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# 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 developed a pooled Adenine Base Editing (ABE) screen to assess the functional impact of genetic variants linked to blood pressure (BP) traits and coronary artery disease (CAD) in a human endothelial cell line (TeloHAEC). Twelve loci identified through Genome-wide association studies (GWAS) were analyzed, including variants in strong linkage disequilibrium ($r^2 \geq 0.8$) across multiple ancestries and fine-mapped (PIP > 0.1) in the UK Biobank. A gRNA library targeting 1,271 variants was designed, incorporating positional effects and nearby genetic variations. This platform enables high-throughput analysis of BP- and CAD-associated variants.

## Troubleshooting

## A. Variant selection for the Adenine-base editing (ABE) screen

1 A pooled ABE screen was designed to characterize the impact of BP-associated or coronary artery disease (CAD)-)-associated GWAS variants. Twelve loci within a 500-kb window, variants associated with SBP, DBP, PP, or CAD (PMID: 30224653 and 30578418) were selected, and their linkage disequilibrium (LD) proxies were retrieved using TOP-LD. All variants in LD ($r^2 \geq 0.8$) with GWAS sentinel variants across European, African, East Asian, and South Asian ancestries (PMID: 35504290) were recovered. Variants with a fine-mapping posterior inclusion probability (PIP) > 0.1 in the UK Biobank https://www.finucanelab.org/data) were then added to this list.

## B. Guide RNA (gRNA) design for the ABE screen

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, 9387 gRNAs were designed to target 1271 variants. Additionally, 111 non-targeting gRNAs and 111 safe gRNAs were added as negative controls, as well as in 257 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 9,387 gRNAs were randomly distributed into two pooled library sets.

## C. Guide RNA library preparation

3 The pooled gRNA library was synthesized by Agilent Technologies.

4 Guide library structure for ABE screen:
5'-ggaaaggacgaaacaccg[20-nt gRNA]
GTTTAAGAGCTATGCTGGAAACAGCATAGCAAGTTTAAATAAGGCT
AGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTT[32-nt target (6-nt upstream, 20-nt gRNA, 6-nt PAM)][7-nt barcode]
AGATCGGAAGAGCACACGNNNNNNNNNNNNN

5 Reconstitute ssDNA oligo pool.

5.1 Spin down lyophilized ssDNA oligo pool.

5.2 Prepare 20 ng/µL stock by resuspending in TE buffer with low EDTA (10mM Tris-Cl pH 8.0, 0.1 mM EDTA).

5.3 PCR1:
Perform 10x PCR1 using the same forward primer and 2 different sublibrary reverse primers to amplify each library half independently.

| | A | B |
|---|---|---|
| | | Sequence |
| | BE_Sensor_Fwd | TAACTTGAAAGTATTTCGATTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCG |
| | BE_Sensor_PCR1_1_Rev | TCACCTTGCTACGTCGTGT |
| | BE_Sensor_PCR1_2_Rev | TAGCTTCTACCCTGCGTGT |

5.4 PCR1 Reaction:

| | | |
|---|---|---|
| | | 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 |

5.5 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 | |

5.6 PCR volume reduction to load in gel:
Pool the 10 reactions and PCR purify using QIAquick PCR purification Kit, QIAGEN Elute in 20ul dd H2O.

