Nov 20, 2024

CRISPRi Screening for Functional Characterization of Blood Pressure (BP) and Coronary Artery Disease (CAD)-Associated Genes in Endothelial Cells

DOI
dx.doi.org/10.17504/protocols.io.5qpvo9ybxv4o/v1

Estelle Lecluze[1], Hicham Belhaj[1], Candice Diaz[1], Chao-Sheng Lo[1], Guillaume Lettre[1,2], Ken Sin Lo[3]

[1]Montreal Heart Institute, Montréal, Quebec, Canada.;
[2]Faculté de Médecine, Université de Montréal, Montréal, Quebec, Canada.; [3]Montreal Heart Institute

IGVF

Chao-Sheng Lo
Montreal Heart Institute

DOI: https://dx.doi.org/10.17504/protocols.io.5qpvo9ybxv4o/v1

Protocol Citation: Estelle Lecluze, Hicham Belhaj, Candice Diaz, Chao-Sheng Lo, Guillaume Lettre, Ken Sin Lo 2024. CRISPRi Screening for Functional Characterization of Blood Pressure (BP) and Coronary Artery Disease (CAD)-Associated Genes in Endothelial Cells. **protocols.io https://dx.doi.org/10.17504/protocols.io.5qpvo9ybxv4o/v1**

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

Created: November 19, 2024

Last Modified: November 20, 2024

Protocol Integer ID: 112416

Keywords: genetic loci critical to endothelial biology, implementation of pooled crispr interference, pooled crispr interference, genes in endothelial cell, endothelial biology, crispr, human endothelial cell, endothelial cell, guide rna, cardiovascular health, coronary artery disease, exploring genetic loci, functional characterization of blood pressure, gene, functional roles of gene, blood pressure, artery

Funders Acknowledgements:
Guillaume Lettre
Grant ID: HG012010

Abstract

This protocol describes the development and implementation of pooled CRISPR interference (CRISPRi) screens to investigate the functional roles of genes associated with blood pressure (BP) regulation and coronary artery disease (CAD) in human endothelial cells (TeloHAEC). A comprehensive library of 6,553 guide RNAs (gRNAs) was designed to target 1,281 variants, enabling precise identification and high-throughput analysis. This protocol provides a robust framework for exploring genetic loci critical to endothelial biology and cardiovascular health.

Troubleshooting

A. Selection of genes for the CRISPRi screening

1 Sentinel variants associated with systolic blood pressure (SBP), diastolic blood pressure (DBP), or pulse pressure (PP) was compiled from large European- or cross-ancestry meta-analyses of genome-wide association study (GWAS) results (PMID: 30224653 and 30578418). All variants in linkage disequilibrium (LD, $r^2 \geq 0.8$) with the blood pressure (BP), GWAS sentinel variants in European, African, East Asian, and South Asian ancestries were recovered using TOP-LD (PMID: 35504290). All genes located within 250 kb of one of these variants were selected as the first set of candidate BP genes. The list of genes was further refined by retaining variant-gene pairs that are eQTL or sQTL in any tissues from the GTEx database. Additionally, genes carrying rare coding variants associated with BP were included (PMID: 33230300). The list of genes was further filtered to retain those expressed in endothelial cells (FPKM ≥ 1) (PMID: 31287004) or those encoding drug targets based on DGIdb (PMID: 33237278). Gene set enrichment was characterized in Gene Ontology Biological Processes, KEGG pathways, and hallmarks of MsigDB using
ShinyGo (PMID: 31882993). Essential genes (31 genes) and genes known to be important for endothelial cell activity (19 genes) were added.

