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18 Loci Variant Installation Protocol (ABE)-LDLuptake

Forked from ChrX Variant Installation Protocol

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We use this protocol and it's working

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Abstract

Genome-wide association studies (GWAS) have identified numerous single nucleotide polymorphisms (SNPs) associated with various traits and diseases, yet understanding the functional consequences of these variants remains challenging. We have chosen a set of 18 loci associated with cholesterol traits (LDL-C and HDL-C) in a recent trans-ancestry GWAS (Graham et al 2021, Nature, GLGC consortium). Genes within these loci have coding burden for these same traits and/or are known monogenic disease genes, and importantly, targeting these genes gives robust phenotypes in CRISPR screens using cholesterol-related phenotypic assays. We have used human genetic evidence to select ~2,500 variants within these 18 loci to evaluate, including variants with strong GWAS evidence and variants with strong evidence as liver eQTLs through fine-mapping and/or linkage to sentinel variants.

This protocol describes pooled LDL uptake screen using CRISPR base editing screening to install all variants in a human hepatocyte cell line, with the goal of gaining insight into causal variants and genes at the selected 18 loci.

Troubleshooting

Guide library Cloning

1 **Library structure**
5'flank-[19-20-nt gRNA]-FEscaffold-terminator-[32-nt target][4-nt barcode]-revcomp-r2seq-[14-nt BE sublibrary primer]

tggaaaggacgaaacaccg[19-20-nt gRNA]
GTTTAAGAGCTATGCTGGAAACAGCATAGCAAGTTTAAATAAGGCTAGTCCGTTATCAACTT
GAAAAAGTGGCACCGAGTCGGTGCTTTTTTT [32-nt target (6-nt upstream, 20-nt gRNA, 6-nt PAM)][4-nt barcode] AGATCGGAAGAGCACACG

2 **Reconstitution of ssDNA oligo pool**

2.1 Spin down lyophilized ssDNA oligo pool

2.2 Prepare 2 ng/µL stock by resuspending in TE buffer with low EDTA (10mM Tris-Cl pH 8.0, 0.1 mM EDTA)

2.3 Store at -20 °C.

3 **Restriction digest of CRISPRv2FE-ABE8e-SpRY-BsrGI library backbone**

3.1 Cut 10 µg CRISPRv2FE-ABE8e-SpRY-BsrGI with BsmBI-V2 and BsrGI-HF

3.2 Restriction digest reaction mix overview:

	A	B
	H2O	ad 100 µl
	NEB 3.1	10 µL
	CRISPRv2FE-ABE8e-SpRY-BsrGI	10 µg
	BsmBI-v2	10 units

	A	B
	BsrGI-HF	10 units

3.3 Incubate at 55 °C for 3-4 hours with BsmBI-V2, then add BsrGI-HF and incubate at 37°C for another 3-4 hours.

3.4 Run digest on 1% agarose gel with SYBR and cut out band at 13.7 kbp.

4 **Amplification of library**

Amplification of library done with the following primers:

010415_sgRNA_60bp_fw
TAACTTGAAAGTATTTCGATTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCG

030322_r2seq_6ntbc_BsrGrv
TTTAAAACTTTATCCATCTTTGCATGTACAGAAGACGGCATACGAGATCTNNNNNNGTGACT
GGAGTTCAGA
CGTGTGCTCTTCCGATCT

4.1 **Determination of optimal PCR cycles for library amplification**

Run a qPCR with 0.5 µl of 1122_18LDLlocus_ABE_gRNAlib library in 15 µl qPCR to determine optimal cycles using the primers above.

qPCR mix

	A	B
	Oligo pool	0.5 uL
	2x Q5 mix	7.5 uL
	F primer @20uM	0.375 uL
	R primer @20uM	0.375 uL
	dd H20	5.5 uL
	20x EVA Green	0.75

qPCR program

	A	B	C	D	E	F
	98 °C	98 °C	65 °C	72 °C	72 °C	4 °C
	30 sec	10 sec	30 sec	60 sec	5 min	remaining

Repeat steps B-D for 20x

Identify the cycle number where the qPCR stops log-linear increase (this is typically 4-5 cycles after the CT), determine how much more input in PCR as in qPCR and subtract the log2 ratio (e.g. if using 0.5 uL, keep as is, if using 1 uL, subtract 1 cycle, if using 2 uL, subtract 2 cycles), to identify PCR1 cycle number.

