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Gryllus Techniques and Protocols

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Please note that protocols were developed and updated by Dr. Taro Nakamura, as well as Extavour Lab members Dr. Ben Ewen-Campen and Seth Donoughe. I have added notes and minor adjustments, but did not pioneer any of the protocols.

Antibody Staining (Immunohistochemistry)

To visualize gene expression in cricket embryos

General Notes:
- Embryos are kept in 1.5mL eppendorf tubes.
- All washes are 5 minutes, room temperature and in ~1.4mL solution unless otherwise stated. Swirl to mix after adding solution unless otherwise stated.
- Be careful not to expose embryos to air when removing washes.
- Pipette solution on side of tube to avoid harshly disrupting embryos.
- Throughout, be careful not to pipette up or destroy embryos.

Day 1*:*

1) Rehydrate embryos that have been stored in methanol. Either leave washes on a rotating tube rack or swirl to ensure appropriate mixing.
    a. 75% MeOH in PBSTw
    b. 50% MeOH in PBSTw
    c. 25% MeOH in PBSTw
2) Wash 3x in PBSTw. Either leave washes on a rotating tube rack or swirl to ensure appropriate mixing.
3) Block in 300µl of 5% NGS/PBSTw for 90min.
4) Incubate in your primary antibody mix at a manufacturer suggested concentration, overnight at 4°C.

Day 2:

1) Wash 3x in PBSTw for 1 minute. After each wash, swirl, let embryos collect in the bottom of tube, and then change wash.
2) Wash 6x in PBSTw for 60 minutes.
3) Block in 300µl of 5% NGS/PBSTw for 90min.
4) Incubate in your secondary antibody mix and a nuclear stain at a manufacturer suggested concentration, overnight at 4°C. Cover with tinfoil to avoid photobleaching. Typically, we have found 1:1,000 dilution for all the Alexa-coupled secondary antibodies and 1:5,000-1:10,000 for Hoeschst works well.

Day 3:

- Embryos should be kept covered with tinfoil from the secondary antibody incubate forward. You can remove tinfoil to change wash solution, but keep covered during washes.

1) Wash 3x in PBSTw for 1 minute. After each wash, swirl, let embryos collect in the bottom of tube, and then change wash.
2) Wash 6x in PBSTw for 60 minutes.
3) Post-fix in 4% formaldehyde in PBSTw for 30 minutes.
4) Wash 3x in PBSTw for 1 minute. After each wash, swirl, let embryos collect in the bottom of tube, and then change wash.
5) Wash 3x in PBSTw.
6) Store embryos in 50µl of Vector Laboratories Vectashield Mounting Medium, at 4°C. Once mounted, embryos on slides can be stored in colder temperatures.

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Recipes

PBS:

Add 2.05g NaH_2PO_4-H_2O and 11.98g NaHPO to 800mL of DEPC-H_2O.
Measure pH. If pH is not 7.4 +/-0.4 adjust with HCl or NaOH.
Add 81.76g NaCl

PBSTw:

Dilute 50mL 10X PBS to 1X with 895mL DEPC-H_2O.
Autoclave and cool.
Add 5mL 20% Tween-20

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gDNA Extraction

To remove the genomic DNA from cricket cells for further use, such as genotyping.

General Notes:
- I have had success using this protocol with cricket eggs, or crickets late instar or older should be used.
- Pestles can be made by briefly heating a p1,000 pipette tip with a Bunsen burner, and then swirling the melty tip in a 1.5mL eppendorf tube. Do not use pestle if there is still an opening in the bottom.

X Instar and Above:
1) Anesthetize crickets using carbon dioxide until they stop moving. Can be done in batches of ~10, depending how quickly you can perform the following steps.
2) Under a dissection scope, lay a KimWipe. Use microscissors to cut off the middle leg from either side of the cricket. Place the leg into a 1.5mL eppendorf tube and return the cricket to its cage to recuperate. Perform serially, cleaning microscissors with 70% EtOH in H_2O in between crickets. Can store continue using fresh, or store frozen at -80°C.