B. Guide RNA (gRNA) design for the CRISPRi screening

2 We aimed to select five gRNA per target genes. First, gRNAs were chosen from the Brunello and Dolcetto libraries (PMID: 30575746). The best gRNAs were selected after evaluating their quality and off-target potential using crispRtool. If fewer than five gRNAs were available, additional gRNAs were selected from the Calabrese library (PMID: 30575746). For coding genes with an insufficient number of high-quality gRNAs in these libraries, gRNAs were retrieved from the GECKO/SAM libraries. For long noncoding RNAs, gRNAs were manually designed based on ATAC-seq peaks. In total, 5817 gRNAs were designed to target 1281 variants. Additionally, 65 targeting gRNAs and 500 safe gRNAs were added as negative controls, as well as in 171 essential genes. Each gRNA in the final library was assigned a unique 7-nucleotide barcode to enable precise identification during downstream analyses, ensuring that these barcodes did not contain repetitive sequences (e.g., AAAA, TTTT, CCCC, GGGG). To increase signal sensitivity, a total of 6553 gRNAs were randomly distributed into two pooled library sets.

C. Guide RNA library preparation

3 The pooled gRNA library was synthesized by Agilent Technologies.
Krab library structure:
First half:
5′-GGCTTTATATATCTTGTGGAAAGGACGAAACACCG[20-nt gRNA]GTTTAAGAGCTATGCTGG-3′

Second half:
5′-GTGGAAAGGACGAAACACCG[20-nt gRNA]GTTTAAGAGCTATGCTGGAAACAGCATAGCAAG-3′

3.1 Reconstitute ssDNA oligo pool:
Spin down lyophilized ssDNA oligo pool.

3.2 Prepare 1 ng/µL stock by resuspending in TE buffer with low EDTA (10mM Tris-Cl pH 8.0, 0.1 mM EDTA).

3.3 Aliquot into 3 tubes to limit chances of contamination and to reduce freeze/thaw cycles.
Store at -80 °C.

3.4 PCR:

	A	B
		Sequence
	kRAB_library1_Fwd	ggctttatatatcttgtgga
	Krab_library1_Rev	cttgctatgctgtttccagcatagctcttaaac
	KRAB_library2_Fwd	ggctttatatatcttgtggaaaggacg
	KRAB_library2_Rev	cttgctatgctgtttccagc

3.5 PCR Reaction:

		Volume (ul)
	Oligo pool (1ng/ul)	1
	NEBNext Ultra II Q5 Master Mix (E7805L)	12.5
	PCR1 forward primer (10uM)	1.25

PCR1 reverse primer (10uM)	1.25
dd H2O	9
Total volume	25

3.6 Cycling:

	Temp	Time	Cycles
Step 1	98°	30 sec	1X
Step 2	98°	10 sec	18X
	66 °	30 sec	
	72°	30 sec	
Step 3	72°	5 min	1X
Step 4	4°	Hold	

3.7 PCR volume reduction to load in gel:
Pool all the reactions and PCR purify using QIAquick PCR purification Kit, QIAGEN.
Elute in 20ul dd H2O

3.8 Run 2% agarose gel, and isolate band using gel extraction method.

Gel extraction, (QIAquick Gel Extraction Kit, QIAGEN)

4 Gel extraction

4.1 Weigh the gel slice

4.2 Add 3 volumes of Buffer QG to 1 volume of gel (100mg~100ul).

4.3 Incubate at 50°C for 15 min with shaker in Thermomix.

4.4 Add 1 gel volume of isopropanol and mix

4.5 Place into a QIAquick spin column

4.6 Centrifuge for 1min

4.7 Discard flow-through

4.8 Add 0.5ml QC buffer and centrifuge for 1min

4.9 Discard flow-through

4.10 To wash, add 0.75ml PE buffer and centrifuge for 1min

4.11 Discard flow-through

4.12 Additional spin 3 min to dry the column

4.13 Place the column into a new clean tube

4.14 Add 20ul dd H2O to elute DNA.

4.15 -Restriction Digest of pHKO9-BsmBI library backbone:

4.16 Add components in the following order:

	Volume (ul)
pHKO9-BsmBI (10ug)	15
dd H2O	65
rCutsmart	10
DTT (20mM)	5
BsmBI	5

4.17 Incubate at 37 °C for 1 hour.

4.18 Run 1% agarose gel and isolate 8K bps band by gel extraction (as described previously).

4.19 Gibson assembly:

4.20 Set up the following reaction for each library half on ice

A	B
	Volume (ul)
Gibson Assembly Master Mix (2X) (E5510)	10

	A	B
	pHKO9-BsmBI (350ng/ul)	2.8
	PCR product from gel extraction (50ngl)	3.5
	dd H2O	3.7
	Total Volume	20

