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Perturb-seq Using ABE8e Base Editing to Functionally Characterize a Subset of Blood Pressure- and CAD-Associated Genetic Variants in TeloHAEC Cells

Forked from Adenine Base Editing (ABE) Screening of Blood Pressure (BP) and Coronary Artery Disease (CAD)-Associated Variants in Endothelial Cells

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Abstract

This study follows a previously developed pooled Adenine Base Editing (ABE) screen that assessed the functional impact of 1,271 genetic variants associated with blood pressure (BP) traits and coronary artery disease (CAD) in human endothelial cells (TeloHAEC). That screen, based on variants identified through genome-wide association studies (GWAS) and fine-mapped using UK Biobank data, enabled high-throughput functional annotation of disease-linked loci.
Building upon this, the current protocol describes a Perturb-seq approach to characterize a selected subset of these variants at single-cell resolution. This method combines ABE8e-mediated base editing with single-cell RNA sequencing of CRISPR perturbations to elucidate the transcriptomic consequences of specific genetic edits in endothelial cells.

Materials

	A	B	C
	Name	**SKU**	**VendorName**
	polybrene (hexadimethrine bromide)	H9268-5G	Merck MilliporeSigma (Sigma-Aldrich)
	Tissue culture plate 6 wells Falcon	CA62406-161	VWR International (Avantor)
	Falcon tube 50 ml	1443222	Thermo Fisher Scientific
	Millipore Steriflip sterile disposable 0.45uM	SE1M003M00	Thermo Fisher Scientific
	pMD2.G	12259	addgene
	Plus reagent	11514015	Life Technologies
	Lipofectamine 2000	11668-019	Life Technologies
	BSA Stock Solution	130-091-376	Miltenyi Biotec
	Falcon tube 15ml	1495949B	Thermo Fisher Scientific
	Opti-MEM	31985070	Life Technologies
	psPAX2	12260	addgene
	Falcon T-175	83.3912.002	Sarstedt
	DMEM High glucose with glutamax and Sodium pyruvate	10569010	Life Technologies
	FBS premium grade 500ml for 293FT	97068-085	VWR International (Avantor)
	TRYPLE express	CC-5002	Cedarlane
	Vascular Cell Basal Medium	PSC-100-030	Cedarlane

	A	B	C
	Vascular Endothelial cell growth kit-VEGF	PSC-100-041	Cedarlane
	Puromycin dihydrochloride	P9620-10ML	Merck MilliporeSigma (Sigma-Aldrich)
	pen/strep 100X, 100mL	450-201-EL	Wisent Bioproducts
	T75 TC FLASK CN VENT CAP/CS100	83.3911.002	Sarstedt
	HEPES buffered saline solution	CC-5024	Cedarlane
	Trypsin-EDTA for Primary cells	PSC-999-003	Cedarlane
	Trypsin neutralizing solution	PSC-999-004	Cedarlane

Protocol materials

- polybrene (hexadimethrine bromide) **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**H9268-5G
- Tissue culture plate 6 wells Falcon **VWR International (Avantor)** **Catalog #**CA62406-161
- Falcon tube 50 ml **Thermo Fisher Scientific** **Catalog #**1443222
- Millipore Steriflip sterile disposable 0.45uM **Thermo Fisher Scientific** **Catalog #**SE1M003M00
- pMD2.G **addgene** **Catalog #**12259
- Plus reagent **Life Technologies** **Catalog #**11514015
- Lipofectamine 2000 **Life Technologies** **Catalog #**11668-019
- BSA Stock Solution **Miltenyi Biotec** **Catalog #**130-091-376
- Falcon tube 15ml **Thermo Fisher Scientific** **Catalog #**1495949B
- Opti-MEM **Life Technologies** **Catalog #**31985070
- psPAX2 **addgene** **Catalog #**12260
- Falcon T-175 **Sarstedt** **Catalog #**83.3912.002
- DMEM High glucose with glutamax and Sodium pyruvate **Life Technologies** **Catalog #**10569010
- FBS premium grade 500ml for 293FT **VWR International (Avantor)** **Catalog #**97068-085
- TRYPLE express **Cedarlane** **Catalog #**CC-5002
- Vascular Cell Basal Medium **Cedarlane** **Catalog #**PSC-100-030
- Vascular Endothelial cell growth kit-VEGF **Cedarlane** **Catalog #**PSC-100-041
- Puromycin dihydrochloride **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**P9620-10ML
- pen/strep 100X, 100mL **Wisent Bioproducts** **Catalog #**450-201-EL
- T75 TC FLASK CN VENT CAP/CS100 **Sarstedt** **Catalog #**83.3911.002
- HEPES buffered saline solution **Cedarlane** **Catalog #**CC-5024
- Trypsin-EDTA for Primary cells **Cedarlane** **Catalog #**PSC-999-003
- Trypsin neutralizing solution **Cedarlane** **Catalog #**PSC-999-004

