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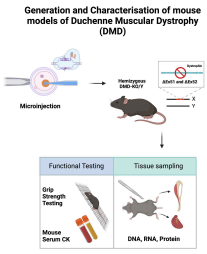
In vitro Transcription (IVT) of Guide RNAs for Cytoplasmic Microinjection



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We use this protocol and it's working

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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: In this section we present a protocol for CRISPR injection into mouse zygotes to create the ΔEx51 DMD model. Here, we successfully generated guide RNAs (sgRNAs) targeting intronic regions flanking the mouse Dmd target region of exon 51 to create a large intervening deletion.

Image Attribution

BioRender was used to generate figures for this manuscript.

Materials

1. NEB HiScribe™ T7 Quick High Yield RNA Synthesis Kit
2. Qiagen RNEasy Mini Kit RNA Cleanup (Qiagen)
3. Purified pX459V2 construct (backbone from Addgene Plasmid #62988)
4. T7 primer + FWD and REV sequences with overhangs
5. NEB Formaldehyde Load Dye
6. QIAquick Gel Extraction Kit (Qiagen)
7. QIAQuick PCR Purification Kit (Qiagen)

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.

Choosing a sgRNA pairing to create the deletion

1 The in-silico design of guide RNAs was conducted through various online guide design tools and narrowed down based on their predicted on-target and off-target activities. Tools that we would recommend for this task include Benchling and CRISPOR (1). The phospho-annealed guide oligos were then cloned into BbsI-linearised pX459V2 plasmid using the Golden Gate Assembly method. Note that 'CACC' and 'AAAC' overhangs allow the oligos to bind the complementary overhanging DNA at the cut sites in the plasmid created by the BbsI digestion (2). This process yielded two recombinant constructs, each housing the left intron and right intron, respectively.

gRNA 1 (Right Intron)
Position is 156bp away from 3' exon 51
Forward: 5' - GGTCAACCTAACTACAATCA - 3'
Rev comp: 5' - TGATTGTAGTTAGGTTGACC - 3'

Forward oligo: 5' - CACCGGTCAACCTAACTACAATCA - 3'
Reverse oligo: 5' - AAACTGATTGTAGTTAGGTTGACC - 3'

gRNA 2 (Left Intron)
Position is 290bp away from 5' exon 51
Forward: 5' - GTTTATAAGCACAAGTATTG - 3'
Rev comp: 5' - CAATACTTGTGCTTATAAAC - 3'

Forward oligo: 5' - CACCGTTTATAAGCACAAGTATTG - 3'
Reverse oligo: 5' - AAACCAATACTTGTGCTTATAAAC - 3'

PCR amplification of guide template

1

Note

The oligos used for Forward and Reverse PCRs are seen in Supplementary Table S1. Here is an expanded form:

gRNA 1 (Right Intron)
T7 Guide Primer for Forward PCR:
5' - TTAATACGACTCACTATAGGGTCAACCTAACTACAATCA - 3'

gRNA 2 (Left Intron)
T7 Guide Primer for Forward PCR:
5' - TTAATACGACTCACTATAGGTTTATAAGCACAAGTATTG - 3'

tracrRNA Reverse Primer: 5’ - AAAAGCACCGACTCGGTGCC - 3’

2 Prepare PCR mix using the T7 Guide Primer for Forward PCR and tracrRNA reverse primer. The T7 promoter is introduced into the template. Add 1 µL of each of the miniprepped recombinant guide plasmids (at ~1-3 ng/µL) to 1 tube each and 1 µL Ultrapure water to the final tube.

	A	B
	Reagent	Amount
	Ultrapure water	12.3 µL
	NEB Phusion HF Reaction Buffer (5x)	4 µL
	T7 guide primer (10 µM)	1 µL
	tracrRNA reverse primer (10 µM)	1 µL
	Roche PCR Grade Nucleotide Mix (10 mM)	0.5 µL
	NEB Phusion HF DNA Polymerase (2 U/µL)	0.2 µL
	Total	19 µL

3 Place the tubes in a thermocycler with the following parameters:

	A	B	C
	Steps	T7 PHUSION	
	1	98°C	3 min
	2	98°C	15 s
	3	60°C	20 s
	4	72°C	15 s
	5	Go to step 2	32 times
	6	72°C	5 min
	7	4°C	∞

PCR amplification of guide template

4 Make a 1% agarose gel and run 5 µL of the PCR products 40 min @100 V.

Note

Testing to see if the plasmid has the correct insert. Band should be present at approximately 100 bp

5 Combine the remainder of all PCR reactions with the correct band and perform Qiagen PCR Purification on the mixture. Use NanoDrop to measure concentration of DNA. A260/A280 values should fall in the range of 1.8-2.0.

Note

This is to confirm if the DNA is still present and determine the amount needed for the IVT reaction.

In vitro transcription (IVT)

6 Transfer 2 µL to a PCR tube for testing later.

Note

We are using the purified PCR product as the template for IVT with the NEB HiScribe™ T7 Quick High Yield RNA Synthesis Kit.

7 Mix the following reagents in a PCR tube:

	A	B
	Reagent	Amount
	Ultrapure H2O	Up to 60 µL
	IVT gRNA Product	40 µL
	NEB DNase I (RNAse-free) (2 U/µL)	4 µL
	Total	104 µL

Note

Degrades DNA

8 Incubate 15 min @ 37 °C.

9 Transfer 2 µL to a separate PCR tube for testing later.

10 Perform Qiagen RNEasy Mini Kit RNA Cleanup, eluting in 30 µL water.

11 Transfer 2 µL to a separate PCR tube for testing later.

12 Add 2 µL NEB Formaldehyde Load Dye to each of the 2 µL RNA aliquots set aside.

13 Place the mixture in a thermocycler with the following parameters:

	Steps	Reagent	Amount
	1	85°C	3 min
	2	4°C	∞

14 Make an RNase-free 1% agarose gel and run RNA aliquots 30 min @ 100 V.

Note

Testing the gRNA has been produced correctly. Bands should be present at >100 bp (RNA will run higher on the gel than DNA when run against a DNA ladder).

Cytoplasmic Injection

15 On the day of zygote injection, prepare the injection mix as follows:

	A	B
	Reagent	Amount
	Ultrapure H2O	10.125 µL
	Injection buffer (10x)	1.5 µL
	gRNA 1	To give 50 ng/µL
	gRNA 2	To give 50 ng/µL
	SpCas9 mRNA	To give 100 ng/µL
	Total	15 µL

Protocol references

1. Concordet JP, Haeussler M. CRISPOR: intuitive guide selection for CRISPR/Cas9 genome editing experiments and screens. Nucleic acids research. 2018;46(W1): W242–W245. **doi: 10.1093/nar/gky354**

2. Adikusuma, F., Pfitzner, C., & Thomas, P. Q. (2017). Versatile single-step-assembly CRISPR/Cas9 vectors for dual gRNA expression. PloS one, 12(12), e0187236. https://doi.org/10.1371/journal.pone.0187236