4.2 Library amplification

Once optimal cycle number is determined, run a 100 µl PCR with the following conditions:

	A	B
	Oligo pool	0.5 uL
	2x Q5 mix	50 uL
	F primer @20uM	2.5 uL
	R primer @20uM	2.5 uL
	dd H20	44.5 uL

	A	B	C	D	E	F
	98 °C	98 °C	65 °C	72 °C	72 °C	4 °C
	30 sec	10 sec	30 sec	60 sec	5 min	remaining

Repeats steps B-D with the determined optimal cycle numbers.

Load PCR onto a 2% agarose gel with SYBR and gel purify band at 299 bp.

5 Gibson assembly

	A	B
	2xNEB HiFi assembly mix	50 ul
	Digested CRISPRv2FE-ABE8e-SpRY-BsrGI	2100 ng (0.25 pmol)
	Purified library PCR	140 ng (0.75 pmol)
	Water	ad 100 ul

Incubate at 50°C for 1 hour.

6 **Cleanup/concentration**

6.1 Add 1 ul GlycoBlue, 2 ul 50mM NaCl, 100 ul Isopropanol to the Gibson reaction system

6.2 Vortex, incubate at room temperature for 15 min

6.3 SPIN > 15.000 g for 15 min

6.4 Carefully remove liquid without disturbing pellet

6.5 Wash with 300 ul 80% EtOH and SPIN >15.000 g for 5 min

6.6 Remove most liquid with P1000 and spin at >15,000 g for 1 min

6.7 Carefully remove all liquid with p200, making sure tube has no liquid left, and air-dry pellet 3-5 min by keeping cap open and leaving at room temp

6.8 Add 8.25 ul EB, warming at 55°C for 10min to fully resuspend

7 **Determination of Coverage and Diversity of Cloned Library**
Transformation of a small amount of the assembly mix for the determination of diversity and coverage of the cloned library.

7.1 Add 0.25 µl of the purified assembled mix into a 1.5 ml tube and gently mix with 25 µl of NEB Stable cells. Incubate for 30 minutes on ice.

7.2 Heat shock at 42°C for 30 seconds and put back on ice for 1 minute.

7.3 Add 975 µl of NEB 10-beta/Stable Outgrowth Medium and incubate at 30°C for 1 hour while shaking.

7.4 Plate 1/10, 1/100, and 1/1,000 of transformation mix onto LB agar plates (supplied with 100 µg/ml ampicillin). Incubate at 30°C overnight.

7.5 **Calculation of the Coverage**
Choose the dilution plate with colony numbers in the range between 20-200 and count the colonies. Calculate the coverage as follows:

((Counted colonies * dilution factor) / Number of library members) * 1600

A coverage of >100x is ideal.

Note: 0.25 µl of the remaining 8 uL purified assembly mix is 32x used in this test. Electroporation with Endura typically yields in a 50x higher transformation efficiency compared to NEB Stable cells, meaning that the entire remaining 8 ul of the assembly mix has 32×50=1600x the number of colonies as the test transformation with 0.25 µl.

7.6 **Determination of the Diversity**
Perform colony PCR on 16 colonies from any of the dilution plates to determined the diversity of the cloned library, using the following conditions:

pX330_seqfw
GAGGGCCTATTTCCCATGAT
111219_postPT_rv
CTAGGCACCGGATCAATTGC

PCR mix for one reaction:

	A	B
	NEB OneTaq 2X Master	12.5 µl

	A	B
	Mix with Standard Buffer	
	Forward Primer @20 µM	0.25 µl
	Reverse Primer @20 µM	0.25 µl
	Water	ad 12 µl

PCR program:

	A	B	C	D	E	F
	94 °C	94 °C	55°C	72 °C	72 °C	4 °C
	30 sec	15 sec	30 sec	60 sec	5 min	remaining

Repeat steps B-D for 35x

7.7 Load 5 µl of each PCR onto a 2% agarose gel with ethidium bromide and expect bands at 635 bp for successful cloning, while 850 bp indicates plasmid background. If >2 colonies have 850 bp, then re-clone.

7.8 Purify rest of the colony PCR via QIAquick PCR Purification Kit and send in for Sanger sequencing with primer pX330_seqfw (GAGGGCCTATTTCCCATGAT). Check the cloned sgRNAs for diversity and move on to the electroporation of the assembly mix into Endura cells.