Variant selection

1 **Input variants**
All 1281 variants analysed in the previous Base Editing Screen (dx.doi.org/10.17504/protocols.io.5qpvo9ybxv4o/v1)

Variants annotation
All variants were annotated for their presence in:

- a coding sequence of gene of interest for endothelial cells (top expressed genes in endothelial cell (Dai et al,PMID: 35610053) , differentially expressed gene in teloHAEC +/- TNFa, differentially expressed gene in HAECs depending flow exposure (Wu et al, PMID: 28556776)
- an endothelial cell or teloHAEC active +enhancer, characterized with the ENCODE rE2G and scE2G model

Variants were also annotated with their:

- finemapping results for Systolic Blood Pressure and Diastolic Blood Pressure (Keaton et al, PMID: 38689001)
- base editing results from the previous base editing Screen

Variants selection
Firstly, all variants were filtered based on their BEAN results from the previous Base Editing Screen: variants with an editing rate >= 10% and an effect size, abs(mu_z_adj) > 5, were considered.
Then, variants were filtered based on their relative genes. A few loci were eliminated:
- AIDA : the causal variant that we proposed in Lalonde et al is not tested (it is not an A-target variant)
- ALDH2 : the gene maps to the SH2B3/ATXN2 locus. SH2B3 is very pleiotropic and has a validated role in immune cells.
- CALCRL: the gene is expressed at low levels in teloHAEC. And the putative causal variant (rs880890, PMID: 38602103) is tested and not significant.
- FHL3: expressed at low levels in teloHAEC
- MAT2A: In our PLoS Genet paper, we identified 3 promising variants at this locus. 1 is not screened and the 2 others are not A-to-G variants. Following up on these 5 variants would be difficult to reconcile with our previous results.

At the end, 31 variants present in 6 loci (CDKN1A,ERG,PLPP3,PECAM1,TGFB1,SMAD3) were selected.

Guide RNA (gRNA) design

2 The teloHAEC genome sequence was used to design the gRNA library, with genetic variation in the editing window accounted for when appropriate. Alleles at non-A (or T) variants cannot be installed using the ABE
editing strategy. However, because other A's may be present in the editing windows and bystander edits could provide useful information, all selected variants were targeted by the gRNA library, independent of the variant genotype in teloHAEC. With bedtools and the teloHAEC genome sequence, the genomic sequence around the targeted variants was retrieved, and five 20-bp gRNAs were designed for each variant, shifting the variant targeted for editing from position #4 in the editing window (gRNA1) to position #5 (gRNA2), #6 (gRNA3), #7 (gRNA4), and #8 (gRNA5). After this initial design, the gRNA sequences were re-aligned to the teloHAEC genome to account for other nearby genetic variants (i.e., variants not associated with BP or CAD but residing near the targeted variants). When appropriate, these variants were introduced into the gRNA sequences to minimize mismatches.
The strategy developed by Ryu et al. (PMID: 38658794) was adopted, adding a reporter sequence for each gRNA. This reporter includes the same 20 nucleotides as the gRNA and is used to measure editing efficiency. In total, **104** gRNAs were designed to target **31** variants.

Pertub-seq library cloning

3 **The pooled gRNA library was synthesized by Agilent Technologies.**
Guide library structure for ABE screen:
5'-ggaaaggacgaaacaccg[2-nt gRNA]GTTTAAGAGCTATGCTGGAAACAGCATAGCAAGTTTAAATAAGGCT AGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTT[32-nt target (6-nt upstream, 20-nt gRNA, 6-nt PAM)][7-nt barcode] AGATCGGAAGAGCACACGNNNNNNNNNNNNNN-3'

4 **Reconstitute ssDNA oligo pool:**
1- Spin down lyophilized ssDNA oligo pool.
2- Prepare 20 ng/µL stock by resuspending in TE buffer with low EDTA (10mM Tris-Cl pH 8.0, 0.1 mM EDTA).