4.21 Incubate samples in a thermocycler at 50°C for 1 hour

4.22 . Isopropanol precipitation

		Volume (ul)
	Gibson reaction mix	20
	Isopropanol	20
	GlycoBlue	0.5
	5M NaCl	0.5

4.23 Vortex, incubate at room temperature for 15 min

4.24 Centrifuge top speed for 15 min

4.25 Carefully remove liquid without disturbing pellet

4.26 Wash twice with 1ml of ice cold 80% EtOH

4.27 Carefully remove all liquid, and air-dry pellet 2-3 min by keeping the cap open and leaving
at room temperature

4.28 Add 6ul ddH2O and warming at 55C 10min to fully resuspend

4.29 Electroporation:

4.30 Add 2uL of Gibson assembly(~50ng) to 25uL of Lucigen Endura electrocompetent cells

4.31 Electroporate using the following parameters: 1mm cuvette, 10 µF, 550 Ohms, 1700 Volts

4.32 Immediately add 1mL S.O.C recovery media

4.33 Shake at 37 °C for 1 hr

4.34 Prepare 1ml of 1000x dilution of the bacterial culture by adding 10ul to 990ul of LB. Plate 100ul of the 1000x dilution (which will be our 10 000x dilution) in agar plate and incubate at 37 °C overnight. Keep the rest at 4 °C for Midiprep.

4.35 Count colonies present in 10000x dilution and subtract colonies from control to assess cloning
efficiency and estimate total colonies. We aim for a coverage of at least 20x.

4.36 Incubate the remaining 990ul of library liquid culture in 50 ml LB and incubate overnight at 37°C

4.37 Perform Midiprep on the bacterial culture.

D. Generate TeloHAEC- dCas9-KRAB cell line

5 TeloHAEC cell line expressing dCas9-KRAB was generated using immortalized human aortic
endothelial cells (ATCC CRL-4052) as previously described (PMID: 36928188).

E. Lentivirus Production

6 -Lentivirus production:

One day before: T175 flasks at a density of 8×10^4 HEK293FT cells/cm^2 (14M cells total), in a total of 25ml DMEM (+10% FBS) no p/s.

The next day, in the morning, transfect the cells with the plasmids as indicated below,

6.1 (A)Lentiviral target mix- **Always measure the concentration of the plasmid before and adjust the volumes accordingly.**

	A	B
	Component	Amount per T175 flask
	Opti-MEM	1750 µl
	pMD2.G (lentiviral helper plasmid) (Addgene#12259)	11.9 µg
	psPAX2 (lentiviral helper plasmid) Addgene#12260)	18.2 µg
	Lentiviral target plasmid (plasmid library)	23.8 µg

6.2 (B)PLUS reagent mix

	A	B
	Component	Amount per T175 flask
	Opti-MEM	1750 µl
	PLUS reagent	231 µl

Add the (B)PLUS reagent mix to the (A)lentiviral target mix, invert, and incubate at room temperature for 5 min.

6.3 (C)Lipofectamine reagent mix

	A	B
	Component	Amount per T175 flask
	Opti-MEM	3500 µl
	Lipofectamine 2000	210 µl

Add the (A)lentiviral target and (B)PLUS reagent mix to the(C)Lipofectamine reagent mix, invert, and incubate at room temperature for 5 min. Pipette 9 ml of the lentiviral transfection mix into T175 flask and shake the flasks gently to mix.

6.4 Change the medium 4 hours later with 50ml DMEM(+10%FBS+1%BSA). **Also, when changing**
the medium, should notice small black dots between cells.

6.5 Two days after transfection, collect the supernatant in a 50ml tube, centrifuge at 1000rpm for 1 minute, pool the supernatant in Steriflip, and use the vacuum to pass it through the filter. Aliquot 5ml into the required 15ml conical tubes and 1ml into a 3X microcentrifuge tube.