8 Electroporation of Assembly Mix in Endura Cells

8.1 Add 2 uL of assembly mix to 25uL of Lucigen Endura electrocompetent cells, repeat 4x

8.2 Electroporate using the following parameters:
- 1mm cuvette
- 10 µF
- 600 Ohms
- 1800 Volts

8.3 Immediately add 1mL recovery media from Lucigen into the electroporation cuvette.

8.4 Gently pipet bacterial suspension into a 14 ml culture tube that already contains 1 ml of Lucigen recovery media

8.5 Incubate at 30°C for 1 hour while shaking.

8.6 In a 50 ml centrifugation tube, pool all 4 electroporation mixes together and mix well by swirling.

8.7 Take out 10 µl of the pooled electroporation mix and add to 1 ml of recovery medium. Plate out 20 and 100 µl of that dilution onto pre-warmed plates (40,000 and 8,000-fold dilution, respectively). Incubate at 30°C overnight and count colonies to determine the coverage (see step 7.5 for calculation).

8.8 Transfer the pooled electroporation mix into 400 ml LB medium supplied with 100 µg/ml ampicillin. Incubate at 30°C overnight while shaking.

8.9 Centrifuge bacterial suspension as 2×200 ml aliquotes. Maxi prep on one bacteria pellet and keep the other one as backup at -20°C.

Lentivirus Production

9 **Production and Titration of Lentivirus**

9.1 **Day -1: Plate HEK293 cells for Transfection**
Plate 4×15 cm plates with HEK293FT cells at 1.625*10^7 cells per 15-cm plate in 20 mL DMEM + 10% FBS each

9.2 **Day 0: Transfection of Lentiviral Plasmid Library**

Prepare 2 separate tubes with the following components (mix for 1×15 cm dish transfection).

Tube A

	A	B
	OptiMEM	4 ml
	pMDLg/pRRE	9.7 µg

	A	B
	pRSV-Rev	6.5 µg
	pcDNA3-VSV-G	3.3 µg
	Lentiviral Plasmid Library	13 µg

Tube B

	A	B
	OptiMEM	4 ml
	TransIT-Lenti Transfection Reagent	98 µl

Combine tube A and B and mix by gently inverting. Incubate for 15 minutes at room temperature.

9.3 Gently add transfection mix dropwise to the cells.

9.4 **Day 1: First Harvest**
Collect lentiviral supernatant and store at 4°C. Replace medium with 16 ml of DMEM+10% FBS.

9.5 **Day 2: Second Harvest**
Collect lentiviral supernatant and store at 4°C. Replace medium with 16 ml of DMEM+10% FBS.

9.6 **Day 3: Final Harvest and Lentivirus Concetration**
Collect lentiviral supernatant and pool all lentivirus harvest together.

9.7 Centrifuge pooled lentiviral supernatant at 300xg for 5 minutes. Filter supernatant through a 0.45 µm filter and add 1/3 volume of Lenti-X Concentrator. Mix gently by inversion and incubate for 30 minutes or overnight at 4°C.

9.8 Centrifuge at 1,500xg for 45 minutes at 4°C.

9.9 Gently pour out the supernatant and resuspend pellet in 2 ml of DMEM+10% FBS per 15 cm. Aliquote 500 µl per cryotube and store at -80°C.

9.10 **Titration of Lentivirus**

Titrate the lentivirus on a 24 well plate using 8*10^4/well (4*10^4/cm2). Eventual experiment will use 6.25M (6.25*10^6) cells on a 15-cm (4*10^4/cm2). Each 24-well of titration uses ~1/75 as many cells as
the 15-cm plate will.

Below is the standard lentivirus titration dose chart, although you can alter if necessary. Volumes are calculated for 4 mL total lenti and should be adjusted as necessary to account for fraction of lenti produced:

- 1/150 = 26.66 uL
- 1/300 = 13.33 uL
- 1/600 = 6.66 uL
- 1/1200 = 3.33 uL
- 1/1800 = 2.22 uL
- 1/2400 = 1.67 uL
- 1/4800 = 0.83 uL

9.11 **Day 0: Plating and Infection of HepG2 Cells**

Plate 80,000 HepG2 cells per well in a 24-well supplied with polybrene in a final concentration of 8 µg/ml. Add lentivirus in the dilution steps as stated above. Have two extra wells seeded with no virus as selection control.

9.12 **Day 2: Start of Puromycin Selection**

Replace medium with DMEM+10% FBS supplied with 500 ng/ml puromycin

9.13 **Day 3: First Passage**

Wash cells with PBS and add 75 µl of trypsin. Incubate for 5 minutes at 37°C and add 500 µl of medium supplied with puromycin. Mix detached cells with a P1000 and transfer cell suspension to a new 24-well.