Genomic Prep:
1) Add 100µl Buffer A to each sample.
   → Set dry heat block to 65°C to begin warming.
2) Grind by pestle until sample is visibly disrupted.
3) Add 300µl Buffer A to each sample; rinse pestle over tube as you pipette, to collect sample stuck to pestle.
4) Incubate 30 min at 65°C.
5) Add 800µl LiCl/KAc solution, invert repeatedly to mix.
6) Incubate 10min on ice. Do not incubate over 10 minutes.
7) Spin 15min at 12,000rpm in a microfuge room temperature.
8) Transfer 1mL supernatant to new Eppendorf tube, excluding floating crud.
9) Add 600µl isopropanol and invert several times to mix.
10) Spin 20min at 12,000rpm in a microfuge at room temperature.
11) Aspirate and discard s/n. Quick spin. Aspirate again.
12) Wash pellet with 500µl cold 70% EtOH in H_2O.
13) Spin 10min at 12,000rpm in a microfuge at room temperature.
14) Aspirate and discard s/m. Quick spin. Aspirate again.
15) Air dry pellets ~10min.
16) Resuspend in 50µl TE (or ~10µl per embryo) for several hours at room temperature or overnight at 4°C.
17) Measure concentration with spectrophotometer.
18) Store at -20°C.

Recipes:
Buffer A

	Per prep (µl)
1M Tris-HCl, pH 7.5	40
500 mM EDTA, pH 8.0	80
5M NaCl	8
10% SDS	20
H_2O	To 400

LiCl/KAc Solution

	Per prep (µl)
5M KAc	230
6M LiCl	570

Total RNA Extraction & cDNA Synthesis

To extract RNA from embryos and make a library of cDNA from cricket embryos, as a more stable alternative to mRNA.

General Notes:
- Be certain to clean bench and pipettes first with water and 70% ethanol.
- Use ~20 embryos per tube.
- Can repeat cDNA reaction with 2x, 3x, 4x etc volume if the reaction fails.

1) Add 200µl Trizol.
   *Note: Steps involving Trizol must be performed under a fume hood and with caution, as Trizol is toxic.
2) Homogenize with pestle, by hand.
3) Add 800µl Trizol; rinse pestle over tube as you pipette, to collect sample stuck to pestle.
4) Spin 10min at 12,000rpm at 4°C in a microfuge.
5) Incubate 5min at room temperature.
6) Add 200µl chloroform.
   * Note: Steps involving chloroform must be performed under a fume hood and with caution, as chloroform is toxic.
7) Vortex for 15sec.
8) Incubate 3min at room temperature.
9) Spin 15min at 12,000rpm at 4°C in a microfuge.
10) Collect supernatant and add 500µl isopropanol.
11) Spin 15min at 12,000rpm at 4°C in a microfuge.
12) Aspirate and discard s/n.
13) Wash with 500µl 70% EtOH in MilliQ H_2O.
14) Spin 15min at 12,000rpm at 4°C in a microfuge.
15) Aspirate and discard s/n.
16) Air dry 2-3min. Do not fully dry, as it can be difficult to dissolve completely dried RNA.
17) Resuspend in 10µl MilliQ H_2O.
    Use water from a new tube, that is certainly clean.
18) Measure concentration with spectrophotometer.
19) Use Invitrogen SuperScript III First-Strand Synthesis System for RT-PCR to generate cDNA.
20) Measure concentration with spectrophotometer. Can also perform a PCR and gel electrophoresis with reliable primers/conditions to test cDNA quality.

***in situ* Hybridization Probe Synthesis**

To generate probes for use visualizing mRNA in Gryllus.

General Notes:

1) Amplify your target gene.
2) Assemble the following reaction in a 1.5mL eppendorf tube, using your target gene as template and either T7 or Sp6-T7 primers for sense and antisense probes. Seal tube lids with parafilm.

Reagent	1x (µl)
10x RNA polymerase buffer	2
Dig labeling mix	2
RNAse inhibitor	1
RNA Polymerase	2
500ng template	x
MQ H_2O	To 20µl

3) Incubate 3-4h at 37°C.
4) Add 30µl TE, 5µl 3M NaOAC, and 2.5volumes (125µl) of cold 100%EtOH.
5) Incubate on ice at least 20min.
6) Spin 15min at max speed at 4°C in a microfuge.
7) Aspirate and discard supernatant.
8) Add 500µl; cold 75% EtOH in H_2O.
9) Invert several times to mix.
10) Spin 5min at max speed at 4°C in a microfuge.
11) Aspirate and discard supernatant.
12) Air dry 2-3 (and certainly under 5) minutes.
13) Dissolve in 20µl TE. Do not pipette or vortex.
14) Dilute 1:10 in TE to check reaction by measuring the concentration on a spectrophotometer and running in gel electrophoresis.
    *Wash box and comb, and change running buffer before making gel to decrease RNAses.
15) Adjust to 100ng/µl in hybridization solution. Store at -20°C.