6.6 -Titrating:
Before cell infection, the virus must be titrated to determine the volume required to achieve a Multiplicity of Infection (MOI) of approximately 0.3.
TeloHAEC- dCas9-KRAB cells were thawed from liquid nitrogen storage and passaged twice before infection. The cells were trypsinized and prepared at a density of 2.5 × 10^5 cells/ml.

6.7 The volumes of cells, medium, virus, and polybrene required for virus titration in a 6-well plate are provided.

	A	B	C	D	E
	Well ID	Cell suspension (ul)	VCBM (ul)	Virus (ul)	Polybrene (ul)
	1	800	800	0	1.6
	2	800	750	50	1.6
	3	800	700	100	1.6
	4	800	600	200	1.6

	A	B	C	D	E
	5	800	400	400	1.6
	6	800	0	800	1.6

6.8 Twenty-four hours after infection, the infected cells were passaged and split 1:3 into T25 flasks containing selection medium (Vascular Vell Basal Medium, VCBM (PCS-100-030) + VEGF-kit (PCS-100-041) + puromycin (P9620) at 0.3 µg/ml + Geneticin (G418, #10131035) at 350 µg/ml).

6.9 Five days post-infection, the cells were counted using a hemocytometer. The MOI was then established by dividing the number of viable cells in each virus-infected and antibiotic-selected condition by the number of viable cells in the control condition without antibiotics.

F. CRISPRi screens for endothelial cell proliferation

7 -Transduction of guide RNA library in TeloHAEC- dCas9-KRAB cells.
Four batches per set of lentiviruses were produced. TeloHAEC-dCas9-KRAB cells were thawed from liquid nitrogen storage and passaged twice before infection. For each batch needs 3xT225 culture flasks, each T225 culture flask contains 5 million cells in 26.75 ml VCBM and 25ul polybrene (1mg/ml) with the volume of virus (MOI=0.3) obtained from titration.
After 24 hr infection, the infected cells were passaged and split 1:3 into T25 flasks. Cells were selected using 0.3 µg/ml puromycin and 350 µg/ml G418 for 5 days in VCBM. After the selection period, 2 million cells were collected for proliferation screening.

G. CRISPRi screens for NFKBIA expression by HCR-FlowFISH method

8 Following selection on day 6, cells were stimulated with TNFα (10 ng/ml) for four hours to induce a stress response. NFKBIA gene expression was detected using HCR-FlowFISH pipeline (PMID: 34326544). The HCR-FlowFISH method was utilized to capture signals corresponding to the TOP 20%, TOP 20–40%, Bottom 20–40%, and lowest 20% of NFKBIA gene expression in 4 bins. HCR probes and fluorescently labeled hairpins were purchased from Molecular Instruments, using probe sequences targeting the NFKBIA gene (NM_020529.3) and the housekeeping gene TBP (NM_003194). For TBP detection, 20 probes were designed, while for NFKBIA detection, 18 probes were used.
Hybridization and signal amplification were carried out according to the HCR-FlowFISH protocol provided by Molecular
Instruments.

8.1 Day-1
After 4hr TNF-a stimulation, 60 million cells were processed to HCR-FlowFISH protocol.

8.2 Paraformaldehyde fixation-
Resuspend cells in 1ml 4% PFA in PBST.

8.3 Fix cells for 30 min at room temperature with gentle agitation. Darkness (Aluminium foil).

8.4 Centrifuge for 5 min at 700g and aspirate supernatant.

8.5 Resuspend in an equal amount of PBST and without incubation, spin down the cells for 5 minutes at 500g. Aspirate supernatant.

