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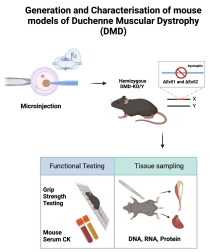
RNA Extraction and RT-PCR on Mouse tissues



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We use this protocol and it's working

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Keywords: RNA, RT-PCR, Dystrophin, transcript, Phenotyping, Knockout, DMD, murine models of duchenne muscular dystrophy, crispr microinjection of embryo, crispr microinjection, duchenne muscular dystrophy, quality rna for subsequent molecular analysis, crispr, mrna transcript expression in mouse tissue, cas9 gene, molecular testing at the dna, quality rna, broader landscape of genetic disorder investigation, rna extraction, molecular testing, rna, mouse tissue, pcr on mouse tissues paper, isolated rna into complementary dna, using reverse transcription pcr, cdna synthesis, serum creatine kinase, reverse transcription pcr, genetic disorder investigation, subsequent molecular analysis, mouse tissues paper, isolated rna, using mouse model, molecular analysis, mrna transcript expression, permanent modifications to dna, generation of diverse animal model, mouse model, protein, protein level

Disclaimer

These protocols are for research purposes only.

Abstract

Paper abstract: CRISPR-Cas9 gene-editing technology has revolutionised the creation of precise and permanent modifications to DNA, enabling the generation of diverse animal models for investigating potential treatments. Here, we provide a protocol for the use of CRISPR-Cas9 to create murine models of Duchenne Muscular Dystrophy (DMD) along with a step-by-step guide for their phenotypic and molecular characterisation. The experimental procedures include CRISPR microinjection of embryos, molecular testing at the DNA, RNA, and protein levels, forelimb grip strength testing, immunostaining and serum creatine kinase (CK) testing. We further provide suggestions for analysis and interpretation of the generated data, as well as the limitations of our approach. These protocols are designed for researchers who intend on generating and using mouse models to study DMD as well as those seeking a detailed framework of phenotyping to contribute to the broader landscape of genetic disorder investigations.

Protocol summary: To obtain high-quality RNA for subsequent molecular analyses, specifically to assess mRNA transcript expression in mouse tissues. Following this, cDNA synthesis is employed to convert the isolated RNA into complementary DNA (cDNA). This cDNA serves as a stable template for downstream analyses, where we look at using reverse transcription PCR (RT-PCR) or quantitative real-time PCR (qPCR).

Image Attribution

BioRender was used to generate figures for this manuscript.

Materials

- TRIzolTM Reagent (Invitrogen).
- Chloroform.
- MagNA Lyser Green Beads (Roche)
- Ethanol.
- Isopropanol.
- DNase/RNase-free distilled water.
- RNEasy Mini Extraction kit (Qiagen)
- High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems)
- QuantStudio Real-Time PCR software v1.3 (Applied Biosystems)

Safety warnings

Wear proper PPE (gloves, safety goggles, enclosed shoes and lab coat) and prepare solvents in a chemical fume hood. Dispose used solvents or waste material in an appropriate biohazard waste containers.

Ethics statement

Animal work described in this manuscript has been approved and conducted under the oversight of the Animal Ethics Committee of South Australian Health and Medical Research Institute (SAHMRI) and The University of Adelaide.

Preparation Phase

1 Keep the tissue samples on ice

2 Set the centrifuge to cool at 4°C

3 Mince the heart and skeletal muscle tissues in a few drops of water and place the minced portions into a magNA lyser tube for bead homogenisation step

4 Homogenise samples in the Precellys bead-based homogeniser at 6500 RPM for 2 cycles at 20s each

TRIzol Treatment

5 Add 500 µL of Invitrogen TRIzol reagent to the supernatant

6 Incubate for 5 minutes at room temperature (RT) to dissociate the nucleoprotein complex

Chloroform Extraction

7 Add 100 µL chloroform

> Note
>
> Use 0.2 ml chloroform/ 1 ml TRIZOL reagent

8 Shake the tubes vigorously by hand for 15 seconds to mix

9 Incubate for 2 min at RT.

10 Centrifuge at 12,000 RCF in a 4°C cooled centrifuge for 15 minutes.

RNA Precipitation and Collection

11 Label new microcentrifuge tubes and keep on ice

12 Transfer approximately 175 µL of the colourless liquid layer to a new eppendorf tube.

13 Add an equal volume of 70% ethanol. Mix by pipetting. Invert the tube and mix to disperse any visible precipitate that can form after adding ethanol.

14 Transfer a maximum of 700 µL to the QIAGEN spin column with a collection tube.

15 Centrifuge for 30 seconds at 8,000 RCF (room temperature centrifuge).

16 Place the flow-through back into the spin column and centrifuge again for 30 seconds at 6,500 RCF. Discard the flow-through and place the column back into the collection tube.

17 Add 700 µL Buffer RW1 to the RNeasy spin column. Centrifuge for 15 seconds at 8,000 RCF.

18 Add 500 µL Buffer RPE to the RNeasy spin column. Centrifuge for 15 seconds at 8,000 RCF.

19 Discard the flow-through and place the spin column in a new collection tube.

20 Add 500 µL Buffer RPE to the RNeasy spin column. Centrifuge for 2 minutes at 8,000 RCF to wash the spin column membrane.

21 Discard the flow-through and place the spin column in a new collection tube.

22 Place the RNeasy spin column in a new collection tube. Centrifuge for 1 minute at max speed.

23 Place the RNeasy spin column in a new QIAGEN RNase-free 1.5 ml tube.

24 Add 30-50 µL RNase-free water directly to the spin column membrane.

25 Centrifuge for 1 minute at 8,000 RCF to elute the RNA.

> Note
>
> For <100 µg starting tissue, use one 30-100 µL elution volume. For >100 µg, use 2–3 sequential 100 µL elutions.

26 Measure concentration of the RNA solution using NanoDrop Spectrophotometer

> Note
>
> The A260/A280 ratio should be approximately 2.0, but values between 1.8 and 2.1 are considered to be of acceptable purity.

Reverse Transcription Reaction for cDNA Generation

27 Follow the protocol provided by the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) to convert 1-2 µg of RNA into cDNA

28 Dilute cDNA in 50 µL ultrapure water prior to running downstream analysis

Reverse-transcription polymerase chain reaction (RT-PCR)

29 The New England Biolabs (NEB) website can be consulted for the finer details of reaction volumes and components when amplifying using a standard NEB Taq DNA Polymerase and the 10X Standard Taq Reaction Buffer. The primers we used are listed in Supplementary Figure S2. We recommend using cDNA synthesised from 1-2 µg of RNA, as well as assembling all rection components on ice prior to transferring to the preheated

thermocycler. After the RT-PCR, confirm the expected band sizes by carefully visualizing the amplified products on a 1% agarose gel.