Aug 08, 2024

CRISPRi screen, MYH9 locus

DOI
dx.doi.org/10.17504/protocols.io.yxmvm3b46l3p/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. CRISPRi screen, MYH9 locus. **protocols.io https://dx.doi.org/10.17504/protocols.io.yxmvm3b46l3p/v1**

Manuscript citation:
Mechanosensitive genomic enhancers potentiate the cellular response to matrix stiffness
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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: December 21, 2023

Last Modified: August 08, 2024

Protocol Integer ID: 92636

Keywords: ECM stiffness, extracellular matrix, CRISPRi, MYH9, regulatory elements

Funders Acknowledgement:
NIH
Grant ID: HG012053

Abstract

This protocol describes methods for a CRISPR interference screen to identify open chromatin regions that function as stiffness-responsive modulators of *MYH9* expression.

Library design and cloning

1 Open chromatin regions within 440 kb of the MYH9 TSS are used to generate the oligo pool (identified from ATAC-seq of primary human fibroblasts cultured on soft and stiff ECM). For each ATAC-seq peak, any gRNA with a GuideScan specificity score of > 0.2 is included [which has previously been shown to increase the quality of non-coding screens (1)]. 114 peaks are represented in the library, with an average of ~41 gRNA/peak.

2 500 non-targeting gRNA are included as negative controls (2). The combined gRNA library of 5,192 gRNA is synthesized as an oligo pool by Twist Biosciences with common overhangs for cloning into our lentiviral backbone.

3 PCR amplify the oligo pool.

4 A hU6-driven lentiviral gRNA vector (pBDC119) is then digested with Esp3I, gel purified, and ligated along with the amplified oligo pool by Gibson assembly.

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

6 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 of MYH9 locus library

7 gRNA library plasmid is co-transfected into ~18M HEK293T cells along with two lentiviral packaging plasmids using Lipofectamine 3000 (ThermoFisher).

8 20 hours post-transfection, the transfection media is removed and fresh growth media added.

9 One day later, media containing viral particles is removed (at 48 hours post-transfection) and stored at 4C. Fresh media is added to the cultures and collected one day later for storage at 4C.

10 The media containing viral particles is combined and filtered using 0.45 µm low-protein binding filters. The filtered media is concentrated using Lenti-X Concentrator (Takara Bio) according to the manufacturer’s protocol.

11 Functional titering to determine MOI is performed by transducing HFF cells across a 50x-10,000x 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.

CRISPRi locus screen

12 A stable HFF (primary human neonatal foreskin fibroblasts) cell line created using a lentiviral dCas9-KRAB construct [pLV-hUbC-dCas9-KRAB-2A-Blast (pJB289)]. The gRNA library is transduced at an MOI of ~0.33 followed by Puro selection for four days at 1 ug/mL.

13 Cells are maintained for an additional four days, prior to 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 Count HFF cells and remove approximately 2 million to use as unsorted controls. Set aside another approximately 500,000 cells to use as control samples for single channel compensation controls.

17 Immunostaining is performed on the remaining cells using a AlexaFluor-488 conjugated Rabbit monoclonal anti-NMMIIA antibody (clone EPR8965, Abcam, #ab204675) at a ratio of 0.5 uL antibody per 300k HFF cells per 100uL of Perm Buffer.

18 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.

19 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.

20 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.

gDNA recovery and library preparation

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

22 Recovery digests are performed for 20 hours at 65C using up to 1.5M HFF cells per reaction volume. All gDNA is split between sample-indexed 100uL Q5 PCR reactions (up to ~340ng max input per 100uL reaction) to amplify out the gRNA protospacer from HFF cells.

23 gDNA PCR reaction conditions:
98C for 30s / 25x: 98C for 10s, 60C for 30s, 72C for 15s / 72C for 2 min.

