Aug 08, 2024

MYH9 intron 3 saturation mutagenesis screening

DOI
dx.doi.org/10.17504/protocols.io.8epv5x5qdg1b/v1

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Andrea R Daniel: This protocol was adapted from the work of Brian Cosgrove and Lexi Bounds in the Gersbach lab at Duke University.

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Protocol Citation: Brian D. Cosgrove, Lexi Bounds, Carson Key Taylor, Alan L. Su, Anthony J. Rizzo, Alejandro Barrera, Andrea R Daniel, Gregory E. Crawford, Brenton D. Hoffman, Charles A. Gersbach 2024. MYH9 intron 3 saturation mutagenesis screening. **protocols.io https://dx.doi.org/10.17504/protocols.io.8epv5x5qdg1b/v1**

Manuscript citation:
Mechanosensitive genomic enhancers potentiate the cellular response to matrix stiffness
Brian D. Cosgrove, Lexi R. Bounds, Carson Key Taylor, Alan L. Su, Anthony J. Rizzo, Alejandro Barrera, Gregory E. Crawford, Brenton D. Hoffman, Charles A. Gersbach
bioRxiv 2024.01.10.574997; doi: https://doi.org/10.1101/2024.01.10.574997

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

Created: January 09, 2024

Last Modified: August 08, 2024

Protocol Integer ID: 93205

Keywords: CRISPR, MYH9, enhancer, mechanoenhancer, extracellular matrix

Funders Acknowledgement:
NIH
Grant ID: HG012053

Abstract

This protocol describes methods for a CRISPR screen to identify motifs in the MYH9 intron 3 mechanoenhancer that control MYH9 expression.

Materials

pLV_hU6-sgRNA_hUbC-GFP-P2A-PuroR (Addgene plasmid #162335)

Library design and cloning

1 For the MYH9 intron 3 pRE saturation mutagenesis library, we include any gRNA that are within the hit pRE from the MYH9 locus library (from our previous CRISPRi stiffness-responsive screen), which resulted in 64 gRNA across the library.

2 Include 25 non-targeting gRNA (1) and 11 safe-targeting gRNA (2) as negative controls. This combined gRNA library of 100 gRNA is synthesized as an oligo pool by Twist Biosciences with common overhangs for cloning into a lentiviral backbone.

3 This oligo pool is PCR amplified. pLV_hU6-sgRNA_hUbC-GFP-P2A-PuroR (Addgene plasmid #162335) is then digested with Esp3I, gel purified, and then ligated along with the amplified oligo pool by Gibson assembly.

4 Following a 1x SPRI cleaning, the Gibson assembly is transformed into Endura competent cells (Lucigen) according to the manufacturer's protocol, and cultured overnight before maxi-prepping the
gRNA-library plasmid.

5 A PCR amplicon across the gRNA region of the resulting plasmid is sequenced to a depth of ~100k-1M read pairs on an Illumina miSeq in order to verify coverage across the entire gRNA library.

Lentiviral generation and functional titering

6 gRNA library plasmid pool is co-transfected into ~7.8M HEK293T cells along with two lentiviral packaging plasmids using Lipofectamine 3000 (ThermoFisher).

7 20 hours post-transfection, the transfection media is removed and fresh growth media added. Media containing viral particles is removed one day later at 48 hours post-transfection and stored, replaced with fresh media and collected one day later and stored at 4C.

8 Combined media containing viral particles is filtered through 0.45 µm low-protein binding filters, and then concentrated using Lenti-X Concentrator (Takara Bio) according to the manufacturer’s protocol.

9 Functional titering to determine MOI is performed by transducing HFF (human neonate fibroblast) cells across a 0.75x-100x dilution range of the viral stock, and then subjecting the cells to FACS-based cell sorting to identify what percent of the population is mCherry+ for each viral stock dilution.

MYH9 saturation mutagenesis screen

10 HFF cells are transduced with a lentiviral SpCas9 construct [FUGW-SpCas9-2A-HygroR (pVG54)], selected with 100ug/mL hygromycin for 4 days with hygromycin in order to make a

stable line.

11 Following four passages the cells are frozen and used for subsequent screening experiments and validations. 600k HFF cells are transduced with lentivirus encoding the MYH9 intron 3 saturation pool.

12 For screening cells are cultured for 8 days.

13 Cells are trypsinization with 0.25% Trypsin-EDTA for 5 minutes at 37C. Trypsin is neutralized with 1X volumes of complete growth media, cells are centrifuged at 300g for 5 minutes, and the supernatant aspirated. The cells are rinsed once with 1X volume PBS followed by another centrifugation and aspiration leaving 200uL of PBS above the pellet.

14 The eBioScience ICC Fixation kit (ThermoFisher) is used to fix/permeabilize cells according to manufacturer's instructions. Note, equilibrate reagents to room temp prior to usage. To fix cells, add 500uL eBioSciences Fix/Perm Buffer (ThermoFisher) to the 200uL PBS and pellet, and incubate at room temperature for 20 minutes.

15 Add 1X Permeabilization Buffer up to 8mL total volume, spin at 600g for 5 minutes, and add perm buffer rinse.

16 HFF cells are incubated for 30 minutes at room temperature in the dark on a nutating rocker, spun 600g for 5 minutes spin, followed by two repeats of 3mL 1X Perm Buffer rinse/spin cycles.

17 Cells were resuspended in FACS Buffer [1X PBS supplemented w/ 1% BSA (Sigma) and 0.5mM EDTA (Sigma)] at a density of ~9M cells/mL and sorted. A SH800 Cell Sorter (Sony Biotechnologies) is used to separate out the top/bottom-expressing MYH9 fractions following immunostaining.

18 Single channel expressing cell populations are used to set up compensation panels. These include untreated cells, antibody-only cells, mCherry-only cells. The top 10% and lower 10% of the MYH9 population is sorted and used for downstream gRNA-enrichment analysis and sequencing.

19 Sorted cells are counted and DNA extracted form the fixed cells using the PicoPure DNA extraction kit (ThermoFisher) according to the manufacturer's protocol.

20 Next, gRNA PCR and processing for enrichment across the low and high MYH9 expression bins is performed.

Protocol references

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