds to the polyA tail of mRNA

random hexamers, which bind randomly through the transcriptome

gene specific primers, which are complementary to specific genes

In this course, we will use the Promega **GoScript™ Reverse Transcription System.** The Promega GoScript™ Reverse Transcription System is a convenient kit that includes a reverse transcriptase and an optimized set of reagents for efficient synthesis of first-strand cDNA optimized in preparation for PCR amplification. This can be used to reverse transcribe RNA templates starting with either total RNA or poly(A)+ mRNA.

To watch a video of how to conduct reverse transcription with GoScript™, click on the QR code to the right. The full protocol can be access with the QR code, but an abbreviated version is included on the following page.

**Controls:** Always include a negative control of “no RT” from your cDNA synthesis reaction for each primer set to control for genomic DNA or other sources of contamination.

Note: Reverse transcription followed by quantitative real-time PCR can be performed in one or two steps. In this course, we will only use the 2 step approach, which allows for a more flexible workflow. In two-step RT-qPCR using GoTaq® qPCR Master Mix, samples of the GoScript™ Reverse Transcription System can be added directly, up to 20% v/v. Depending on the concentration of your cDNA, you may want to dilute you template before running qPCR.

Promega GoTaq 2-Step RT qPCR System

1. Thaw buffers, salts, and nucleotide primers and mixes at room temperature (but leave the RNasin® or GoScript in the freezer). Vortex and centrifuge. Confirm that no salts are present.
2. Calculate the number of RNA samples to be reverse transcribed. **N = \_\_\_\_\_\_**
3. Obtain samples from the -80°C and RNA on ice.
4. Obtain enough PCR tubes for **N+1 rxns = \_\_\_\_\_\_\_.** Label side with sample name/number

|  |  |  |
| --- | --- | --- |
| **Primer Master** | μL per rxn | (N+1) x 1.1 |
| Oligo(dT)15 primer | 1 |  |
| random primers | 1 |  |

1. Prepare Primer Master mix, with 10% extra (x1.1).
2. Pipette **2 μL Primer Master** into each PCR tube.
3. Add **9.5 μL RNA sample** to PCR tube. (Or, add volume containing 2 μg RNA then add water to bring volume added to 8 uL).
4. Add **8 μL RNA** (can be a pool of RNA or from a single sample) to tube for the noRT control sample
5. Vortex and centrifuge briefly. Place RNA back on ice.
6. Denature the RNA at 70°C for 5 minutes then chill at 4°C for 5 minutes using the thermal cycler program “Denature”. Place RNA back on ice.

|  |  |  |  |
| --- | --- | --- | --- |
| **GoScript™ Reaction Mix** | 1 rxn (μL) | N x 1.1 | **NoRT** |
| Nuclease-Free Water | 0 |  | 1.5 |
| GoScript™ 5X Reaction Buffer | 4 |  | 4 |
| MgCl2 | 2 |  | 2 |
| PCR Nucleotide Mix, 10mM | 1 |  | 1 |
| RNasin® Ribonuclease Inhibitor | 0.5 |  | 0.5 |
| GoScriptTM Reverse Transcriptase | 1 |  | 0 |

1. NOTE: **Do NOT vortex enzymes** or they can become inactivated. Mix by inverting the tube 3 times to mix and centrifuge briefly before use. Place on ice. Place Eppendorf tubes needed for making master mixes on ice before adding components.
2. Prepare the GoScript™ Reaction Mix on ice for the samples (with 10 % excess) and a No-RT rxn. On ice, add all reagents in the order listed. Mix by inversion. Centrifuge briefly.
3. Add **8.5 μL of GoScript Reaction Mix** to each RNA sample.
4. Add **8.5 μL NoRT** to NoRT PCR tube.
5. Synthesize cDNA with GoScript™ Reverse Transcriptase using the thermal cycler program “synthesis” which is programed for the following: 25°C for 5 min, 42°C for 60 min, 70°C for 15 min, and 4°C for infinity.
6. Label a 0.5 or 1.5 mL tube with the sample name/number AND the date for storing the sample.
7. When the thermal cycling is complete, place samples on ice.
8. Proceed immediately to PCR or store samples at -20°C.

Chapter 6

Quantitative Real-time PCR (qPCR)

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Overview

Quantitative Real-time PCR (qPCR) is a technique used to amplify and quantify specific DNA molecules. In order to amplify small amounts of DNA, one needs a gene-specific primer pair, deoxyribonucleotides, a suitable buffer solution, a thermo-stable DNA polymerase, and fluorophores. Amplified DNA fragments are detected using either **dye-based approaches** (such as SYBR green) or **probe-based approaches** (such as TaqMan).

**COMPAISON OF DETECTION METHODS**

Dye-based Detection

SYBR green, a commonly used florescent dye, binds to dsDNA. dsDNA quantification is monitored by measuring an increase in fluorescence throughout the cycle. Since SYBR binds all dsDNA in the sample, it is important to analyze the melting curve to check for the presence of multiple products (including primer dimers and non-specific amplification).

Probe-based Detection

Probe-based approaches use forward and reverse primers, as well as a probe. TaqMan probes are complementary to the DNA sequences and are bound to a fluorophore. When the polymerase starts copying the DNA fragment bound to the probe, the fluorophore is cleave and the fluorescent dye is excited. With this approach, primer dimers to not give off signal, so a melting curve is not needed.