5 **Insert preparation**

1- Perform a first PCR:
Primers for PCR1 :

	A	B
		Sequence

	A	B
	BE_Sensor_Fwd	TAACTTGAAAGTATTTCGATTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCG
	BE_Sensor_PCR1_1_Rev	TCACCTTGCTACGTCGTGT

PCR1 Reaction:

	A	B
		Volume (ul)
	Oligo pool (20ng/ul)	1
	NEBNext Ultra II Q5 Master Mix (E7805L)	10
	PCR1 forward primer (10uM)	1
	PCR1 reverse primer (10uM)	1
	dd H2O	7.5
	Total volume	20

Cycling:

		Temp	Time	Cycles
	Step 1	98°	30 sec	1X
	Step 2	98°	10 sec	7X
		68 °	30 sec	

	72°	30 sec	
Step 3	72°	5 min	1X
Step 4	4°	Hold	

2- Pool the 10 reactions and purify the PCR product using the **QIAquick PCR Purification Kit**, following the manufacturer's instructions. **Elute in 20 µL of nuclease-free (ddH_2O) water.**

3- Run the PCR product on a **2% agarose gel** and excise the **241 bp band**. Purify the DNA using the **Qiagen Gel Extraction Kit**, following the manufacturer's instructions. **Elute in 50 µL of nuclease-free (ddH_2O) water.**

4- Perform a second PCR using all the product from PCR1:
Primers for PCR2:

A	B
	Sequence
BE_Sensor_Fwd	TAACTTGAAAGTATTTCGATTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCG
BE_Sensor_PCR2_1_Rev	ATTTGTCTCAAGATCTAGTTACGCCAAGCTTCAGAAGACGGCATACGAGATCTATAGCTTCACCTTGCTACGTCGTGTGCTCTTCCGATCT

PCR2 reaction – Amplify the PCR2 reaction in 10 tubes:

A	B	C
	Volume (ul)	10X volume (ul)
PCR1 product from gel extraction	5	50

	A	B	C
	NEBNext Ultra II Q5 Master Mix (E7805L)	10	100
	PCR2 forward primer (10uM)	1	10
	PCR2 reverse primer (10uM)	1	10
	dd H2O	3	30
	Total volume	20	200

Cycling:

	A	B	C	D
		Temp	Time	Cycles
	Step 1	98°	30 sec	1X
	Step 2	98°	10 sec	10X
		70°	30 sec	
		72°	30 sec	
	Step 3	72°	5 min	1X
	Step 4	4°	Hold	

5- Pool the 10 PCR reactions and purify the product using a PCR purification column, as previously described. Run the purified product on a **2% agarose gel** and excise the **299 bp band**. Perform gel extraction using the **Qiagen Gel Extraction Kit**, following the manufacturer's instructions. **Elute in 20 µL of nuclease-free (ddH_2O) water.**

6 **Vector preparation**

Restriction Digest of pHKO9-BsmBI library backbone.

1- Add components in the following order:

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

2- Incubate at 37 °C for 1 hour.

3- Run 1% agarose gel and isolate 8kb band by gel extraction (as described previously).

7 **Gibson assembly:**

1- Set up the following reaction on ice:

A	B
	Volume (ul)
Gibson Assembly Master Mix (2X) (E5510)	10
pHKO9-BsmBI (350ng/ul)	1
PCR2 product from gel extraction (75ng/ul)	0.7
dd H2O	8.3
Total Volume	20

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

8 **Perform isopropanol precipitation:**

	Volume (ul)
Gibson reaction mix	20
Isopropanol	20
GlycoBlue	0.4
5M NaCl	0.4

1. Vortex, incubate at **room temperature** for **15 min**.
2. Centrifuge at **top speed** for **15 min**.
3. Carefully **remove liquid** without disturbing the **pellet**.
4. Wash **twice** with **1 mL** of **ice-cold 80% EtOH**.
5. Carefully **remove all liquid**, and **air-dry pellet** for **2-3 min** by keeping the cap open at room temperature.
6. Add **6 µL ddH_2O** and **warm at 55°C for 10 min** to fully resuspend.

9 **Electroporation Protocol**

1. **Transformation**
Add **2 µL** of Gibson assembly product (~50 ng) to **25 µL** of **Lucigen Endura electrocompetent cells**.
Electroporate using the following settings:
- **Cuvette:** 1 mm
- **Capacitance:** 10 µF
- **Resistance:** 550 Ω
- **Voltage:** 1700 V

2. **Recovery**
Immediately add **1 mL of S.O.C. recovery medium** to the cuvette.
Transfer to a culture tube and **shake at 37 °C for 1 hour**.

