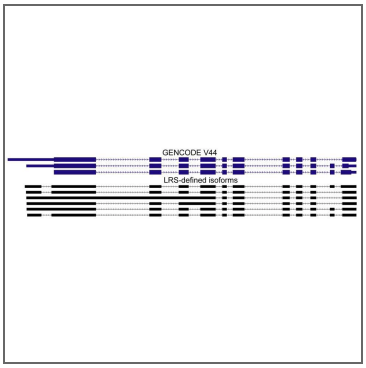

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Protocol status: Working
We use this protocol and it's working

Protocol for long read sequencing of dorsal root ganglia from human organ donors

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ABSTRACT

In this protocol, we describe how to extract RNA from dorsal root ganglia sourced from human organ donors, and subsequently perform long read sequencing with PacBio IsoSeq.

MATERIALS

RNase*Zap* RNase Decontamination Solution - Invitrogen, Catalog #:AM9780
RNeasy Plus Universal Mini Kit - Qiagen, Catalog #:73404
Precellys Tissue Homogenizing Mixed Beads Kit - Cayman Chemical Company, Catalog #10409
PacBio Iso-Seq Express SMRTbell Library Template Preparation Kit 2.0: PacBio, Catalog #: 100-938-900

BEFORE START INSTRUCTIONS

Required PPE: All work must be done wearing appropriate PPE including lab coat and gloves. Any work with TRIzol/QIAzol should be carried out inside of a fume hood. Clean pipettes and lab bench area with 70% ethanol, followed by RNase decontamination solution, such as RNaseZap, prior to carrying out RNA extraction.

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Harvesting and storing human dorsal root ganglia

1 Lumbar dorsal root ganglia (DRGs) are recovered from human organ donors with no known history of chronic pain, through a collaboration with the Southwest Transplant Alliance. DRGs are recovered within 4 hours of cross-clamp and immediately frozen in powdered dry ice.

2 Human DRG (hDRGs) are stored in a -80C freezer until use.

RNA extraction

3 <50mg hDRG tissue is isolated and submerged in 1ml QIAzol from RNeasy Plus Universal Mini kit. The sample is homogenized using Precellys Tissue Homogenizing Mixed Beads Kit and 1min shake in the Bertin Technologies Minilys, until no visible pieces appear. Pipette lysate into new tube.
The sample is kept on ice between each step.

4 RNA extraction is performed with reagents from the RNeasy Plus Universal Mini kit from Qiagen including RNeasy mini spin-columns:

4.1 100ul gDNA eliminator is added, followed by rigorous shaking for 15 seconds

4.2 180ul chloroform is added, followed by rigorous shaking for 15 seconds

4.3 2-3minute incubation at room temperature

4.4 Centrifuge samples at 12,000g for 15 minutes at 4C using a Bio-Rad Model 16K Microcentrifuge

4.5 Transfer lysate to new tube and add 500ul 70% EtOH

4.6 Add 700ul sample at a time to RNeasy mini spin-column placed in 2ml collection tube

4.7 15 second centrifuge at full-speed (16,000g) until all lysate has been passed through spin-column and flow-through is discarded

4.8 Add 700ul Buffer RWT to spin-column

4.9 Centrifuge for 15 seconds at full-speed and discard flow-through from collection tube

4.10 Add 500ul RPE to spin-column

4.11 Centrifuge for 15 seconds at full-speed and discard flow-through from collection tube

4.12 Repeat step 4.10-4.11 two times but centrifuge for 2 minutes

4.13 Place spin-column in new collection tube and centrifuge for 1 minute at full-speed

4.14 Place spin-column in new Eppendorf for RNA storage (1.5ml tube with cap) for final storage

4.15 Add 20-30ul RNAse free water

4.16 Centrifuge for 1 minute at full-speed

4.17 Repeat step 4.15-4.16 with the same elution water

4.18 Measure RNA quantity/quality with Qubit 3.0 Fluorometer and confirm with Agilent 5200 Fragment analyzer system

4.19 RNA is stored in -80C freezer until shipment to core facility at UC Davis. Samples are shipped on dry ice

Library preparation and long read RNA sequencing

5 The library is created using the PacBio Iso-Seq Express SMRTbell Library Template Preparation Kit 2.0

6 Circular consensus sequencing (CCS) is carried out using the Sequel II equipment for 3plex on 1 SMRT Cell 8M

Processing of long read RNA sequencing data

7 Raw sequencing data is processed through the PacBio recommended Iso-Seq pipeline which carries out the following steps:

7.1 Use *lima* to remove cDNA primers

7.2 Use *isoseq refine* to polyA tail and artificial concatemers

7.3 Use *isoseq cluster2* to carry out de novo isoform-level clustering scalable to large number of reads

7.4 Use *pbmm2* to align reads to the reference genome (GRCh38)

7.5 Use *isoseq collapse* to collapse redundant transcripts based on exonic structures

8 A GFF file is obtained which contains information for each sequence identified in the pooled samples as well as csv abundance files with information for each transcript in each sample