8.6 Repeat for a total of 4 washes with PBST.

8.7 Resuspend cells in 1 ml cold fresh 70% ethanol.

8.8 Store cells solution at 4 °C for 10 minutes.

8.9 Signal Detection-
Start pre-warming Probe Hybridization to 37°C.

8.10 Spin down the cells for 5 minutes at 500g. Aspirate supernatant.

8.11 Resuspend cells in 500µl of PBST (per 5 million cells).

8.12 Spin down the cells for 5 minutes at 500g. Aspirate supernatant.

8.13 Repeat for a total of 2 washes.

8.14 Resuspend the cell pellet in 400µl of pre-warmed Probe Hybridization (per 5 million cells).

8.15 Transfer to ependorfs as compatible with Thermomixer.

8.16 Incubate at 37°C for 30 minutes.

8.17 In the meantime, prepare a DNA probe solution by adding 2 µl (2 pmol) of DNA probe to 100µl of Probe Hybridization buffer. (1uM stock = 2µl per 100µl)

	DNA probe	Number of samples	Volume of DNA probe needed	Volume of Hybridization needed
	TBP	12	24 µl	2400 µl
	NFKBIA	12	24 µl	2400 µl

8.18 Add 100 µl of DNA probe solution to 400 µl of cells.

8.19 Incubate the sample overnight at 37°C in Thermomixer.

8.20 Day-2
Add 2500 µl Probe Wash to cells and spin down for 15 minutes at 850g. Ensure minimal cell loss and aspirate supernatant.

8.21 Resuspend cells in 500µl of Probe Wash solution, spin down cells at 650-750g for 5 minutes and aspirate supernatant. Repeat for a total of 4 washes.

8.22 Resuspend the cell pellet in 500µl of 5x SSCT (per 5 million cells).

8.23 Incubate at room temperature for 5 minutes.

8.24 Centrifuge at 500g for 5 minutes. Aspirate supernatant.

8.25 Resuspend the cell pellet in 150 µl pre-warmed Amplification Buffer.

8.26 Transfer to ependorf as compatible with Termomixer.

8.27 Pre-amplify for 30 minutes at room temperature with rotation.

8.28 Signal Amplification-

Prepare 15 pmol of each fluorescently labeled hairpin h1 and h2. h1 and h2 should be snap cooled in separate tube. Snap cooling 5 µl of 3 uM stock (per 5 million cells). This is achieved by boiling the hairpin solution for 90 seconds at 95°C and cooling to room temperature in a dark environment for 30 min. (Tip: Prepare extra hairpins to account for any potential loss during snap cooling.)

	A	B
	HCR amplifier	Volume of hairpin
	HCR amplifier B1-488	5 µl x 12 of h1-488
		5 µl x 12 of h2-488
	HCR amplifier B2-546	5 µl x 12 of h1-546
		5 µl x 12 of h2-546

8.29 Prepare hairpin mixture by adding all snap-cooled hairpins to 100µl of Amplification Buffer (per 5 million cells) at room temperature.

	A	B	C
	HCR amplifier	Volume of hairpin	Volume of amplification buffer

	A	B	C
	HCR amplifier B1-488 AND HCR amplifier B2-546	5 µl x 12 of h1-488	1200 µl (for 12 samples. 100 µl each)
		5 µl x 12 of h2-488	
		5 µl x 12 of h1-546	
		5 µl x 12 of h2-546	

8.30 Add 100 µl of hairpin mixture directly to the 150 µl of sample to reach a final hairpin concentration of 60 nM.

8.31 Incubate the sample for 3 hours in a dark room at room temperature in the Thermomixer.

8.32 Hairpin Removal and Flow Cytometry Preparation-
Add 5x volume (so 1250 µl) of 5x SSCT and spin down cells at 850g for 15 minutes to remove hairpin amplification solution and pellet cells. Aspirate supernatant.

8.33 Resuspend cell pellet in 500µl of 5x SSCT.
Spin down cells at 500g for 5 minutes and aspirate supernatant. Repeat for a total of 6 washes.

8.34 Resuspend cell pellet in 500 µl of PBS.

8.35 Store in the dark and at 4°C until ready for flow cytometry.

8.36 Day-3
Fluorescence-activated cell sorting (FACS)-

8.37 Centrifuge and aspirate the supernatant and resuspend in 300 µl of FACS Buffer. Do 5-10 up
down to avoid aggregates.

8.38 Pass the 300 µl through a 70 µM filter. Hit the tube.

8.39 Collect in a plastic FACS tube.

8.40 Keep on ice before sorting by FACS.

8.41 FITC channel for TBP-fluorophore 488: excitation 490 nm, emission 525 nm.
YG582 channel for NFKBIA-fluorophore 546: excitation 556 nm, emission 573 nm.