3. **Dilution and Plating**
- Prepare a **1:1000 dilution** by adding **10 µL** of the recovered culture to **990 µL of LB**.
- Plate **100 µL** of this dilution (i.e., a **1:10,000 final dilution**) onto an agar plate.
- Incubate the plate **overnight at 37 °C**.

4. **Library Expansion**
- Incubate the remaining **990 µL** of the undiluted culture in **50 mL of LB broth**.
- Grow **overnight at 37 °C with shaking**.

5. **Colony Counting & Cloning Efficiency**
- Count the colonies on the 1:10,000 dilution plate.

- Subtract any colonies observed on the **negative control plate** (no insert) to assess **cloning efficiency**.
- Estimate **total colony count** by multiplying by the dilution factor.
- **Goal:** Achieve **≥20× coverage** of your library.

6. **Plasmid Extraction**
   - If coverage requirements are met, proceed with a **Midiprep** on the overnight bacterial culture.

Sequencing and QC

10 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**, targeting a read coverage of more than **500 reads per sgRNA per sample**.

TeloHAEC Thawing

11 For each vial, prepare a **15 mL tube** with **10 mL** of warm complete **VCBM media** supplemented with **0.15 µg/mL puromycin** and **P/S**.

Vascular Cell Basal Medium **Cedarlane Catalog #**PSC-100-030

Vascular Endothelial cell growth kit-VEGF **Cedarlane Catalog #**PSC-100-041

Puromycin dihydrochloride **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**P9620-10ML

pen/strep 100X, 100mL **Wisent Bioproducts Catalog #**450-201-EL

12 Take a **cryovial of cells** out of liquid nitrogen storage and thaw in a **37°C water bath** until only a tiny bit of ice remains. Vials should contain **1 million cells**.

13 Mix cells by **gentle shaking**, spray off the outside with **70% EtOH**, flick down to bring all **cells/media** to the bottom of the tube, and transfer to a **tissue culture (TC) hood**.

14 Transfer the content of one vial into a prepared **15 mL tube** containing **10 mL of warm complete VCBM**.

15 Centrifuge for **5 minutes at 200g, room temperature (RT)**, and discard the supernatant.

16 Resuspend in **1 mL** of complete **VCBM**, then count and assess viability.

17 Plate cells in a **T75 flask** with **15 mL of complete media**.
T75 TC FLASK CN VENT CAP/CS100 **Sarstedt Catalog #**83.3911.002

18 Incubate in a sterile tissue culture incubator at **37°C** with **5% CO_2**, maintaining humidity by placing a pan of water at the bottom.

19 Allow cells to recover for **1 week** before performing any assay.

TeloHAEC culture in T75 format.

20 Take the flask out of the incubator and **aspirate the media**.

21 Wash with **3 mL of HEPES**, making sure it **covers the entire surface area of the dish**.
HEPES buffered saline solution **Cedarlane Catalog #**CC-5024

22 Aspirate the **HEPES**.

23 Add **2 mL** of **trypsin/EDTA** solution and incubate for **3 minutes**.
Trypsin-EDTA for Primary cells **Cedarlane Catalog #**PSC-999-003

24 Take the flask out of the incubator, add **2 mL** of **TNS**, and gently shake the plate.
Trypsin neutralizing solution **Cedarlane Catalog #**PSC-999-004

25 Transfer the cell suspension into a **15 mL tube** and centrifuge at **200 g** for **5 minutes** at **room temperature (RT)**.

26 Resuspend the pellet in **1 mL of VCBM** and count the cells.

27 Seed **5,000 cells/cm²** in complete media to reach **80% confluence** within 3 days.

28 Freeze mix for TeloHAEC cells is complete VCBM supplemented with 10% DMSO.

HEK293FT cells culture

29 **HEK293FT cells** were purchased from **ThermoFisher (Cat# R70007)**, thawed, split, and maintained the same as for **TeloHAEC**, with the following exceptions:

1. Use **DMEM High glucose** with **GlutaMAX** and **Sodium pyruvate** + **10% premium grade FBS** + **1x Penn/Strep** as a growth medium.
2. During passage, no need to wash cells with HEPES; simply **aspirate the media** and use **Trypsin low EDTA express** instead of Trypsin/EDTA.
3. Plate at ~**20,000 cells/cm²** and split when they reach **80% confluence**.
4. Freeze mix for 293FT cells is **DMEM + 30% FBS + 10% DMSO**.

