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Total RNA Extraction from Transgenic Flies Misexpressing Foreign Genes to Perform Next Generation RNA Sequencing

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1 *Works for me* dx.doi.org/10.17504/protocols.io.5bng2me

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BEFORE STARTING

Before starting any step it is recommended to clean working space (RNA isolation bench), and all the equipments(e.g. scalpels, forceps, pippets) with RNase decontamination solution e.g. use RNaseZap® (Thermofisher scientific Cat No. AM9780).Simply spray RNaseZap® Solution onto the surface to be decontaminated and rinse it off with RNase-free water.

1 Sample collection and Storage

Transgenic Drosophila melanogaster were generated by genetivision (https://www.genetivision.com/). Briefly, transgenic flies were generated using microinjection-based ϕC31 integrase mRNA-mediated method. A cloned candidate gene using pUAST-attB plasmid containing both a transgene and donor sequence (attB) is coinjected along with ϕC31 integrase mRNA into attP-containing recipient embryos, resulting in the site-specific insertion of the transgene (Fish et al., 2007; Groth et al., 2004). Following this procedure 5 independent transgenic flies were generated with insertion of newt gene at Chromosome III.Foreign gene of interest was misexpressed ubiquitously using tubulin Gal-4. The third instar larvae were selected and were stored in RNAlater (Thermo Fisher, Cat. No. # AM7024) solution. The samples were then stored at 4°C. For long term storage we isolated larvae into empty RNase free tubes and store them at -70°C.

2 RNA isolation and Purification

The tubes were briefly centrifuged and RNAlater solution was completely removed. We added 500 µl acids, and are compatible with downstream analysis . Tissue was homogenized in TRIzol using a 150 handheld homogenizer Motor (Fisher scientific, Cat. No. # 15-340-167). The solution was incubated for 30 minutes in ice. Centrifuged for 10 minutes at 4°C and supernatant was transferred to clean tube. Chloroform (Sigma Catalog #: 319988/W205702)was added and homogenate was allowed to separate into a clear upper aqueous layer. 200 µl of the aqueous phase was transferred to RNA clean and Concentrator^{TM} (Zymo research, Cat. No. R1080) columns, and the recommended protocol was followed, i.e., solution was passed through the RNA binding buffer, which binds RNA to the desired columns. Then RNA wash buffer was added to remove all the impurities from the column. Finally RNA was eluted in 20 µl of molecular grade water (DNase/RNase free) and collected in a separate tube. The molecular grade water serves as the elution buffer. It releases the RNA from the column.

3 **RNA quantitation, and Storage**

RNA quantitation is an important and necessary step prior to most RNA analysis methods. Quality of RNA as well as concentration of RNA was determined by calculating absorbance at 260 nm (A260) and 280 nm (A280) wavelengths using Nanodrop 2000 spectrophotometer (Thermo Scientific). Good quality samples had A260/ A280 ratio greater than 2 and a peak at 260 nm. High quality total RNA was shipped in Dry ice to our collaborator for Next generation RNA sequencing.

4 **RNA Sequencing and Analysis**

Illumina reads were mapped to the *Drosophila*genome dm6 using TopHat splice-aware aligner (Kim et al., 2013). Expectation-Maximization (EM) approach was used to estimate transcript abundance (Jiang and Wong, 2009). Reads per kilobase per million mapped reads (RPKM) approach was applied for within sample normalization (Mortazavi et al., 2008). Between sample normalization and differentially expressed test were performed by BioConductor DESeq package (v 1.20) which allows analysis of non-replicate experiments (Anders and Huber, 2010). The significant criteria were the detected transcript in at least one sample (RPKM>1), fold change over 2 and adjusted p-value less than 0.05.

Enriched gene ontology (GO) terms were identified using gene ontology enrichment analysis and visualization tool (GOrilla) (Eden et al., 2009). Single ranked list of gene was chosen as a running mode criteria, and for enriched GO terms searched P-value threshold was equal to 10^{-3}. Using reduce + visualize gene ontology (REVIGO) tool long lists of gene ontology terms obtained by running Gorilla were summarized by removing redundant GO terms (Supek et al., 2011). The remaining terms were visualized as tree map/ bar graph. We also used protein analysis through evolutionary relationships (PANTHER) 14.1 version as a tool to select set of enriched gene ontology terms for classifications by molecular function, cellular component, and Protein class (Mi et al., 2019).