Promega [GoTaq® qPCR Master Mix](https://www.promega.com/resources/protocols/technical-manuals/101/gotaq-qpcr-master-mix-protocol/) and [GoTaq® Probe qPCR Master Mix](https://www.promega.com/resources/protocols/technical-manuals/101/gotaq-qpcr-master-mix-protocol/) are provided as a simple-to-use, stabilized 2X master mixes that include all components for quantitative PCR except sample DNA, primers and water. qPCR experiments are typically run in 384 or 96 well plates. We will use 96 well plates.

Life Technologies has combined many resources for qPCR experimental design and troubleshooting into a “Real-time PCR handbook”. Quantitative PCR (qPCR) is a very sensitive measurement technique. Therefore, optimization of reaction conditions, consistency in technique, and care to avoid contamination are of utmost importance. While some optimization can be accomplished in an endpoint PCR reaction, the final optimization should be done with a qPCR reaction.

**Important Controls**

Always include a negative control, “no DNA template” for each primer set on each plate to control for non-specific binding. Always include a negative control of “no RT” from your cDNA synthesis reaction for each primer set to control for genomic DNA or other sources of contamination.

**Quantification Instruments**

At MBL, Applied Biosystems has generously loaned two StepOnePlusTM Real Time PCR instruments, which are located on the 3rd floor in Loeb 326. Please refer to the manuals in the lab for instructions on operation the instrument and the software.

Primer Design for qPCR

Note: these guidelines were used when designing primers used in this course

1. Identify exon-exon boundaries using BLAT
2. Go to an online primer design website such as Primer3, Primer3Plus, or IDT PrimerQuest
3. Paste nucleotide sequence where indicated
4. Mark the exon-exon boundary so that primer pairs will span this junction
5. Provide a name indicating species
6. Select the output to give a forward and reverse primer
7. Select the following primer specifications
   1. GC content of primers: 50-60%
   2. Select a melting temperature: 60 – 63 (with no more than 1C difference).
   3. Select min primer length 18, max length 24, optimal length 20
   4. pair separated by an exon-exon boundary (reduces, or at least detects genomic background)
   5. amplicon should be about 100 bp, usually 60-150 bp
   6. min length: 18, max length 24 (best: 20 nt) (not different by more than 3 bp)
   7. max 3' self-complementary: 1 bp
   8. max poly-x: 3
8. Once you have the output, select pairs that are:
   1. towards 3' end of gene (often more specific) or in UTR
9. Paste the output primer sequences with all the relevant info (Tm, length, etc.) for all the primer pairs listed into an excel spreadsheet.
10. Save on the NS&B shared folder, adding your initials to the file name.

Making primers solutions for dye-based PCR:

Synthesized primers are delivered as lyophilized powders. To use these primers, one first must make a stock primer solution (100 μM) that can be stored in freezer (in a separate box away from sources of genomic DNA or cDNA contamination) for many years. Two use primers in PCR, however, one must make working primer solutions (10 μM).

1. Record the manufacturer’s primer information in your notebook (e.g. Tm, sequence).
2. Spin down primer stocks.
3. To create a stock solution of the primer (100 μM), multiple # of nmols of primer by 10. Add that volume of water or 1X TE buffer. Vortex and spin.
4. Label the top of each stock primer solution with a shorthand name and the concentration.
5. Obtain a clean 1.5 mL tube to make a working 10 μM primer solution. Label with name and conc. (10 μM)
6. Add 225 μL water to each 1.5 mL tube.
7. Transfer 25 μL of 100 μM stock primer solution to labeled tube with water.
8. Store stock primer solution at -20°C and working primer solution at 4°C.

Making A Standard Curve

Creating a standard curve is necessary for calculating the concentration of each sample without respect to a control sample. This will also tell us important information about the efficiency (a topic we will cover during data analysis) of our qPCR reactions, an important piece of information needed for accurate DNA quantification.

1. Synthesize a large amount of cDNA from tissue representative of your samples.
2. Pool your cDNA. This is your 1:1 dilution, or your most concentrated standard
3. Calculate the number of genes you will be measuring. Multiply the number by three to determine the minimum volume of each standard you need (since each is run in triplicate). Since you will need to test your standard curve and maybe rerun some samples, multiply that number by three to estimate the volume needed.
4. Create a 10 point two-fold dilution series of cDNA from your species. (Refer to page 16 for an example of making 1:2 dilution series).
5. Test the efficiency of your standard curve by running a qPCR experiment in the “Standard Curve” method.
6. Store your samples at 4°C for short term or -20°C for long term storage.

qPCR Plate Layouts

Use the StepOnePlusTM software to design your plate layout. Below is an example plate layout for quantifying gene expression of one gene in twenty samples with ten standards, including both a NoRT and no template (H20) controls. Note that all reactions are run in triplicate.

If you make an pipetting errors, record those in your notebook and make the change in your save experimental setup before staring the run.

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | 1 | | | 2 | | | 3 | | | 4 | | |
| B | 5 | | | 6 | | | 7 | | | 8 | | |
| C | 9 | | | 10 | | | 11 | | | 12 | | |
| D | 13 | | | 14 | | | 15 | | | 16 | | |
| E | 17 | | | 18 | | | 19 | | | 20 | | |
| F | NoRT | | | Std 1 | | | Std 2 | | | Std 3 | | |
| G | Std 4 | | | Std 5 | | | Std 6 | | | Std 7 | | |
| H | Std 8 | | | Std 9 | | | Std 10 | | | H20 | | |

Protocol: Dye-Based Quantitative Real-time PCR (qPCR)

