Ribosome profiling of Drosophila third instar larvae body wall muscle (with ribosome affinity purification)

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Abstract

Citation: Xun Chen,Dion Dickman Ribosome profiling of Drosophila third instar larvae body wall muscle (with ribosome affinity purification). **protocols.io**
dx.doi.org/10.17504/protocols.io.j89crz6
Published: 12 Nov 2017

Protocol

Step 1.
Sample preparation and generation of ribosome protected mRNA fragments (ribosome footprints): Drive expression of RpL3-3xFlag using a muscle driver (BG57>UAS-RpL3-3XFlag). Pick wandering 3rd instar larvae and dissect larvae in HL3 to collect body wall tissue with all internal organs removed, after dissection, immediately place tissue in a 1.7ml centrifuge tube and freeze on dry ice.

Step 2.
Collect a total of 8 body wall in one tube for each sample. Add 240 µl lysis buffer (10 mM HEPES, PH 7.4, 150 mM KCl, 5 mM MgCl2, 100 µg/ml Cycloheximide, 1X protease inhibitor (EDTA-free, Sigma, COEDTAF-RO), 1U/µl ANTI-RNase (ThermoFisher scientific, AM2690)) and thoroughly grind tissue using pellet pestles (Z359971, Sigma-Aldrich). Add 12 µl 10% TritonX-100. Then rotate the tube at 4 °C for 30 min.

Step 3.
Centrifuge the lysate at 4 °C, 15000g for 10 min to clear the lysate and then transfer supernatant to a new tube.

Step 4.
Coat 100 µl magnetic beads (10004D, ThermoFisher Scientific) with 4 µg of anti-Flag antibody (F1804, Sigma-Aldrich). Mix cleared lysate from step 3 with the beads, add 10µl RNase T1 (EN0542, ThermoFisher Scientific). Rotate the mixture at 4 °C for 6 hours.

Step 5.
Place the tube on a magnet and discard all liquid. Wash beads with 200 µl wash buffer (10 mM HEPES, PH 7.4, 150 mM KCl, 5 mM MgCl2, 100 µg/ml Cycloheximide, 0.1% Triton-X100, 0.1 U/µl SUPERase.in RNase Inhibitor (ThermoFisher scientific, AM2696)) by pipetting up and down about 10 times, perform a total of three washes then discard all liquid.

Step 6.
Extract RNA with 500 µl Trizol (15596026, ThermoFisher Scientific), precipitate RNA with 5 µl linear acrylamide (AM9520, ThermoFisher Scientific), dissolve pellet in 7 µl nuclease-free water.

Step 7.
Mix RNA sample from step 6 with 2XTBE urea sample buffer (LC6876, ThermoFisher Scientific). Heat the mixture at 70 °C for 2 min then immediately place on ice.

Step 8.

Run the sample from step 7 on a 15% TBE-Urea PAGE gel along with 30nt and 50 nt DNA oligo markers for 90 min at 200 V. Stain the gel with SYBR Green II RNA gel stain (S7564, ThermoFisher Scientific, use at 1X in TBE buffer) for 15 min.

Step 9.
View gel under a transilluminator, cut the gel region corresponding to 30 nt to 50 nt as indicated by oligo markers and place the gel piece in a 1.7 ml centrifuge tube.

Step 10.
Add 200 µl gel elution buffer (10 mM Tris-HCl, PH 7.5, 250 mM NaCl, 1 mM EDTA). Crush gel with pellet pestle. Add another 300 µl gel elution buffer, 22 µl RNAsecure (AM7005, ThermoFisher Scientific) and 5 µl 20% SDS. Mix well and then heat at 60 °C for 10 min.

Step 11.
Rotate the tube at 4 °C overnight. Transfer eluate with gel pieces to a gel filter (CLS8162, Sigma-Aldrich) and centrifuge at 16000g for 5 min at 25 °C. Transfer the cleared eluate to a new 1.7 ml centrifuge tube.

Step 12.
Perform RNA precipitation by adding 500 µl isopropanol, 7 µl linear acrylamide to the tube and mix well. Incubate at room temperature for 30 min then centrifuge at 15000g at 25 °C for 15 min. Wash the pellet with 75% ethanol once, discard all liquid and air dry the pellet for 5 min. Dissolve the pellet in 16 µl nuclease-free water, this is the gel purified ribosome footprints (RFs) sample.

Step 13.
Next Generation Sequencing Library generation: Perform Phosphatase treatment by combining 15 µl Gel purified RFs, 10 µl 5XrSAP buffer (20 mM Tris, PH 8.0, 250 mM NaCl, 1 mM MgCl2), 2.5 µl rSAP (M0371S, NEB), 2.5 µl SUPERase.In (AM2696, ThermoFisher Scientific), 20 µl Nuclease-free water in a PCR tube. Mix and then incubate at 37 °C for 30 min.

Step 14.
Add 2 µl RNAsecure, 0.5 µl EDTA (500 mM). Mix and then heat at 65 °C for 10 min to inactivate rSAP.

