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Techniques and Protocols

Sequencing mRNA from Cryo-Sliced *Drosophila* Embryos

Rationale: *This protocol describes how to prepare Drosophila embryo for cryo-slicing, and how to prepare total RNA sample from each of the slices. The protocol for library preparation, TruSeq RNA Sample Prep V2 Kit LS protocol, is quite standard and easy to obtain from Illumina website, which is not included in this protocol.*

Drosophila Embryo Preparation

1. Set up *Drosophila melanogaster* adults for egg laying, collect eggs after 2 hours.
2. Transfer embryos to a 20 ml glass scintillation vial containing 10 ml of heptane and 10 ml of PEM buffer (100mM PIPES, 2mM EGTA, 1mM MgSO4). Mix gently.
3. Swirl vials to collect embryos at center of interphase, remove all the aqueous phase (lower phase). Add heptane to maintain at least 8 ml volume if needed.
4. Add 10 ml of methanol and shake vigorously for 15-30 seconds by hand. Devitellinized embryos will fall to the bottom of vial.
5. Remove heptane phase (upper phase) and the interphase. Pipette out the embryos from the bottom and transfer them to a 1.5ml Eppendorf tube.
6. Wash the embryos several times with methanol to remove heptane residue.
7. Embryos can be stored in methanol at -20 °C for several years.

Embryo Mounting and Cryo-Slicing

1. Wash embryos with 1X PBS several times to remove methanol.
2. Stain embryos with DAPI in 1X PBS for 5 minutes at room temperature.
3. Wash off DAPI by 1X PBT (1X PBS with 0.1% TWEEN® 20) several times. Leave embryos in PBT.
4. Transfer embryos on a slide and keep the embryos wet. Identify embryos at early blastoderm stage by DAPI under compound microscope.
5. Transferred one embryo at right stage to a methanol pre-washed embedding mold (Peel-A-Way® Embedding Mold (Rectangular - R30), Polysciences, Inc.).
6. Stain embryo with bromophenol blue solution (Saturated bromophenol blue (Sigma-Aldrich) in 100% Methanol), and wash off excess dye by methanol. Remove all the color on the bottom of mold in order to avoid confusion when slicing.

7. Adjust embryo positions to make it close to the edge of the mold and its AP axis perpendicular to the edge.
8. Add Tissue freezing medium slowly at least half way of the mold. Adjust embryo positions like above if needed.
9. Freeze mold at -80°C. Embryo in frozen tissue freezing medium can be stored at -80°C for several months.
10. Pre-chill Leica CM1950 cryostat at -20°C.
11. Apply a thin layer of tissue freezing medium on the scaffold. Then put the opposite side of the embryo on the scaffold with tissue freezing medium. Make sure the side with embryo is on top. Freeze the liquid medium in between the scaffold and block in the chamber of cryostat to give a firm attachment.
12. Set the slicing thickness at 60 um on cryostat. Put the scaffold in the slot for cryo-slicling, and orientate the block edge with embryo away from the blade.
13. Slice off the extra medium until the little blue dot (the embryo) is getting close to the blade, and then stop slicing.
14. Use razor blade to make two deep cuts at the two sides of embryo. Make sure the cuts are deeper than the length of the embryo.
15. Turn around the block to make the block edge with embryo close to the blade.
16. Start slicing. For each slicing, only collect the part with embryo between the two cuts by pre-chilled tweezers; transfer the sliced sample in a 1.5 ml nonstick, RNase-free microfuge tube (Cat. NO. AM 12450, Life Technologies). Put the tube with sliced sample on dry ice.

TRIzol RNA Extraction

Use RNase-free technique to perform RNA extraction.

1. Prepare TRIzol/Glycogen solution (TRIzol® Reagent, Cat. NO.15596-026, Life Technologies; 20 mg/ml Glycogen Solution, VWR Cat. NO. 97063-256, AMRESCO) by mix 20ul 20mg/ml glycogen in 1ml TRIzol.
2. Pre-chill centrifuge at 4°C.
3. Add 40ul TRIzol/Glycogen solution to the sample in each tube.
4. Add 960ul TRIzol to bring the volume up to 1ml. Make sure all the tubes are tightly closed. Shake tubes vigorously on a VWR vortex mixer with full speed for 1minute. Briefly spin down. Stand at room temperature for 5 minutes.
5. Add 200ul Chloroform to each tube. Shake sample by hand for 15 seconds. Stand at room temperature for 5 minutes.
6. Spin for 15 minutes at 12,000 x g, at 4°C. This spin separates above mixture into a lower red phenol-chloroform stage (protein), an interphase (DNA), and a clear upper aqueous phase (RNA).
7. During spin, prepare tubes each with 500ul isopropanol.
8. Carefully transfer upper phase to its corresponding new tube by pipetting it slowly without disturbing the interphase.

9. Mix the aqueous phase with isopropanol gently by hand. Let RNA precipitate at -20°C at least 30 minutes.
10. Spin for 15 minutes at 12,000 x g, at 4°C. Remove supernatant by pipette. Be careful, as RNA pellet will be loose in nonstick tube and difficult to see.
11. Add 1ml 75% ethanol to each tube. Mix by hand.
12. Spin for 5 minutes at 12,000 x g, at 4°C. Remove supernatant.
13. Repeat 75% ethanol wash.
14. Spin for 15 minutes at 12,000 x g, at 4°C. Carefully remove all the supernatant by pipette. Let RNA samples dry at room temperature for 5 minutes if necessary.
15. Add 20ul RNase-free water. Refrigerate at 4°C for at least 4 hours up to over night. If desired, samples can be stored at -80°C.
16. Run samples on 2100 Bioanalyzer (Agilent Technologies) to check the quality of RNA sample.
17. If all the RNA samples are of sufficient quality, proceed to library preparation. Otherwise discard all the samples, and start over.

Library Preparation

1. Before start, we should know that the amount of total RNA sample in each tube is not enough for library preparation. In order to increase the amount the RNA sample, we can pool 60ng total RNA from a divergent specie that has a sequenced genome. Therefore, we can map the reads to corresponding insects later on.
2. Perform DNase treatment for the pooled RNA sample in 50ul reaction.
3. Follow TruSeq RNA Sample Prep V2 Kit LS protocol to prepare library. Make sure to carefully read the guide, and get reagents or equipment ready before starting the library preparation.

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Reference

Combs PA, Eisen MB (2013) Sequencing mRNA from cryo-sliced Drosophila embryos to determine genome-wide spatial patterns of gene expression. PLOS one 8: p. e71820