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Scientific Protocol for Western Blotting

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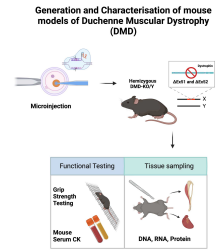
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Protocol status: Working

We use this protocol and it's working

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Keywords: Protein, CRISPR, DMD, Mouse, PVDF, Blot, Western, Knockout, murine models of duchenne muscular dystrophy, crispr microinjection, crispr microinjection of embryo, duchenne muscular dystrophy, crispr, broader landscape of genetic disorder investigation, cas9 gene, molecular testing at the dna, molecular testing, genetic disorder investigation, serum creatine kinase, protein, generation of diverse animal model, subsequent staining with dystrophin, scientific protocol for western blotting paper abstract, molecular characterisation, using mouse model, murine model, permanent modifications to dna, diverse animal model, protein level, embryo, dystrophin, mouse model

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.

Paper summary: The following protocol describes the separation of proteins through SDS-PAGE, the transfer onto a PVDF membrane and subsequent staining with dystrophin-specific antibodies.

Image Attribution

BioRender was used to generate figures for this manuscript.

Materials

- CompleteTM, Mini, EDTA-free Protease Inhibitor Cocktail tablet (Roche)
- PierceTM BCA Protein Assay Kit (ThermoFisher)
- MagNA Lyser Green Beads (Roche)
- PierceTM Protease Inhibitor Mini Tablets, EDTA-free (ThermoFisher)
- 96-well plates
- SureLockTM Tandem Midi Gel Tank (Invitrogen)
- Trans-Blot TurboTM Transfer System (Bio-Rad)
- Sample loading buffer: 75 mM Tris-HCl at pH 6.8, 5 mM EDTA, 15% SDS, 20% glycerol, 0.004% w/v bromophenol blue, and 5% β -mercaptoethanol
- NuPAGETM NovexTM 3–8% Tris-Acetate Midi 20-well gel (Invitrogen)
- NuPAGETM Tris-Acetate SDS running buffer (Invitrogen)
- HiMarkTM pre-stained protein standard (ThermoFisher)
- Trans-Blot Turbo Midi 0.2 μ m PVDF Transfer Packs, 0.20 μ m pore size (Bio-Rad).
- Wash Buffer: PBST (phosphate-buffered saline, 0.1% Tween 20)
- Blocking: PBST and 5% skimmed milk
- Primary antibodies: MANDYS8 Monoclonal Ant-Dystrophin antibody (Sigma-Aldrich) and V9131 Anti-Vinculin antibody (Sigma-Aldrich)
- Secondary antibodies: Goat anti-mouse immunoglobulin G (IgG) (H+L) secondary antibody, horseradish peroxidase (HRP) (ThermoFisher).
- Supersignal West Femto kit (ThermoFisher)
- Blot Shaker
- Skim milk powder (Diploma)
- Blotting Tweezer (Invitrogen)
- Blot roller (Bio-Rad)
- Heat block
- Centrifuge
- Incubation Tray 10 $\times$ 14 cm (ThermoFisher)
- Bio-Rad ChemiDoc MP Imaging System
- RestoreTM PLUS Western Blot Stripping Buffer (ThermoFisher)



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.



Protein Extraction and BCA

- 1 Transfer isolated skeletal muscle and heart tissues to magna lyser bead tubes
- 2 Add 480 μ L Disruption Buffer (15% SDS, 75 mM Tris HCl pH 6.8) + 20 μ L 25x PI cocktail (optional).
- 3 Homogenise samples in the Precellys bead-based homogeniser at 6500 RPM for 2 cycles 20 s each
- 4 Spin down the supernatant in a 4°C cooled centrifuge at 13,500 RCF for 10 minutes
- 5 Collect the supernatant and place into a new 1.5 ml tube
- 6 Perform BCA Assay and quantitation steps according to manufacturer's protocol, using the disruption buffer as a blank.