1. Determine number of samples (including negative controls) **N=\_\_\_\_\_**
2. Design plate layout to include a standard curve, NoRT and NO template control all run in triplicate.
3. Thaw the GoTaq® qPCR Master Mix on ice. Vortex and spin. Place on ice.
4. **CXR Note**: Check the top of the GoTaq® qPCR Master Mix tube for a black dot, which indicates that 17μLCXR dye has been added. If there is no dot, thaw CXR at room temperature and add 17μLCXR to the 1mL tube of GoTaq® qPCR Master Mix. Vortex, spin, and mark the tube with a dot to indicate that CXR was added
5. Calculate the volume of each master mix ingredient needed by multiplying the volume listed in the table by N and 1.1 (to give 10% extra)

|  |  |  |
| --- | --- | --- |
| **qPCR Master Mix** | **1 rxn ( uL)** | **x N x 1.1 (uL)** |
| 2X GoTaq® qPCR Master Mix | 5 |  |
| Nuclease-Free Water | 1.4 |  |
| Forward primer, 10μM | 0.3 |  |
| Reverse primer, 10μM | 0.3 |  |

1. Prepare a PCR Master Mix for each gene on ice. Vortex briefly and spin.
2. Obtain a 96 well optically clear plate designed for qPCR
3. Using a multichannel pipette, add **7 μL PCR Master Mix** to the appropriate wells.
4. Obtain your cDNA for the 4°C, vortex, spin, and place on ice.
5. Using a single channel pipette, add **3 μL cDNA** sample or control to the appropriate wells.
6. Seal the plate with optically clear adhesive film. Spin at 1000 rpm for 1 min.
7. Execute the “fast” thermal cycling reaction in the Applied Biosystems StepOnePlus instrument.

1x 95°C 2 min (denature)

37x 95°C 3 sec (denature)

­­60°C 30 sec (anneal and extend)

1x 60-95°C dissociation

Protocol: Probe-Based Quantitative Real-time PCR (qPCR)

1. Determine number of samples (including negative controls) **N=\_\_\_\_\_**
2. Design plate layout to include a standard curve, NoRT and NO template control all run in triplicate.
3. Thaw the GoTaq® Probe qPCR Master Mix on ice. Vortex and spin. Place on ice.
4. **CXR Note**: Check the top of the GoTaq® qPCR Master Mix tube for a black dot, which indicates that 17μLCXR dye has been added. If there is no dot, thaw CXR at room temperature and add 17μLCXR to the 1mL tube of GoTaq® qPCR Master Mix. Vortex, spin, and mark the tube with a dot to indicate that CXR was added
5. Calculate the volume of each master mix ingredient needed by multiplying the volume listed in the table by N and 1.1 (to give 10% extra)

|  |  |  |  |
| --- | --- | --- | --- |
| **Probe Master Mix** | 1 rxn (uL) | x N x 1.1 | Final Conc. |
| 2x GoTaq Probe qPCR master mix | 5.0 |  | 1x |
| Water | 1.5 |  |  |
| 20X TaqMan Assay | 0.5 |  | 900nM/900nM/250nM |

1. Prepare a **master mix** for each gene. Vortex briefly and spin.
2. Obtain a 96 well optically clear plate designed for qPCR
3. Using a multichannel pipette, add **7 μL PCR Master Mix** to the appropriate wells.
4. Obtain your cDNA for the 4°C, vortex, spin, and place on ice.
5. Using a single channel pipette, add **3 μL cDNA** sample or control to the appropriate wells.
6. Seal the plate with optically clear adhesive film. Spin at 1000 rpm for 1 min.
7. Execute the “fast” thermal cycling reaction in the Applied Biosystems StepOnePlus instrument.

1x 95°C 2 min (denature)

37x 95°C 3 sec (denature)

­­60°C 30 sec (anneal and extend)

Data Analysis

After running a qPCR experiment, you must determine the concentration of your candidate genes in each sample. One of the most popular methods is the Delta Delta Ct method, which involves normalization to a reference or control gene and a reference sample. Two methods that do not require normalization to a control gene are the normalization by RNA or cDNA quantity and MCMC.qpcr. We will briefly describe those here, but we will use the latter two in this course.

**DELTA DELTA CT METHOD**

This method involves comparing the Ct values of the samples of interest with a control sample. The Ct values of both the control and the samples of interest are normalized to an appropriate endogenous housekeeping gene. The name comes from the math:

[delta][delta]Ct = [delta]Ctsample - [delta]Ctreference

Here, “[delta] Ctsample” is the Ct value for any sample normalized to the endogenous housekeeping gene and “[delta] Ctreference” is the Ct value for the control also normalized to the endogenous housekeeping gene. For the [delta][delta]Ct calculation to be valid, the amplification efficiencies of the target and the endogenous reference must be approximately equal. If a housekeeping gene cannot be found whose primer amplification efficiency is similar to the target, other methods are preferred.

**NORMALIZATION BY RNA OR cDNA INPUT QUANTITY**

The use of control genes is not always recommended. In those cases, the user can quantify the cDNA input and either use the same concentration of cDNA for every reaction or the user can divide the relative concentration of the sample (calculated from a standard curve) by the sample concentration.

**MCMC.qPCR**

MCMC.qPCR is an R package for analyzing raw qPCR data (represented as molecule counts) using generalized linear mixed models under Poisson-lognormal error. This method can incorporate prior knowledge about the expected degree of control gene stability can be directly incorporated into the model, but the model can also be run in the absence of control genes. Scan the QR code to the right to read the 2013 PLOS ONE manuscript “No Control Genes Required: Bayesian Analysis of qRT-PCR Data” by Matz, Wright, and Scott.

In order to use this method, we will first teach you the basics of R, the free software environment for statistical computing and graphics. One of R's strengths is the ease with which well-designed publication-quality plots can be produced, including mathematical symbols and formulae where needed. R is highly extensible through the use of user-submitted packages for specific functions or specific areas of study.

