RNA Isolation – Stallion tissues

Tissues were provided by the FAANG biobank (Donnelly et al., 2021). The methods used to collect and store tissues are described in Donnelly et al., (2021); in brief, samples utilized for RNA-sequencing were flash frozen in liquid nitrogen and stored at -80C until RNA isolation. Of the 102 tissues in the biobank, 8 tissues were chosen for study including abdominal adipose, parietal cortex, left ventricle of the heart, lamina, liver, left lung, *Longissimus dorsi* muscle, and left testis. Approximately 70mg of each tissue (100mg of adipose) was minced with a razor on dry ice. Tissue samples were then homogenized in 1ml of Trizol™ (Invitrogen, Waltham, MA, USA) using a Kinematic Polytron™ (Luzern, Switzerland) on ice in 30 second bursts. After homogenization, the samples were incubated at room temperature for 5 minutes before the addition of 200µL of chloroform. Tough tissues, like adipose and testis, were incubated for 10 minutes or more at room temperature before undergoing a dirty spin. For the dirty spin, the adipose and testis samples were centrifuged at 12,000 x g for 10 minutes, and the supernatant was transferred to a clean 2.0mL tube before the addition of chloroform. After the addition of chloroform, the samples were vortexed, incubated for 2-3 minutes at room temperature, and spun at 12,000 x g for 15 minutes. The clear supernatant of each sample was added to 600mL of ethanol and placed on a spin column. RNA was isolated using the Zymo Research Direct-zol RNA Miniprep kit (Irvine, CA, USA) according to manufacturer guidelines with minor revisions as described. DNA was removed from samples using a 15 minute on-column DNase I treatment. In addition to the 1-minute spin outlined in the Zymo protocol, the columns were spun for an additional two minutes before elution in DNase/RNase free water to remove any residual wash buffers. The speed and number of bursts used to homogenize samples, their elution volume, and the presence of additional steps are recorded in **Table 1**. RNA was quantified and integrity (RIN values) determined using an Agilent Bioanalyzer 2100 Eukaryote Total RNA Nano chip (Santa Clara, CA, USA).

Library Preparation

RNA from the stallion tissues was sent to Admera Health (South Plainfield, NJ, USA), prepped using the TruSeq kit (Illumina, San Diego, CA, USA), and sequenced on a NovaSeq 6000 Sequencing System (Illumina, San Diego, CA, USA). Libraries include stranded, poly(A+) selected 150bp paired-end reads.

Table 1. Tissue Homogenization and RNA Elution Specifications

Tissue	Homogenization		RT Incubation/ Dirty Spin	Elution Volume
	Speed (kRPM)	Duration		
Abdominal Adipose (Adipose)	26-28	30 sec (x3)	10 min: Yes	50uL
Parietal Cortex (Brain)	10-12	20 sec (x2)	5 min: No	50uL
Heart Left Ventricle (Heart)	22-24	30 sec (x3)	5 min: No	50uL
Lamina	22-24	30 sec (x3)	5 min: No	100uL
Liver	22-24	30 sec (x3)	5 min: No	80uL
Left Lung (Lung)	22-24	30 sec (x3)	5 min: No	50uL
Longissimus dorsi (Muscle)	18-20	30 sec (x3)	5 min: No	100uL
Left Testis (Testis)	22-24	30 sec (x4)	20 min: Yes	100uL

Donnelly, C. G., Bellone, R. R., Hales, E. N., Nguyen, A., Katzman, S. A., Dujovne, G. A., Knickelbein, K. E., Avila, F., Kalbfleisch, T. S., Giulotto, E., Kingsley, N. B., Tanaka, J., Esdaile, E., Peng, S., Dahlgren, A., Fuller, A., Mienaltowski, M. J., Raudsepp, T., Affolter, V. K., Petersen, J. L., … Finno, C. J. (2021). Generation of a Biobank From Two Adult Thoroughbred Stallions for the Functional Annotation of Animal Genomes Initiative. *Frontiers in Genetics*, 12, 650305. https://doi.org/10.3389/fgene.2021.650305