5.7 Run 2% agarose gel, and isolate 241 bp band using gel extraction method.

## Gel extraction, (QIAquick Gel Extraction Kit, QIAGEN)

6

6.1 Weigh the gel slice.

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

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

6.4 Add 1 gel volume of isopropanol and mix.

6.5 Place into a QIAquick spin column.

6.6 Centrifuge for 1min.

6.7 Discard flow-through.

6.8 Add 0.5ml QC buffer and centrifuge for 1min.

6.9 Discard flow-through.

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

6.11 Discard flow-through.

6.12 Additional spin 3 min to dry the column.

6.13 Place the column into a new clean tube.

6.14 Add 20ul dd H2O to elute DNA.

## PCR2:

7

7.1 Primers for PCR2

| | A | B |
|---|---|---|
| | | Sequence |
| | BE_Sensor_Fwd | TAACTTGAAAGTATTTCGATTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCG |
| | BE_Sensor_PCR2_1_Rev | ATTTGTCTCAAGATCTAGTTACGCCAAGCTTCAGAAGACGGCATACGAGATCTATAGCTTCACCTTGCTACGTCGTGTGCTCTTCCGATCT |
| | BE_Sensor_PCR2_2_Rev | ATTTGTCTCAAGATCTAGTTACGCCAAGCTTCAGAAGACGGCATACGAGATCTATAGCTTAGCTTCTACCCTGCGTGTGCTCTTCCGATCT |

7.2 PCR2 reaction – Amplify the PCR2 reaction in 10 tubes

| | | Volume (ul) | 10X volume (ul) |
|---|---|---|---|
| | PCR1 product from gel extraction | 5 | 50 |
| | NEBNext Ultra II Q5 Master Mix | 10 | 100 |

| | | |
|---|---|---|
| (E7805L) | | |
| PCR2 forward primer (10uM) | 1 | 10 |
| PCR2 reverse primer (10uM) | 1 | 10 |
| dd H2O | 3 | 30 |
| | | |
| Total volume | 20 | 200 |

7.3 Cycling:

| | Temp | Time | Cycles |
|---|---|---|---|
| Step 1 | 98° | 30 sec | 1X |
| Step 2 | 98° | 10 sec | 10X |
| | 68 ° | 30 sec | |
| | 72° | 30 sec | |
| Step 3 | 72° | 5 min | 1X |
| Step 4 | 4° | Hold | |

7.4 Pool 10 PCR reactions PCR purify, run its in 2% gel and isolate band by gel extraction, as described
previously. Product should be 299 bp.

## Restriction Digest of pHKO9-BsmBI library backbone:

8

8.1 Add components in the following order:

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

8.2 Incubate at 37 °C for 1 hour.

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

## Gibson assembly:

9

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

| A | B |
|---|---|
| | Volume (ul) |
| Gibson Assembly Master Mix (2X) (E5510) | 10 |
| pHKO9-BsmBI (350ng/ul) | 1 |

| | A | B |
|---|---|---|
| | PCR2 product from gel extraction (75bg/ul) | 0.7 |
| | dd H2O | 8.3 |
| | Total Volume | 20 |

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

9.3 Isopropanol precipitation

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

9.4 Vortex, incubate at room temperature for 15 min.

9.5 Centrifuge top speed for 15 min.

9.6 Carefully remove liquid without disturbing pellet.

9.7 Wash twice with 1ml of ice cold 80% EtOH.

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

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

## Electroporation:

10

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

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

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

10.4 Shake at 37 °C for 1 hr.

10.5 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.

10.6

10.7 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.

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

10.9 Perform Midiprep on the bacterial culture.

## D. Generate TeloHAEC-SFFV-ABE8e cell line

11 The TeloHAEC model expressing dCas9-SFFV-ABE8e was generated via lentiviral transduction using the pLenti-SFFV-ABE8e-(D10A) SpRY-P2A-Blast vector. Cells were then subjected to selection for 7 days in 8 µg/mL puromycin. The expression of the ABE8e-(D10A) SpRY-P2A-Blast protein in the population was subsequently validated by Western blotting.

## E. Lentivirus Production

12

12.1 Lentivirus production:
One day before: T175 flasks at a density of $8\times10^4$ HEK293FT cells/$cm^2$ (14M cells total), in a total of 25ml DMEM (+10% FBS) no p/s.

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

12.3 (B)PLUS reagent mix

| | A | B |
|---|---|---|
| | Component | Amount per T175 flask |
| | Opti-MEM | 1750 µl |

| | A | B |
|---|---|---|
| | 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.

12.4 (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.

12.5 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.**

12.6 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 tubes and also aliquot 1ml into a 3X microcentrifuge tube.