Chapter 7

Immunohistochemistry

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For sectioning protocol, see Chapter 2, page 8

Immunohistochemistry is a technique used to detect proteins in cells of a tissue using primary (1°) antibodies specific to proteins of interest and secondary (2°) antibodies specific to the primary antibody. Visualizing an antibody-protein interaction can be accomplished in a number of ways. One of the most common techniques for visualization is the use of an antibody that is conjugated to an enzyme, such as peroxidase, that can catalyze a color-producing reaction that can be visualized with a light microscope. Alternatively, the 2°antibody can also be tagged to a fluorophore that can be visualized with a fluorescent microscope.

Tissue Processing

**Overview**

When performing an immunohistochemistry experiment, the tissue must be **fixed** most commonly with 4% paraformaldehyde (PFA) to maintain cell morphology and tissue architecture. Next, the tissue is **cryoprotected** in a sucrose solution to protect it from freezing damage. The tissue can then be **embedded** in a mounting medium (O.C.T) and sectioned. Sections can be made on a variety of instruments, most commonly a microtome or cryostat, and are sliced at a range of 4-40 μm.

**Protocol**

1. Obtain cold, fresh 4% paraformaldehyde (PFA) from the fridge.
2. Dissect tissue.
3. Fix. Place whole brain in cold 4% paraformaldehyde and store at 4°C overnight (~16-24 hours post-harvest).
4. Cryoprotect.
   1. Carefully pour out PFA into hazardous waste container.
   2. Add cold 1X PBS (fill vial half way) to the vial with the brain and wait 5 min.
   3. Carefully pour off 1X PBS and repeat step b two more times (for a total of 3 washes).
   4. Pour in cold 30% sucrose solution.
   5. Store at 4°C overnight (or until tissue no longer floats).
5. Embed.
6. Obtain a tube or cryomold of appropriate size for your tissue.
7. Fill tubes ¾ of the way with O.C.T. compound. Using the spinal cord to grip the brain, place it into the O.C.T. compound. Straighten out as much as possible. Mark the dorsal midline with a marker.
8. Place on dry ice until frozen then store upright in the -80˚C.
9. Sectionthe brain according to the protocol in Chapter 2 on page 8.

Immunohistochemistry Antibodies

This year, we have five primary antibodies available for use with weakly electric fish tissues:

|  |  |  |
| --- | --- | --- |
| **Primary Antibodies** | | |
| **Protein Target** | **IgG** | **Supplier** |
| Androgen receptors (AR) | Rabbit Anti AR | Millipore (06-680) |
| c-Fos | Rabbit Anti-c-Fos | Santa Cruz (sc-253) |
| c-Fos | Goat Anti-c-Fos | Santa Cruz (sc-253-G) |
| Estrogen receptor alpha (ERα) | Rabbit Anti ERα | Millipore (06-935) |
| Tyrosine hydroxylase (TH) | Mouse Anti-TH | Millipore (AB152) |

|  |  |  |  |
| --- | --- | --- | --- |
| **Secondary Antibodies** | | | |
| **Target IgG** | **IgG** | **Visualization** | **Supplier** |
| Goat IgG | Alexa Fluor 568 Donkey Anti-Goat | Orange-Red Fluorescence | Invitrogen (A-11057) |
| Mouse IgG | Alexa Fluor 488 Goat Anti-Mouse | Green Fluorescence | Invitrogen (A-11001) |
| Rabbit IgG | Alexa Fluor 647 Goat Anti-Rabbit | Far Red Fluorescence | Invitrogen (A-21244) |
| Rabbit IgG | Alexa Fluor 488 Donkey Anti-Rabbit | Green Fluorescence | Invitrogen (A-21206) |
| Rabbit IgG | Biotinylated Goat Anti-Rabbit | Brown Precipitate | Vector Labs (BA-1000) |

Secondary (2°) antibodies from Vector labs: Use the secondary that matches your primary antibody animal. For example, if you are using a rabbit anti-tyrosine hydroxylase, use the goat anti-rabbit.

Sera: Use serum from the species that your 2° was raised in. For example, if you are using a goat anti-rabbit secondary, then you want to use normal goat serum in your antibody solutions.

Immunohistochemistry Solutions

Label all solutions with name, date, and your initials.

1. 10X Phosphate Buffered Saline PBS. Prep time ~5 min.
   1. Dissolve 1 tablet in 200 mL Ultrapure water.
   2. Store at room temperature
2. 1X PBS. Prep time ~5 min.
   1. In small bottles, add 50mL 10X PBS and 450 ml Ultrapure water.
   2. Store at 4˚C.
3. 2X PBS. Prep time ~5 min.
   1. In small bottles, add 100mL 10X PBS and 400 ml Ultrapure water.
   2. Store at room temperature
4. Paraformaldehyde (PFA). Prep time ~2 hours
   1. In a small flask, add 20mL Ultrapure water, 5μL10N NaOH, and 1.6 grams PFA
   2. Cover the mouth with Parafilm, then heat and stir at 60˚C for 1 hour (or until PFA is completely dissolved).
   3. Cool the solution to room temperature
   4. Add 20mL of 2X PBS
   5. Adjust pH to 7.2-7.4. Label vial flask with pH and today’s date
   6. Store at 4˚C for up to 24 hours. Unused PFA can be stored at -20°C.
5. 30% sucrose. Prep time ~5 min.
   1. In a flask, add 30g sucrose to 100 mL 1X PBS
   2. Stir until dissolved.
   3. Filter via vacuum filtration.
   4. Store at 4˚C
6. Citric Acid. Prep time ~5 min.
7. 1.92g citric acid (anhydrous)
8. 950mL nanopure water
9. Adjust pH to 6.0 with 2.6mL 10N NaOH.
10. Add water to 1L.
11. Add 0.5mL Tween20

|  |  |  |  |
| --- | --- | --- | --- |
| Quench Master Mix | Final Conc. | Vol. per slide | Vol. per \_\_ slides |
| 30% H2O2 | 10% | 30 µL |  |
| TritonX | 0.3% | 0.9 µL |  |
| 1X PBS | to vol | 269.1 µL |  |
| Final Volume |  | 300 µL |  |