Note

25 μ g of total protein will be used from each tissue per run

- 7 Store protein sample as multiple low volume aliquots at -80°C until needed for SDS-PAGE

SDS-PAGE

- 8 Load equal parts of sample loading buffer with sample to make up 25 μ g protein

Note

We use a 20-combed 3-8% Polyacrylamide gel that is pre-cast. The maximum load volume in each well is 25 μ L.



- 9 Denature samples at 98°C for 5 minutes

Note

While samples are left on heating block, prepare the gel tank with midi-well immersed in running buffer. Ensure the buffer is above the electrode line.

- 10 Briefly centrifuge samples to collect the mixture

- 11 Load samples into the gel and run the gel at 100 V for 15 minutes, followed by 120 V for 1 hour and 45 minutes.

Note

In alignment with the recommendation from ThermoFisher, we have also found that running at 150 V for 1h worked as effectively.

Semi-dry transfer (TransBlot Turbo System) and Block

- 12 Transfer the protein from the gel to the membrane and place into Trans-Blot Turbo Transfer System cassette.

Note

Ensure that the PVDF membrane is kept moist on the top and bottom filter paper stack. Once gel is neatly stacked on the membrane, firmly roll out the air bubbles with a roller.

- 13 Ensure that the whole transfer stack is firm when closing the cassette lid to ensure no additional air bubbles form
- 14 Place transfer stack inside the Trans-Blot Turbo Transfer System machine and run using the 'HIGH MW' protocol in the standard Bio-Rad setting.
- 15 Place the membrane in a blotting container and add enough blocking solution (10% Skim milk in PBST) to cover the entire membrane surface

Note

Recommended to pour solutions gently into the corner of the blotting container and not directly onto the membrane so that the proteins on the membranes are not disturbed.

16 Incubate the membrane on a blot shaker at 50 – 60 RPM for 1h

17 Pour out the blocking solution

Primary and Secondary Antibody Incubations

18 Incubate the membrane with primary antibody at a dilution of 1:1000 (MANDYS8) in blocking buffer (2% Skim milk in PBST), for an overnight incubation at 4°C

19 Wash the membrane in three washes of PBST on a blot shaker for 5 min each

20 Incubate the membrane with HRP-conjugated secondary antibody (Goat anti-mouse IgG HRP) in blocking buffer (2% Skim milk in PBST) at a dilution of 1:8000, for 1 hour at room temperature.

21 Wash the membrane in three washes of PBST on a blot shaker for 5 min each

Detection and Imaging

22 Detect the signal following the Supersignal West Femto Maximum Sensitivity Substrate kit recommendations

Note

For a Midi (8.5 x 13.5 cm) membrane, we use 2 ml of West Femto Working Solution mix. In a yellow-capped tube, mix equal parts of West Femto Stable Peroxide agent (1 mL) and West Femto Luminol/Enhancer reagent (1 mL). This Working Solution forms the substrate for chemiluminescence. Pipette enough of the substrate onto a parafilm paper. Incubate the membrane under dark conditions for 5-min before removing excess reagent.

- 23 Arrange the membrane on the imaging surface, taking care not to create bubbles on the surface.
- 24 Image the blot using your machine of choice

Note

We use the Bio-Rad Chemidoc MP Imaging system using the Chemi Hi Sensitivity setting. The exposure time can be manually adjusted to obtain the best image prior to saturation. This system enables you to take a multichannel image with the colorimetric and either the Chemi Hi Sensitivity or Hi-Resolution setting to get an image of the chemiluminescent dystrophin bands overlaid with the protein size marker – evident from the colorimetric setting. Verify the molecular weight of the band of interest (should be close to 460kDa mark) based on the multichannel image.

Stripping and Re-probe with Vinculin

- 25 Strip the membrane using the Restore PLUS Western Stripping Buffer, following manufacturer's instructions. This includes a membrane washing and subsequent blocking step prior to re-probing.
- 26 Re-probe by incubating the membrane with vinculin-targeting primary antibody at a dilution of 1:1000 (V9131) in blocking buffer (2% Skim milk in PBST) at room temperature for 1h.
- 27 Follow steps 17-22 until imaging of the blot