Primers:

PCR primers for gDNA recovery and library preparation.		
PCR number	Primer name	Primer sequence
1	gRNA Lib Fwd	AATGATACGGCGACCACCGAGATCTACACAATTTCTTGGGTAGTTTGCAGTT
1	gRNA Lib Rev, BC 21	CAAGCAGAAGACGGCATACGAGATCTTTTGGACTCGGTGCCACTTTTTCAAGTTGATAAC
1	gRNA Lib Rev, BC 22	CAAGCAGAAGACGGCATACGAGATtagttgGACTCGGTGCCACTTTTTCAAGTTGATAAC
1	gRNA Lib Rev, BC 23	CAAGCAGAAGACGGCATACGAGATccggtgGACTCGGTGCCACTTTTTCAAGTTGATAAC
1	gRNA Lib Rev, BC 24	CAAGCAGAAGACGGCATACGAGATatcgtgGACTCGGTGCCACTTTTTCAAGTTGATAAC
1	gRNA Lib Rev, BC 25	CAAGCAGAAGACGGCATACGAGATcgcctgGACTCGGTGCCACTTTTTCAAGTTGATAAC
1	gRNA Lib Rev, BC 26	CAAGCAGAAGACGGCATACGAGATctgatcGACTCGGTGCCACTTTTTCAAGTTGATAAC
2	Oligo Lib Fwd	AATGATACGGCGACCACCGAGATCTACACTAACTTGAAAGTATTTCGATTTCTT
2	gRNA Lib Rev, BC 22	CAAGCAGAAGACGGCATACGAGATtagttgGACTCGGTGCCACTTTTTCAAGTTGATAAC

24 Individual PCRs are pooled together and subjected to a double-sided 0.65X/1X SPRI clean-up.

25 Quality control is performed by running the amplified libraries out on a High Sensitivity D1000 Tapestation (Agilent) to confirm expected size and Qubit dsDNA HS assays were performed to determine a final concentration.

26 All libraries are pooled to an effective concentration of 4 nM and combined in equal volumes prior to sequencing on an Illumina MiSeq, using a v2 50 cycle reagent kit with Read1 being 21 cycles (protospacer) and index read 1 being 6 reads (sample barcoding).

MYH9 locus library analysis

27 Resulting FASTQ files are aligned to a custom reference sequence corresponding to the given gRNA library using bowtie2 and all downstream analyses performed in R.

28 All gRNA are verified to be represented in the baseline untreated library data Day 8 post-transduction, and counts+1 for each gRNA are taken (to normalize for samples that dropped out in one condition) and normalized by sequencing depth for each library before downstream analysis (in counts per million reads sequenced, 'CPM').

29 Due to the highly-apparent strand bias in the positive-strand when targeting the MYH9 gene body only include the non-interfering gRNA from the negative strand.

30 Take a ratio of the CPM for each gRNA of the low MYH9 expression group to high MYH9 expression group to identify whether the gRNA perturbation led to increases in enrichment in either expression bin.

31 Next, for each screen replicate the Z-score is calculated for each gRNA relative to the control non-targeting gRNA population using similar methodologies as previously described in (3).

32 First, each sample's ratio is converted to a log2 fold-enrichment, and population statistics for the negative control non-targeting gRNAs (median, standard deviation, gRNA number) are calculated.

33 For each individual gRNA, the median of the negative control fold-enrichment is subtracted from each individual gRNA's log2 fold-enrichment value, and this value is further divided by the standard deviation of the negative control non-targeting gRNA population to get an individual Z-score relative to the negative control population.

34 Raw Z-score values from both replicates are pooled to calculate pRE-wide effects. Phenotype scores (t-score based) are calculated as:

$$Phenotype\ Score\ =\ U\ (pRE)\ -\ U\ CTL\ /\ \sqrt{(\frac{S\ var}{Nexp}\ +\frac{S\ var}{NCTL})}$$

$$Svar\ =\ Var\ (pRE)\ *\ (N(pRE)-1)\ +\ Var(CTL)*(Nctl\ -1)$$

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