Brightfield Immunohistochemistry

**Day 1 IHC:**

1. Thaw slides at low temp on warmer for 3-4 hours
2. Pap pen perimeter of slides
3. Fix slides in chilled PFA for 10 min
4. Wash 2x in 1X PBS for 5 min each
5. Add 300 μL quenching solution to each slide – incubate 20 min at room temperature
6. Wash 2x in 1X PBS bath for 5 min each
7. Antigen retrieval: Boil citric acid in microwave, add to slides in bath chamber and incubate 2 min. Repeat with fresh citric acid for 5 min. Repeat again for 5 min.
8. Wash 2x in 1X PBS bath for 5 min each (make blocking solution during second wash)
9. In hybridization chamber, add 300 μL blocking solution to each slide – incubate 1 hour at room temperature (make 1˚Ab when 10 min remaining)
10. Add 300 μL1˚Ab solution to each slide (on top of blocking solution) – incubate overnight at room temperature in hybridization chamber

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  | Blocking Solution | | | 1˚Ab | | | no 1˚Ab ctl |
|  | Final | Vol. per slide | Vol. for \_\_\_ slides | Final | Vol. per slide | Vol. for \_\_\_ slides | Vol. for \_\_\_ slides |
| 1˚Ab | - | - |  |  | 0.6 µL (1:500) |  | - |
| Normal Goat Serum | 5% | 15 µL |  | 2% | 6 µL |  |  |
| TritonX-100 | 0.3% | 0.9 µL |  | 0.3% | 0.9 µL |  |  |
| 1X PBS | to vol | 284.1 µL |  | to vol | 292.5 µL |  |  |
| Final Volume |  | 300 µL |  |  | 300 µL |  |  |

|  |  |  |  |
| --- | --- | --- | --- |
| 2˚Ab | | | |
|  | Final | Vol./ slide | Vol.\_\_\_ slides |
| 2˚Ab | 1:200 | 1.5 µL |  |
| Serum | 2% | 6 µL |  |
| TritonX-100 | 0.3% | 0.9 µL |  |
| 1X PBS | to vol | 290.1 µL |  |
| Final Volume |  | 300 µL |  |

**Day 2 IHC:**

1. Wash 2x in 1X PBS for 10 min each
2. Add 300 μL2˚Ab solution to each slide – incubate 2 hours at RT (make ABC when 20-30 min left)
3. Wash 2x in 1X PBS for 10 min each
4. ABC: 300uL/slide – 1hr incubation at RT
5. Wash 2x in 1X PBS for 10 min each
6. Apply DAB substrate: 500uL/slide – 2-3min RT
7. Rinse in H2O. Ethanol dehydrate and coverslip

**ABC:**

2.5mL of 1X PBS

1 drop reagent A

1 drop reagent B

**DAB:**

2.5mL H2O

1 drop buffer

2 drops DAB

1 drop H2O2

Caution with DAB – anything that touches DAB MUST be neutralized with bleach before it goes down the sink

Fluorescent Immunohistochemistry

|  |  |  |  |
| --- | --- | --- | --- |
| Quench Master Mix | Final Conc. | Vol. per slide | Total vol. needed |
| 30% H2O2 | 10% | 30 µL |  |
| TritonX | 0.3% | 0.9 µL |  |
| 1X PBS | to vol | 269.1 µL |  |
| Final Volume |  | 300 µL |  |

**Day 1**

1. Thaw slides on warmer 3-4 hours
2. Pap pen the perimeter of the slides
3. Fix in **chilled** 4% PFA at RT for 10m
4. Wash 2x in 1X PBS at RT for 5m
5. Add 300 μL quenching solution to each slide – incubate at RT for 20m (Optional step for fluorescent staining)
6. Wash 2x in 1X PBS bath at RT for 5m
7. Antigen Retrieval: Boil citric acid in microwave (microwave 25-50mL more than you need, boil 75mL for Coplin jars holding <10 slides, and 550mL for a large 50 slide chamber). Add to slides and incubate 2m. Repeat with *fresh* citric acid for 5m. Repeat againfor 5m.
8. Wash 2x in 1XPBS at RT for 5m [make blocking solution during 2nd wash]
9. Blocking: 300uL/slide – 1 hr. at RT [make 1˚Ab when 10m left]
10. 1˚Ab: 300uL/slide – overnight at RT in hybridization chamber

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  | Blocking Solution | | | 1˚Ab | | | no 1˚Ab ctl |
|  | Final | Vol. per slide | Vol. for \_\_\_ slides | Final | Vol. per slide | Vol. for \_\_\_ slides | Vol. for \_\_\_ slides |
| 1˚Ab | - | - |  |  | 0.6 µL (1:500) |  | - |
| Serum | 5% | 15 µL |  | 2% | 6 µL |  |  |
| TritonX-100 | 0.3% | 0.9 µL |  | 0.3% | 0.9 µL |  |  |
| 1X PBS | to vol | 284.1 µL |  | to vol | 292.5 µL |  |  |
| Final Volume |  | 300 µL |  |  | 300 µL |  |  |

|  |  |  |  |
| --- | --- | --- | --- |
| 2˚Ab – **Prepare in the dark** | | | |
|  | Final | Vol. per slide | Vol. for \_\_\_ slides |
| 2˚Ab\_\_\_\_\_\_\_ | 1:200 | 1.5 µL |  |
| 2˚Ab \_\_\_\_\_\_\_ | 1:200 | 1.5 µL |  |
| Serum | 2% | 6 µL |  |
| TritonX-100 | 0.3% | 0.9 µL |  |
| 1X PBS | to vol | 290.1 µL |  |
| Final Volume |  | 300 µL |  |