8.42 Cells were sorted into four bins, with 2.5 million cells per bin, based on the ratio of NFKBIA-YG582/TBP-FITC in the histogram. The bins corresponded to the TOP 20%, TOP 20–40%, Bottom 20–40%, and lowest 20% of NFKBIA gene expression.

H. DNA Extraction and Sequencing Preparation

9 Following flow cytometry, DNA was extracted from the fixed cells in each bin using DNeasy Blood and Tissue Kits (Cat. 69504, QIAGEN) and the guides in each bin were specifically amplified for sequencing.

9.1 Add 180 µl Buffer ATL with 20ul Proteinase K. Mix thoroughly by vortexing and incubate samples at 60°C for 1 hour.

9.2 Then Incubate at 90°C for 1 hour.

9.3 Cool for 5 min and add 4 uL RNase A (per 2M cells).

9.4 Add 200 µl Buffer AL and Mix thoroughly by vortexing.

9.5 Add 200 µl ethanol (96–100%). Mix thoroughly by vortexing.

9.6 Pipet the mixture into a DNeasy Mini spin column placed in a 2 ml collection tube. Centrifuge at 14000 x g for 1 min. Discard the flow-through and collection tube.

9.7 Place the spin column in a new 2 ml collection tube. Add 500 µl Buffer AW1. Centrifuge for 1 min at 14000x g. Discard the flow-through and collection tube.

9.8 Place the spin column in a new 2 ml collection tube, add 500 µl Buffer AW2 and centrifuge for 3 min at 20,000 x g (14,000 rpm). Discard the flow-through and collection tube.

9.9 Transfer the spin column to a new 1.5 ml microcentrifuge tube.

9.10 Elute the DNA by adding 75 µl Buffer AE to the center of the spin column membrane. Incubate for 1 min at room temperature (15–25°C). Centrifuge for 1 min at 14000x g.

9.11 Repeat previous step for increased DNA yield.

9.12 Measure the DNA concentration by Nanodrop.

9.13 Store DNA at 4°C until PCR amplification.

9.14 PCR amplification-
Set up the following PCR reaction in a sterile environment. Use the most diverse barcode primers found in the Sequencing Table. A maximum of 500ng of DNA per reaction is suggested. Set up as many reactions as necessary to use all the available DNA from (high, low, and unsorted). Include one negative control reaction per reaction set.

9.15

	A	B
	Reagent	1x Reaction
	NEBNext High Fidelity 2X PCR Master Mix	25ul
	sgRNA_barcodePCR_F (10uM)	1.25ul
	sgRNA_barcodePCR_R (10uM)	1.25ul
	Spermine (10mM)	1ul
	Template gDNA (500ng)	500ng
	dH2O	up to 50ul

	A	B
	TOTAL	50 ul

9.16 Cycling:

		Temp	Time	Cycles
	Step 1	98°	2 min	1X
	Step 2	98°	10 sec	25X
		63 °	15 sec	
		72°	45 sec	
	Step 3	72°	5 min	1X
	Step 4	4°	Hold	

9.17 All PCR reactions should be pooled together and purified in the column (Qiagen kit). Note that each column can only purify 6 PCR reactions, so if having more than 6 PCR reactions use multiple columns.

- The elution volume for each column should be minimal (21ul)
- Pool all purified PCR reactions.
- Run 2ug of the pool on 2% agarose gel and extract the right band
- Elute in 21 ul of water
- Run a high sensitivity Bioanalyzer ship to look at the quality of the library
- Quantify using Qubit before sending for sequencing

I. Sequencing

10 Sequencing libraries were prepared using Illumina TruSeq adapters according to the manufacturer's protocols. Sequencing was performed at the Centre d'expertise et de services Génome Québec, located at CHU Sainte-Justine. Libraries were sequenced on an Illumina NovaSeq PE150 platform. All fractions/replicates were multiplexed within a

sequencing lane, targeting a read coverage of approximately 500 reads per sgRNA per sample.