DMEM High glucose with glutamax and Sodium pyruvate **Life Technologies Catalog #**10569010

FBS premium grade 500ml for 293FT **VWR International (Avantor) Catalog #**97068-085

TRYPLE express **Cedarlane Catalog #**CC-5002

Lentivirus production in T175 format

30 **Day 0:**

- Plate **80,000 cells/cm²** in a **T175 flask** (14 million cells total) in 24.5 mL of **DMEM + 10% FBS**, no P/S.
- Four batches of **lentivirus** will be produced, so four **T175 flasks** were seeded with 14 million **293FT cells** each.

Falcon T-175 **Sarstedt Catalog #**83.3912.002

31 **Day 1:**

A: Prepare lentiviral target mix:

In a sterile 15 ml falcon tube add:

1. **1.75 mL of Opti-MEM**.
2. **11.9 µg of pMD2.G**.
3. **18.2 µg of psPAX2**.
4. **23.8 µg of transfer plasmid** expressing the **library of guide RNA** and **puromycin resistance**.

Falcon tube 15ml **Thermo Fisher Scientific Catalog #**1495949B

Opti-MEM **Life Technologies Catalog #**31985070

psPAX2 **addgene Catalog #**12260

pMD2.G **addgene Catalog #**12259

B: Prepare PLUS reagent mix:

1. **In another 15 mL tube**, **add 231 µL of PLUS reagent** to **1.75 mL of Opti-MEM**.
2. **Add** the **PLUS reagent mix** to the **lentiviral target mix in A**, **mix** by **inverting three times**, and **incubate** at **room temperature for 5 minutes**.

Plus reagent **Life Technologies Catalog #**11514015

C: Prepare Lipofectamine mix:

1. **In another 15 mL tube**, **add 210 µL of Lipofectamine 2000** to **3.5 mL of Opti-MEM**.
2. **Add** the **A+B mix** to **C (Lipofectamine mix)**, **mix** by **inverting three times**, and **incubate** at **room temperature for 5 minutes**.
3. **Add 7 mL** of the final mixture to the **T175 flask dropwise**, **ensuring** the entire surface of the flask is covered.
4. **After 4 hours**, **replace** the media with **40 mL of DMEM + 10% FBS + 1% BSA**.

Lipofectamine 2000 **Life Technologies Catalog #**11668-019

BSA Stock Solution **Miltenyi Biotec Catalog #**130-091-376

32 **Day 3:**

1. **Transfer** the media containing virus to a 50 mL Falcon tube **48 hours post-transfection**.
2. **Centrifuge** at **1,000 × g** for **1 minute** at **room temperature**.
3. **Filter** the supernatant using a **Steriflip filter unit**. **Keep** the virus at **4 °C** until ready to **freeze**.
4. **Aliquot** and **store** at **-80 °C**.

Falcon tube 50 ml **Thermo Fisher Scientific Catalog #**1443222

Millipore Steriflip sterile disposable 0.45uM **Thermo Fisher Scientific Catalog #**SE1M003M00

Lentivirus titration

33 **Thaw TeloHAEC cells** as described above and allow them to **recover for a few days** in complete VCBM. Throughout the recovery period, ensure that cells are **maintained below 80% confluence**.

34 Before infection, **remove the virus stock from the -80 °C freezer** and **thaw it slowly on ice** in a **biosafety level 2 (BL2) culture room**.

35 **Trypsinize and count** TeloHAEC cells. Based on the cell count, **distribute 2 × 10^5 cells per well** into **six wells** of a 6-well plate. Ensure that the **cell suspension volume per well is ≤ 800 µL**.
Additionally, **prepare one extra control well** that will **not undergo selection**.

Tissue culture plate 6 wells Falcon **VWR International (Avantor) Catalog #**CA62406-161

36 Example of volumes of cells, medium, virus and polybrene needed for a virus titration in a 6-well plate

	A	B	C	D	E
	Well ID	**Cell suspension (ul)**	**VCBM compl.**	**Virus (ul)**	**Polybrene (ul)**
	1	800	300	0	1
	2	800	290	10	1
	3	800	270	30	1
	4	800	210	90	1
	5	800	30	270	1
	6	800	0	600	1

polybrene (hexadimethrine bromide) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**H9268-5G

37 **The next day**, passage the cells into a larger culture dish and replace the medium with **VCBM complete media supplemented with penicillin/streptomycin (p/s) and 8 µg/mL puromycin** to begin selection. For the **control well (no virus)**, maintain cells in **regular VCBM complete media without puromycin**.
It is crucial to **retain all cells or split them equally across all conditions**, as this step is essential for **accurate calculation of the multiplicity of infection (M.O.I.)**.