## Titrating:

13 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-SFFV-ABE8e 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.

13.1 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) |

| | A | B | C | D | E |
|---|---|---|---|---|---|
| | 1 | 800 | 800 | 0 | 1.6 |
| | 2 | 800 | 750 | 50 | 1.6 |
| | 3 | 800 | 700 | 100 | 1.6 |
| | 4 | 800 | 600 | 200 | 1.6 |
| | 5 | 800 | 400 | 400 | 1.6 |
| | 6 | 800 | 0 | 800 | 1.6 |

13.2 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).

13.3 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. Pooled ABE screen for endothelial cell proliferation

14 Transduction of guide RNA library in TeloHAEC-ABE8e-SpRY cells.

14.1 Four batches per set of lentiviruses were produced for this library. TeloHAEC-SFFV-ABE8e cells were thawed from liquid nitrogen storage and passaged twice before infection. For each batch in T225 culture flask, 5 million cells in 26.75 ml VCBM and 25ul polybrene (1mg/ml) were cultured with the volume of virus (MOI=0.3) obtained from titration.

14.2 After 24 hr infection, the infected cells were passaged and split 1:3 into T25 flasks. Cells were selected using 8 µg/ml puromycin and 350 µg/ml G418 for 7 and 14 days in VCBM. After each selection period, 2 million cells were collected for proliferation screening.

## G. ABE screening for NFKBIA expression by HCR-FlowFISH method

15 Following selection on day 7 and 14, 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.

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

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

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

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

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

15.6 Aspirate supernatant. Repeat for a total of 4 washes with PBST.

15.7 Resuspend cells in 1 ml cold fresh 70% ethanol. Store cells solution at 4 °C for 10 minutes.

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

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

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

15.11 Spin down the cells for 5 minutes at 500g.

15.12 Aspirate supernatant. Repeat for a total of 2 washes.

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

15.14 Transfer to ependorfs as compatible with Thermomixer. Incubate at 37°C for 30 minutes.

15.15 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 )

| | A | B | C | D |
|---|---|---|---|---|
| | 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 |

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

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

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

15.19 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.

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

15.21 Incubate at room temperature for 5 minutes.

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

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

15.24 Transfer to ependorf as compatible with Termomixer.

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

15.26 Signal Amplification-

Prepare 15 pmol of each fluorescently labeled hairpin h1 and h2. h1 and h2 should be snap-cooled in separate tubes. 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 it 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 |

15.27 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 |
| | 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 | |

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

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

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

15.31 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.

15.32 Resuspend cell pellet in 500 µl of PBS.

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

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

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

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

15.37 Collect in a plastic FACS tube.

15.38 Keep on ice before sorting by FACS.

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

15.40 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

16 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.

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

16.2 Then incubate at 90°C for 1 hour.

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

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

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

16.6 Pipet the mixture into a DNeasy Mini spin column placed in a 2 ml collection tube.

16.7 Centrifuge at 14000 xg for 1 min. Discard the flow-through and collection tube.

16.8 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.

16.9 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.

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

16.11 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.

16.12 Repeat previous step for increased DNA yield.

16.13 Measure the DNA concentration by Nanodrop.

16.14 Store DNA at 4°C until PCR amplification.

16.15 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.

16.16

| | 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 |

| | A | B |
|---|---|---|
| | Spermine (10mM) | 1ul |
| | Template gDNA (500ng) | 500ng |
| | dH2O | up to 50ul |
| | TOTAL | 50 ul |

16.17 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 | |

16.18 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 reaction use multiple columns. The elution volume for each column should be minimal (21ul).**

16.19 Pool all purified PCR reactions.
Run 2ug of the pool on 2% agarose gel and extract the right band.

16.20 Elute in 21 ul of water.

16.21 Run a high sensitivity Bioanalyzer ship to look at the quality of the library.

16.22 Quantify using Qubit before sending for sequencing.

## I. Sequencing

17 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.