9.14 **Day 5: Second Passage**

Wash cells with PBS and add 75 µl of trypsin. Incubate for 5 minutes at 37°C and add 500 µl of medium supplied with puromycin. Mix detached cells with a P1000 and transfer cell suspension to a new 24-well.

9.15 **Day 7: Count**

By now, control well with no virus should be completely dead. Count each well and detemine the lentiviral dose with the highest survival. The lentiviral dose with 50% survival from that is then the desired lentiviral dosage for the screen. Multiply the dosage with 75 to scale the lentiviral dosage to the 15 cm dish format.

10 **Lentivirus Library Infection and LDL**

10.1 **Day 0: Infection of HepG2 Cells**

Trypsinze and count HepG2 cells. Add 6.25×10^6 cells to a 15 ml centrifugation tube, add lentivirus library and mix by gently inverting. Plate mix onto 15 cm dish with a total medium volume of 20 ml supplied with 8 µg/ml polybrene. Repeat this for a total number of 4 replicates.

Seed 410.000 HepG2 cells in a 6-well as selection control.

10.2 **Day 1: VPA Treatment**
Replace media with DMEM+10% FBS and 2mM VPA

10.3 **Day 3: Start of Puromycin Selection**
Replace medium with 20 ml of DMEM+10% FBS supplied with 500 ng/ml puromycin.

10.4 **Day 4: First Passage**
Split cells 1:2 to one new 15 cm dish with 20 ml of DMEM+10% FBS supplied with 500 ng/ml puromycin (ending up with one plate per replicate).

10.5 **Day 6: Second Passage**
Split cells 1:2 to two new 15 cm dish with 20 ml of DMEM+10% FBS supplied with 500 ng/ml puromycin (ending up with two plates per replicate). By now, control 6-well with no virus should be dead.

10.6 **Day 8: Seed for LDL Uptake Screen**
Seed 31.25×10^6 cells per replicate in a 15 cm dish. For bulk, seed $12{,}25\times10^6$ cells in a 10 cm dish. Seed both in DMEM+10% FBS.

10.7 **Day 9: Serum Depravation**
In the late afternoon, change medium to OptiMEM to start the serum depravation.

10.8 **Day 10: LDL Uptake Screen**

1. 4-6 hours prior to the FACS sort, replace medium of each replicate plate with 16 ml of OptiMEM supplied 40 µl with Low Density Lipoprotein from Human Plasma, BODIPY™ FL complex (BODIPY™ FL LDL).
2. Harvest one plate at the time - trypsinze and spin down to collect cell pellet, resuspend in DMEM+10% FBS supplied with DAPI
3. Sort cells based on BODIPY signal in the following bins: Bottom 20%, Bottom 20-40%, Top 20-40%, and Top 20%.
4. Isolate genomic DNA from sorted cells and bulk cells via PureLink Genomic DNA Mini Kit and elute gDNA in 100 µl.

Library Preparation for NGS

11 **Determination of CT Values for Pooling in PCR1**

Perform qPCR1 to determine CT values of each sample. These values will be used to pool samples prior to PCR1 purification. Use the following primers:

101317_U6PE1_BcX(ACTCTTTCCCTACACGACGCTCTTCCGATCT NNNNN GGAAAGGACGAAACACCG)

030322_P7_anchor (CAAG CAGAAGACGGCATACGAGATCT)

11.1 **qPCR for one reaction:**

	A	B
	gDNA	0.5 µl
	2x Q5 Ultra II Master Mix	7.5 µl
	101317_U6PE1_BcX @20 µM	0.375 µl
	030322_P7_anchor @20 µM	0.375 µl
	20x EvaGreen	0.75 µl
	dH20	5.5

qPCR1 program

	A	B	C	D	E	F
	98°C	98°C	65°C	72 °C	72 °C	4 °C
	30 sec	15 sec	30 sec	60 sec	5 min	remaining

Repeat steps B-D for 30x

After collecting CT values for each sample, organize samples into pools such that samples in each pool have CT values within 0.5 cycles of each other. This step is done to minimize the complexity and time of PCR1 purification and further steps of NGS preparation (qPCR2, PCR2). Note that bulk gDNA samples should never be pooled with

sorted samples, regardless of CT similarity. Also note that every sample you wish to pool after PCR1 MUST use a distinct U6PE1 barcoded primer.