The puromycin concentration (8 µg/mL) was established by performing a **kill curve** on **TeloHAEC cells**.

38 **Selection duration** is defined as the number of days required to completely eliminate all cells in the control well (without virus); in this case, it is **6 days**.
After the selection period:
1. **Trypsinize** the cells and perform a **cell count**.
2. **Plot a curve** with:
X-axis: Volume of virus used
Y-axis: Number of cells that survived selection
3. Use the resulting curve to determine the volume (or approximate volume) of virus that results in **~30% cell survival**, relative to the unselected control well. This volume of virus, when used to infect **2 × 10^5 cells**, corresponds to an **MOI of 0.3**. **Scale up accordingly** if a larger number of cells is to be infected.

Generate TeloHAEC-SFFV-ABE8e cell line

39 The TeloHAEC model expressing **dCas9-SFFV-ABE8e-Blast** was generated via **lentiviral transduction** (as described above) using the **pLenti-SFFV-ABE8e-(D10A) SpRY-P2A-Blast** vector. **Transduction of wild-type TeloHAEC cells (TeloHAEC-WT)** was performed to establish a **stable cell line expressing the ABE8e base editor**. Transduced cells were subjected to **selection with 5 µg/mL blasticidin for 7 days**. Expression of the **ABE8e-(D10A) SpRY-P2A-Blast** protein in the resulting population was subsequently **confirmed by Western blot analysis**.
The **maintenance medium** for the **TeloHAEC-SFFV-ABE8e** cell line consists of **Complete VCBM supplemented with penicillin/streptomycin, 0.15 µg/mL puromycin, and 2.5 µg/mL blasticidin**.

Pooled ABE screen for scRNA-seq

40 TeloHAEC-SFFV-ABE8e cells were **thawed from liquid nitrogen storage and passaged twice** prior to infection, as described above. Briefly, cells were seeded into **four T225 culture flasks**, each containing **5 million cells in 26.75 mL of VCBM** supplemented with **25 µL of polybrene (1 mg/mL)**. Infection was performed using the volume of virus corresponding to an **MOI of 0.3**, as determined by prior titration. These four flasks were prepared to allow for subsequent **TNF-α treatment (10 ng/mL for 4 hours) in two flasks**, with the remaining two flasks serving as **untreated controls**.

41 After 24 hours of infection, cells were subjected to **selection with 8 µg/mL puromycin** for **14 days** in **complete VCBM supplemented with penicillin/streptomycin (p/s)**. Throughout the selection period, cultures were **maintained below 80% confluence**.

42 At the end of the selection period, **two flasks of cells were treated with 10 ng/mL of TNF-α for 4 hours**, while **two additional flasks were kept as untreated controls**.

Following treatment, cells were **trypsinized, counted, centrifuged**, and **resuspended in PBS containing 0.04% BSA** to achieve a final concentration of **1,500 cells/µL**.

43 Cells were then **loaded into the 10x Chromium instrument** following the manufacturer's protocol (**Chromium GEM-X Single Cell 5' Reagent Kits v3 with Feature Barcode technology for CRISPR screening**). A total of **four lanes** on the 10x chip were loaded, corresponding to the **four conditions** (two controls and two TNF-α–treated samples). Each lane was loaded with **29,000 cells**, targeting a recovery of **20,000 cells per condition**.

44 After GEM generation, **reverse transcription, library construction, and quality control (QC)** were performed according to the **manufacturer's instructions**.

Sequencing

45 **Eight libraries** were generated using the **10x Genomics protocol** described above: **four gene expression libraries**, one for each condition, and **four corresponding CRISPR libraries** capturing the gRNAs. The libraries were **pooled such that the gene expression libraries received four times more sequencing reads** than the CRISPR libraries. **Sequencing** was carried out at the **Centre d'expertise et de services Génome Québec**, located at **CHU Sainte-Justine**, using an **Illumina NovaSeq PE100** platform. A total of **3,200 million reads** were generated, targeting a **read depth of over 20,000 reads per cell for the gene expression libraries** and **5,000 reads per cell for the CRISPR libraries**.