Aug 08, 2024

Bulk growth and migration functional CRISPRi screens

DOI
dx.doi.org/10.17504/protocols.io.81wgbxoeolpk/v1

Brian D. Cosgrove[1], Lexi Bounds[1], Carson Key Taylor[1], Alan L. Su[1], Anthony J. Rizzo[1], Alejandro Barrera[1], Andrea R Daniel[1], Gregory E. Crawford[1], Brenton D. Hoffman[1], Charles A. Gersbach[1]

[1]Duke University

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

Andrea R Daniel
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. Bulk growth and migration functional CRISPRi screens. **protocols.io https://dx.doi.org/10.17504/protocols.io.81wgbxoeolpk/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: 93209

Keywords: CRISPRi, mechanoenhancer, ECM stiffness, extracellular matrix, growth, migration

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 cell growth and migration.

Materials

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

Library design and cloning

1 A library of 21,458 gRNA targeting the top 1000 non-promoter ATAC-seq peaks ranked by increased accessibility on stiff substrate (50kPa) compared to the soft 1 kPa substrate hydrogels. For each peak, any gRNA that had a GuideScan specificity score of > 0.2 is included, which has previously been shown to increase the quality of non-coding screens *(1)*. 969 peaks are represented in the
library, with an average of ~20 gRNA/peak.

2 sgRNA library also contains 1000 non-targeting gRNA (2) and 249 promoter-targeting gRNA for 83 positive control genes that have previously been shown to be key modulators of transwell migration following RNAi screens (3), with 3 gRNA per gene taken from the Dolcetto library (4).

3 The combined gRNA library is synthesized as an oligo pool by Twist Biosciences with common overhangs for cloning into the lentiviral backbone.

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

5 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.

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.

Functional titering

7 Concentrated lentivirus is generated from this plasmid pool. Functional titering to determine MOI is performed by transducing HFF (Primary Human Neonate Fibroblasts) cells across a 50x-10,000x dilution range of the viral stock, and then subjecting the cells to a qPCR-based titering protocol that has been previously described in detail (5).

Migration/Growth pRE (putative regulatory element) library screen

8 To perform screening, 600k HFF cells are transduced with the lentiviral library virus at 10.8 MOI to achieve a coverage of ~279 cells per gRNA.

9 20 hours after transduction the viral media is removed and replaced with fresh media, and starting 48 hours after transduction HFF cells are selected with 1 ug/mL puromycin for 4 days.

10 Puromycin selection media is then removed and HFF cells were grown out for two additional days until day 8.

11 On day 8, ~11M cells are counted and split between migration and growth screens. Coverage of
at least 279 cells/gRNA is maintained for each group throughout the entire experiment.

Migration screening

12 On day 8, the bottoms of 8 um transwell inserts for 6WP are coated with 10 ug/mL fibronectin at room temp for 45 minutes and then rinsed 1x with PBS for 30 minutes before use.

13 HFF cells are counted, placed into low serum conditions (0.2% FBS) and seeded at 240k cells per transwell insert across 18 inserts (~4.4M cells total).

14 These inserts are placed into 10% serum and cells are allowed to migrate for 24 hours.

15 Following this first day of migration, each side of the membrane is separately trypsinized and counted, cells recovered from the bottom are recognized as migratory cells (~1.2M cells) and non-migrated cells were recovered from the top of the insert.

16 These migratory and non-migratory populations are re-seeded (separately by group) in the same way on new fibronectin-coated transwell inserts, with 4-5 inserts seeded at 240k cells/insert and allowed to migrate overnight.

17 Following these two rounds of migration the cells that are trypsinized and collected are cells that either migrated twice or did not migrate twice. gDNA is isolated from cells using DNeasy kits (Qiagen).

Growth screening

18 HFF cells are counted on day 8 post-transduction, and gDNA from 2M HFF cells is harvested as the “Day 0” reference population using a DNeasy Blood and Tissue Kit (Qiagen).

19 Around 1M HFF cells are reseeded into 15 cm dishes for ongoing culture, and then serially-passaged as normal for 14 doublings (either 21 days post-“Day0” for replicate 1 or 22 days post-“Day0” for replicate 2) while maintaining at least 1M cells per dish during each passaging, prior to the final gDNA harvest using a DNeasy Blood and Tissue Kit (Qiagen).

Library preparation and sequencing

20 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.

21 PCRs from gDNA are run [98C for 30s / 25x: 98C for 10s, 60C for 30s, 72C for 15s / 72C for 2 min] and followed by the pooling of individual PCRs and subjected to a double-sided 0.65X/1X SPRI clean-up.

	A	B	C
	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	CAAGCAGAAGACGGCATACGAGATcgcctgGACTCGGTG

	A	B	C
			CCACTTTTTCAAGTTGATAAC
	1	gRNA Lib Rev, BC 26	CAAGCAGAAGACGGCATACGAGATctgatcGACTCGGTGCCACTTTTTCAAGTTGATAAC
	2	Oligo Lib Fwd	AATGATACGGCGACCACCGAGATCTACACTAACTTGAAAGTATTTCGATTTCTT
	2	gRNA Lib Rev, BC 22	CAAGCAGAAGACGGCATACGAGATtagttgGACTCGGTGCCACTTTTTCAAGTTGATAAC

22 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 are performed to determine a final concentration.

23 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).

Screen analysis

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

25 All gRNA are verified to be represented in the baseline untreated library at 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').

26 For migration screens: A ratio is taken of the CPM for each gRNA of the 2x migrated group to the 2x non-migrated group to identify migratory or non-migratory enrichment.

27 For growth screens: A ratio is taken of the CPM of the Day 0 population relative to the final Day 21/22 population for each replicate.

28 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 (6).

29 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.

30 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.

31 Raw Z-score values from both replicates are pooled to calculate pRE-level effects.

32 An individual gRNA is called as a "hit" if the Z-score is above 2 or below -2.

33 pRE-level stats are generated by performing a Fisher's exact text relative to the non-targeting gRNA population, and a pRE-level is labeled significant for follow-up if the pval is less than 0.1.

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