12 **PCR1 for cell-integrated libraries**

Choose a distinct U6PE1 barcoded primer for each sample to the extent possible (we have 24 distinct primers). This helps to minimize monotemplate because the barcoded primers are staggered, and it allows demultiplexing of each sample with a distinct U6PE1 during NGS analysis. You may also pool samples during PCR1 purification if they have distinct U6PE1s and qPCR1 showed Ct <0.5 cycles apart.

12.1

	A	B
	gDNA	20 µg
	2x Q5 Ultra II Master Mix	400 µl
	101317_U6PE1_BcX @20 µM	20 µl
	030322_P7_anchor @20 µM	20 µl
	dH20	ad 800 µl

Cycling (30 cycles):

	A	B	C	D	E	F
	98 °C	98 °C	65 °C	72 °C	72 °C	4 °C
	30 sec	10 sec	30 sec	60 sec	5 min	remaining

12.2 You may combine samples with similar CTs (typically within 0.5 cycles, pool bulk samples separately from sorted samples) provided that they used distinct U6PE1 primers in PCR1. Add 5x volume of buffer PB and proceed to PCR purify via Qiagen PCR purification kit each pool using 1 column per pool, eluting in 100 uL EB per pool. Note that

you may want to use tube extenders since there can be a lot of volume to go through one column.

13 **Determination of CT Values for Optimal Cycle Number in PCR2**

13.1

	A	B
	PCR1 pool	0.5 µl
	2x Q5 Ultra II Master Mix	7.5 µl
	NEBNext_i5 @20 µM	0.375 µl
	030322_P7_anchor @20 µM	0.375 µl
	20x EvaGreen	0.75 µl
	dH20	5.5

	A	B	C	D	E	F
	98 °C	98 °C	65 °C	72 °C	72 °C	4 °C
	30 sec	10 sec	30 sec	60 sec	5 min	remaining

Repeat step B-D for 20x

13.2 Use 2-3 cycles less than the qPCR Ct for PCR2 (it's OK to use up to 3 cycles less than qPCR Ct and up to qPCR Ct so as to group samples most efficiently). Minimum number of PCR2 cycles is 5. If 2-3 below the CT value is <5, then use less PCR1 product input for PCR2 to compensate for 5 cycles. Ex: if your CT is 5, then 2 cycles below the CT would be 3. In order to run the minimum of 5 cycles, add in 1/4 of the normal amount of PCR1 product (so 6ul) to compensate for the two extra PCR cycles.

14 **PCR2**

14.1 PCR 2 50 uL Reaction system:

	A	B
	PCR1 pool	22.5 µl
	2x Q5 Ultra II Master Mix	25 µl
	NEBNext_i5 @20 µM	1.25 µl
	030322_P7_ anchor @20 µM	1.25 µl

	A	B	C	D	E	F
	98 °C	98 °C	65 °C	72 °C	72 °C	4 °C
	30 sec	10 sec	30 sec	60 sec	5 min	remaining

Repeat step B-D for the determined optimal cycles.

Amplified Library sequence: ~295-300bp

AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNN
ggaaaggacgaaacaccgNNNNNNNNNNNNNNNNNNNNGTTTAAGAGCTATGCTGGAAACAG
CATAGCAAGTTTAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGC
TTTTTTTNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNAGATCGGAAGAGCAC
ACGTCTGAACTCCAGTCACNNNNNNAGATCTCGTATGCCGTCTTCTGCTTG

14.2 PCR purify PCR2 onto 1 column each. Elute in 50 µl of EB buffer.

15 **Tapestation**

Use D1000 tapescreen only, make sure to use D1000 ladder and buffer. In pcr tubes: Add 3 uL of buffer + either 1 uL of ladder or sample

Pooling sampling based on nM from Tapestation into a single tube

Pooling Calculation:

[(desired read per sample)/(desired total read across all samples)]/Tapestation nM*X000(Random number to get a reasonably pipettable amount)

16 **SPRI bead Purification**: to remove any undesirable bands (eg primer dimers) observed on gel. Make a pool of sample, mix well, take ½ for SPRI bead purification. Add dH2O or EB up
to 50 uL. Perform 0.85X bead purification (add 42.5uL beads to pool) because product is ~300 bp. If
there is competing primer dimer close to your expected band (so between 200-250bp), you may want to be more aggressive and use 0.8X SPRI. Elute from beads with 20 µl EB buffer.