**Day 2**

1. Wash 2x in 1XPBS at RT for 10m

**Everything below must be done in the DARK!**

1. 2˚Ab: 300uL/slide at. RT for 2 hr
2. Wash 2x in 1XPBS at RT for 10m
3. Rinse in H2O. Coverslip in DAPI hardset.
4. Allow to dry, then store at 4°C in light-tight box.

Microscopy

Microscopes have greatly enabled our ability to visualize neural structures and to better understand brain function. In this module, you will be using brightfield and fluorescent microscopy to quantify the number of cells expression a given protein.

The Introductory Biology Lab at The University of Austin has developed a number of video tutorials that teach the basics of microscopes. Please view these videos as a refresher for proper microscope techniques.

Stereology

Stereology comprises a number of practical techniques for extracting quantitative information about a three-dimensional material from measurements made on two-dimensional planar sections of the material. Stereology methods utilize random, systematic sampling to provide unbiased and quantitative data. It is an important and efficient tool in many applications of microscopy

The Stereo Investigator system for stereology gives the user accurate, unbiased estimates of the number, length, area, and volume of cells or biological structures in a tissue specimen. It is a key research tool that has helped lead advances in numerous areas of neuroscience. We will be using Stereo Investigator in this module. Scan the QR code to access the Stereo Investigator website.

Chapter 8

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DNA Isolation

DNA isolation is the process of purifying DNA from a biological sample. DNA isolating procedures include steps for cell lysis; lipids, protein, and RNA removal; and elution into a aqueous solution. In the course, students will become familiar with column based and automated extraction approaches.

**COLUMN-BASED RNA EXTRACTION**

The **Wizard® Genomic DNA Purification Kit** provides a simple, solution-based method for isolation of DNA from white blood cells, tissue culture cells, animal tissue, plant tissue, yeast and Gram-positive and Gram-negative bacteria. DNA purified with this system is suitable for a variety of applications, including amplification, digestion with restriction endonucleases and membrane hybridizations.

For the complete Wizard® Genomic DNA Purification Kit Protocol, scan the QR code to the right.

**AUTOMATED DNA EXTRACTION**

The **Maxwell® 16 Tissue DNA Purification Kit** is used with the Maxwell® 16 Instrument to provide an easy method for efficient, automated purification of genomic DNA from up to 50mg of tissue samples. The Maxwell® 16 Instrument is supplied with preprogrammed purification procedures and is designed for use with the predispensed reagent cartridges, maximizing simplicity and convenience. The instrument can process up to 16 samples in 45 minutes. The purified DNA can be used directly in a variety of downstream applications including PCR, restriction enzyme digestion and agarose gel electrophoresis. The plunger action of the Maxwell® 16 Instrument grinds solid tissue samples directly in lysis buffer in the prefilled reagent cartridge. Integrated tissue grinding replaces the time and labor-intensive use of proteases or manual tissue grinding. We have protocols for a variety of DNA samples.

For the complete Maxwell® 16 Tissue DNA Purification Kit protocol, scan the QR code to the right.

Endpoint PCR

The polymerase chain reaction (PCR) is a biochemical technology in molecular biology used to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. In order to conduct PCR, one must first design primers that are specific to the gene of interest. Then, you prepare a solution of polymerase, primers, cDNA template, dNTPs, salts, and buffers. This reaction is placed in a thermal cycler for multiple rounds of denaturing, annealing, and extending the DNA. Products are then visualized using gel electrophoresis.

**PRIMER DESIGN FOR ENDPOINT PCR**

We demonstrate the basics of primer design using the leech vasopressin-like (aka hirudotocin) gene

>Hirudo­\_medicinalis\_vasopressin\_like\_FP628609.1

CCAAATAACTAAGATTTGATAGCTAAAAGATCAGACGTATAGCCGTTTCTACTGACCAAAGTAACAAA**ATG**CGTGTGACCAGCCTTTTCTTGCTCAACAGTTTGTGCTTGATGGCAGTGTCCGTGTTTGTCGCTGAAGCCTGCTTCATCAGAAATTGTCCTCTTGGTGGAAAGAAAAGGTCCCTTGTCGAGTACTTTACCAGTGGACCACGTAGGTGCCCTTCCTGTAGCCTCGAACTGTCAGGCGAACGTCTGACGGGAACATGCGTCTCGGCTGAGTTGTGTTGCCATGGAAATCATGGATGCTTCTCAGACAGAGAAATAACCAGTTCTTGTCGGAGCGAGAACCTCATTTCAACTCCGTGCTTCGTGAAAGGAAAGACGTGCGGCAGCAACGGATCTGGAATTTGCGTGACCAGAGGAGTTTGTTGCGAGGACGAAGGATCTTGTCGTCTGGATGGAGATTGCTCTCACCAA**TGA**ACTCGGAAATCTTTGTGGACGAACTTACTGCGAATTTTTCTCAAACCCGAAATTATATTATATTTTCCATCCGACTTTTACTATCAATAAAATGTTTACTTTTACTATCAATAAACA