Step 15.
Add 50 µl isopropanol. Mix and incubate at room temperature for 10 min, then centrifuge at 15000g at 4 °C for 10 min. Wash pellet with 75% ethanol, discard all liquid and air dry pellet for 5 min. Dissolve the pellet in 7 µl Nuclease-free water.

Step 16.
Perform 3’ adaptor ligation using the corresponding component of NEBNext Mµltiplex Small RNA Library Prep Set for Illumina (E7300S, NEB) and follow manufacturer’s instructions. Briefly, Mix 6 µl phosphatase treated RNA, 1 µl 3’ SR Adaptor for Illumina. Heat the tube at 70 °C for 2 minutes then immediately transfer to ice. Add 10 µl 3’ Ligation Reaction Buffer, 3 µl 3’ Ligation Enzyme Mix and mix well. Incubate at 25 °C for 1 hour.

Step 17.
Perform Kinase treatment by adding 2.5 µl ATP (10 mM), 1.5 µl DTT (50 mM) and 0.5 µl T4 PNK (M0201S, NEB) to the 3’ ligation reaction. Mix and incubate at 37 °C for 30 min.

Step 18.
Perform reverse transcription primer hybridization by adding 1 µl SR RT Primer for Illumina (M0201S, NEB) to the T4 PNK reaction and mix well. Heat the sample for 5 min at 75 °C then allow the sample to slowly cool down in the thermocycler until the temperature drops to below 35 °C.

Step 19.
Perform 5’ SR adaptor ligation using NEBNext Mµltiplex Small RNA Library Prep Set for Illumina (E7300S, NEB) and follow manufacturer’s instructions. Briefly, Add 1µl denatured 5’ SR Adaptor for

Illumina, 1 µl 5’ Ligation Reaction Buffer, 2.5 µl Ligation Enzyme Mix to the hybridization reaction and mix well. Incubate at 25 °C for 1 hour.

Step 20.
Perform reverse transcription using NEBNext Multiplex Small RNA Library Prep Set for Illumina (E7300S, NEB) and follow manufacturer’s instructions. Briefly, add 8 µl First Strand Synthesis Reaction Buffer, 1 µl Murine RNase Inhibitor, 1 µl ProtoScript II Reverse Transcriptase to the 5’ SR adaptor ligation reaction and mix well. Incubate at 50 °C for 60 min.

Step 21.
Perform PCR amplification (15 cycles) using NEBNext Multiplex Small RNA Library Prep Set for Illumina (E7300S, NEB) and follow manufacturer’s instructions. Briefly, add 50 µl LongAmp Tag 2X Master Mix, 2.5 µl SR Primer for Illumina, 2.5 µl Index Primer, 5 µl Nuclease free water to the reverse transcription reaction and mix well. Perform PCR cycling using condition: 94 °C 30 sec, 15 cycles of [94 °C 15 sec, 62 °C 30 sec, 70 °C 15 sec], 70 °C 5 min, hold at 4 °C.

Step 22.
To the 100 µl PCR product, add 3 µl linear acrylamide, 10 µl NaCl (2.5 M), 100 µl Isopropanol. Mix well and then incubate at room temperature for 30 min. Centrifuge at 15000g at 4 °C for 10 min. Wash the pellet with 75% ethanol once, discard all liquid and air dry the pellet for 5 min. Dissolve the pellet in 10 µl nuclease-free water.

Step 23.
Perform size selection of purified library PCR product using the corresponding component of the NEBNext Multiplex Small RNA Library Prep Set for Illumina (E7300S, NEB) and follow manufacturer’s instructions. Briefly, mix 10 µl PCR product with 2 µl Gel Loading Dye, Blue. Run the sample on 6% PAGE gel along with 5 µl of Quick-Load pBR322 for 1 hour at 120 V. Stain the gel with SYBR Gold nucleic acid gel stain (S11494, ThermoFisher Scientific, use at 1X in TBE buffer) for 15 min.

Step 24.
View gel under a transilluminator, cut the gel region corresponding to 140 bp to 170 bp as indicated by the Quick-Load pBR322 markers and place the gel piece in a 1.7 ml centrifuge tube.

Step 25.
Add 200 µl gel elution buffer (10 mM Tris-HCl, PH 7.5, 250 mM NaCl, 1 mM EDTA). Crush gel with pellet pestle. Add another 300 µl gel elution buffer and 5 µl 20% SDS. Mix well and rotate the tube at 4 °C overnight.

Step 26.
Transfer eluate with gel pieces to a gel filter (CLS8162, Sigma-Aldrich) and centrifuge at 16000g for 5 min at 25 °C. Transfer the cleared eluate to a new 1.7 ml centrifuge tube.

Step 27.
Perform DNA precipitation by adding 500 µl isopropanol, 5 µl linear acrylamide to the tube and mix well. Incubate at room temperature for 30 min then centrifuge at 15000g at 25 °C for 15 min. Wash the pellet with 75% ethanol once, discard all liquid and air dry the pellet for 5 min. Dissolve the pellet in 15 µl nuclease-free water, this is the library sample.

Step 28.
Submit the library sample for next-generation sequencing on an Illumina platform or store at -20 °C.