1. Go to an online primer design website such as Primer3, Primer3Plus, or IDT PrimerQuest
2. Paste nucleotide sequence where indicated
3. Provide a name indicating species (i.e. Hm) and gene (i.e. hirudotocin)
4. Select the output to give a forward and reverse primer
5. Select GC content of primers: 40-60%
6. Select a melting temperature: 60 - 63
7. Select min primer length 18, max length 24, optimal length 20
8. View primer details
9. Copy the primer sequences with all the relevant info (Tm, length, etc.) for all the primer pairs listed into a word document.
10. Choose two sets of forward and reverse primers (1F, 1R, 2F, 2R) for purchases
    1. Pick an “outer” pair that is close to the 5’ and 3’ UTR
    2. Pick an “inner” pair that sits inside the outer pair.
11. Paste the primer sequences next to the primer name below as indicated below.
12. Save as on the NS&B shared folder, adding your initials to the file name.

Hm hirudotocin 1F **AGCCGTTTCTACTGACCAAAG**

Start 41 Stop 62 Length 21 Tm 62 GC% 47.6

Hm hirudotocin 1R  **CAATCTCCATCCAGACGACAAG**

Start 445 Stop 467 Length 22 Tm 62 GC% 50

Hm hirudotocin 2F **CTCAACAGTTTGTGCTTGATGG**

Start 93 Stop 115 Length 22 Tm 62 GC% 45.5

Hm hirudotocin 2R  **TCCTCTGGTCACGCAAATTC**

Start 403 Stop 423 Length 20 Tm 62 GC% 50

**REACTION SETUP FOR ENDPOINT PCR**

GoTaq® Green Master Mix is a premixed ready-to-use solution containing bacterially derived Taq DNA polymerase, dNTPs, MgCl2 and reaction buffers at optimal concentrations for efficient amplification of DNA templates by PCR. GoTaq® Green Master Mix contains two dyes (blue and yellow) that allow monitoring of progress during electrophoresis. Reactions assembled with GoTaq® Green Master Mix have sufficient density for direct loading onto agarose gels.

For the complete GoTaq® Green Master Mix Protocol, click the QR code.

**USING THE THERMAL CYCLER FOR ENDPOINT PCR**

PCR stands for Polymerase Chain Reaction. The process centers around heating DNA to a temperature where the double strands will break apart or denature (95°C), cooling the DNA to a temperature where the primers can anneal or base pair (typically 50-60°C), and heating to a temperature where the polymerase can copy the strand bound to the primer (72°C). This procedure of heating and cooling is repeated many times (typically 25-37 cycles) to allow for logarithmic amplification of target DNA fragments. It is called “endpoint PCR” because the PCR productive is quantified at the end of all cycles.

Exercise for Optimizing Thermal Cycler Settings

Optimizing the annealing temperature is an important step for ensuring a successful PCR reaction. This should be done on primers you design and on primers you obtain from the literature or a colleague. This is most efficiently done on a thermal cycler that allows a gradient across the block.

1. Calculated Tm \_\_\_\_
2. Set the thermal cycler to run 7 different annealing temperatures: +0°C +2°C, +4°C, +6°C, -2°C, -4°C, -6°C
3. Prepare 14 reactions using the exact same master mix ingredients.
4. Set the thermal cycler to run only 20-30 cycles so that amplification reactions do not saturate.
5. Set the extension time to be 1 min per 1kb
6. Use band brightness on the gel to select optimal temperature for single band product
7. Confirm optimization using 1 degree steps around determined “optimal”

Exercise: Determine Best Primer Concentration

For your PCR reaction, you want to use the lowest concentration of primer that yields maximal product. A final concentration of 200 nM per primer is effective for most reactions, but optimal results may require a titration of primer concentrations between 100 and 500 nM. To determine this optimal concentration, you can run multiple PCR reactions holding all ingredients constants but varying primer concentration (and subsequently water volume).

1. Set up endpoint PCR reaction using GoTaq® Green PCR Master Mix with following final concentrations of the forward and reverse primer (always keep concentration of forward and reverse equal): 100nM, 200nM, 300nM, 400nM , 500nM.
2. Prepare a negative control that has primers but no DNA template.
3. Run your reaction in the thermal cycler using the anneal temperature identified early in the course or specified by the teacher.
4. Use band brightness on the gel to select the optimally bright band at the lowest primer concentration.

Chapter 9

Integrative Molecular Neuroethology Laboratory Notebook

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Notebook PagesI-XX

Guidelines for Keeping a Laboratory Notebook

The skill of writing the Laboratory Notebook is a vital part of industrial and academic research. It is a difficult but important part of science. It is required by law to establish intellectual and patent rights. The lab notebook is the property of the lab, not the property of the person doing the work. The objective of a laboratory notebook is to:

* record what, why, how (step by step), and where something was done

### allow someone else to recreate your work process.

### Laboratory notebooks should be hardback bound books with numbered pages, and the writing must be done in durable ink. Do not erase. Do not tear out pages. Do not doodle in your lab notebook.

ORGANIZING YOUR NOTEBOOK

* On the first page, describe your project and state the hypothesis. Later, summarize your results and conclusions
* **Keep a table of contents**. Every page should be accounted for in the table of contents.
* Include the date (YYY/MM/DD) on every page!
* Write the title of your on every page (a one word nickname is sufficient
* Write the protocol you are using at the top of the page (i.e. Maxwell® RNA Isolation)

THE PROTOCOL

This is a detailed description of your research **plan**. It is written before you start into your lab work.

This plan is based upon reading the lab handout before coming to lab. This is the part of the account that tells what you think you are going to do.

* Use simple, direct statements or a numbered list of instructions describing the protocol.
* Account for solutions etc. that need to be prepared.
* **Safety**! Make note of any chemicals or practices that may be dangerous proper handling.

THE DAILY EXECUTION

This is the part of the account that tells **what you actually did** that was a modification on the written protocol, and includes any mistakes, missed steps, pipetting errors etc.

* Identify precisely what equipment was used.
* Describe or diagram settings, adjustments or calibrations
* Record changes in incubation times if you deviate from the protocol
* Record lot#, expiration dates, identifying characteristics of organisms, etc…
* Record what parts of the experiment you do and what parts someone else does.

OBSERVATIONS AND DATA

* Record honestly and completely, without bias.
* Record as you go along, in the notebook, in ink, immediately.
* Do not trust valuable information or data to memory, even for a minute
* Do not use odd scraps of paper or the edge of your lab coat to record data, or make calculations. Only if your data is in a notebook will it be available for future analysis. Use good penmanship. Take care with numbers.
* Never over-write, always cross out mistakes with a single line and re-write the correct data.
* If errors are found, or notes are added at a future date, indicate the date that change is made.
* When data are recorded directly into a computer, note the name and location of the file.
* When data are recorded in another notebook, indicate the location of those data.

GRAPHS

* Do not underestimate the value of even a hand drawn graph to summarize data.
* Each graph should have the title and labeled axes, with units.
* Each graph should indicate the data that are included in the graph.
* Any graphs produced on a computer should be attached to the notebook. The name and location of the computer file must be recorded in the lab notebook.

PRIMERS

Keep track of all primers used in your experiments. You WILL need this for publication! Record the purpose of each primer (isolation, qPCR), the sequence, the concentration used, and whether or not it worked. Be sure to highlight in some way the primers that were successful.

CONCLUSION

* Write any calculations out clearly, showing all the steps and units.
* Record which statistical tests were applied to the data, stating which factors were analyzed. Record what computer program was used for the statistical analysis as well as the name of any computer files generated in the process.
* Briefly state your results.
* Briefly, interpret your results in relation to your hypothesis.
* If results were not as expected, explain how were they different, and propose an explanation for why they are different?
* State your conclusions clearly.
* Include brief suggestions for improvement in experimental design.
* Include brief suggestions for future experiments.
* Record any ideas you have, however brief - if you don't write them down, you'll forget them, and no one will know you had them.

**Appendix: Links**

Page 2. *Astatotilapia burtoni* *mc4r* cDNA sequence: <http://www.ncbi.nlm.nih.gov/nuccore/NM_001287403.1>

Page 3. Clustal Omega: <http://www.ebi.ac.uk/Tools/msa/clustalo/>

Page 8. Video: Using the cryostat: <https://www.youtube.com/watch?v=d43LFVV3h6w>

Page 11. ReliaPrepTM RNA Tissue Miniprep System: <https://www.promega.com/resources/protocols/technical-manuals/101/reliaprep-rna-tissue-miniprep-system-protocol/>

Page 11. Maxwell® 16 Tissue LEV Total RNA Purification Kit: <https://www.promega.com/products/dna-and-rna-purification/rna-purification/maxwell-16-system-rna-purification-kits/maxwell-16-tissue-lev-total-rna-purification-kit/>

Page 15. Video: Quantus™ Fluorometer Sample Quantification: <http://www.promega.com/resources/multimedia/instruments/quantus-fluorometer-sample-measurement/>

Page 18. Video GoScript™ Reverse Transcription: <https://www.promega.com/resources/multimedia/pcr/goscript-reverse-transcription-system-video/>

Page 18. GoScript™ RT System Protocol: <https://www.promega.com/products/pcr/rt-pcr/goscript-reverse-transcription-system/>

Page 21. GoTaq® qPCR Master: <https://www.promega.com/products/pcr/qpcr-and-rt-qpcr/gotaq-real-time-qpcr-and-rt_qpcr-systems/>

Page 21. GoTaq® Probe qPCR Master Mix: <https://www.promega.com/products/pcr/qpcr-and-rt-qpcr/gotaq-probe-qpcr-master-mix/>

Page 24. Video: qPCR experiment setup: <http://www.promega.com/resources/multimedia/pcr/gotaq-qpcr-master-mix-video/>

Page 26. MV Matz et al. 2013 PLOS ONE: <http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0071448>

Page 33. Video: Fluorescent microscopy: <http://www.youtube.com/watch?v=fZn6FkixiHI>

Page 33. Stereo Investigator: <http://www.mbfbioscience.com/stereo-investigator>

Page 35. Wizard® Genomic DNA Purification Kit Protocol: <https://www.promega.com/resources/protocols/technical-manuals/0/wizard-genomic-dna-purification-kit-protocol/>

Page 35. Maxwell® 16 Tissue DNA Purification Kit: <https://www.promega.com/products/dna-and-rna-purification/genomic-dna-purification-kits/maxwell-16-system-dna-purification-kits/maxwell-16-tissue-dna-purification-kit/>

Page 37. GoTaq® Green PCR Master Mix: <https://www.promega.com/resources/protocols/product-information-sheets/g/gotaq-green-master-mix-m712-protocol/>

Page XXIII. Feedback Survey: <http://tiny.cc/c91vhx>

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We are also want to acknowledge all the NS&B management, faculty members, teaching assistants, and course assistants who made this module possible.

We hope that this manual will help neuroethologists to integrate these established molecular methods into their own research programs. If you used any of the resources provide herein, we would appreciate your feedback regarding its quality and utility. Please visit <http://tiny.cc/c91vhx> to complete a short survey and leave your specific comments. Your feedback is much appreciated.