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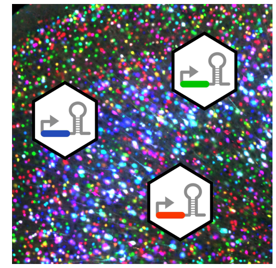
Protocol for CRISPR screening by AAV episome sequencing (CrAAVe-seq) for cell type-specific CRISPR screens in vivo



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We use this protocol and it's working

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Abstract

There is a significant need for scalable CRISPR-based genetic screening methods that can be applied in mammalian tissues in vivo while enabling cell type specificity. Here, we developed an adeno-associated virus (AAV)-based CRISPR screening platform, CrAAVe-seq, that incorporates a Cre-sensitive sgRNA construct for pooled screening within targeted cell populations in the mouse brain. Incorporating a Cre recombinase-based genetic element into the sgRNA library backbone enables the selective screening of phenotypes caused by genetic perturbations only in cell types of interest. Furthermore, CrAAVe-seq exploits the amplification of sgRNA sequences from AAV episomes, rather than genomic DNA, to dramatically increase the scalability and reduce the cost of quantifying sgRNA frequencies from whole brain homogenate. This approach yielded highly reproducible top hits across independent mice. Therefore, CrAAVe-seq enables high-throughput, cost-effective CRISPR screening directly in the mammalian central nervous system, with immediate applicability to other cell types and tissues.

Image Attribution

All images in this protocol were created by Indigo Rose (2025).

Materials

1. Dounce Homogenizers, 7 ml Pyrex (Corning, 7722-7), with Type A pestle (0.0045 nominal clearance) or equivalent
2. TRIzol reagent (Invitrogen, 15596026)
3. 2-Propanol, molecular biology grade (e.g. Sigma, I9516)
4. Chloroform, molecular biology grade (e.g. Sigma, C2432)
5. Ethanol, molecular biology grade, 200 Proof (e.g. Sigma, E7023)
6. DNase/RNase-free water, molecular biology grade (e.g. Gibco, 10977015)
7. RNase A (Thermo, EN0531)
8. 5 ml or 2 ml low-bind centrifuge tubes, DNase/RNase-free (e.g. Eppendorf, 0030119487; Thermo, 5453)
9. PCR strip tubes, sterile, DNase/RNase-free (e.g. Olympus, 27-125U)
10. Q5 2X High Fidelity Master Mix (New England Biolabs, M0492S)
11. SPRI Beads (we home-brew these, see Boswell (2020) *Protocols.io*, DOI: 10.17504/protocols.io.bkppkvmn, but they can also be purchased commercially: e.g. Beckman Coulter, B23317)
12. Elution Buffer, 5 mM Tris-Hcl, pH 8.5 (e.g. Machinery-Nagel Buffer AE, 12716563)
13. 0.5% SDS Solution (for cleaning, dilute in MilliQ water from e.g. Millipore Sigma, 7990-OP), in spray bottle
14. 70% Ethanol (for cleaning, dilute in MilliQ water from e.g. Fisher, BP82014), put in spray bottle
15. MilliQ water, in spray bottle
16. Qubit dsDNA HS Assay Kit (Invitrogen, Q32851) for library quantification
17. TapeStation reagents (Agilent D5000 High Sensitivity Kit), or agarose gel (e.g. Thermo, 16500100) for library quantification
18. Common Fwd Primer oMK732 (order custom DNA oligo, e.g. 25 nmol from IDT, standard desalting); see Appendix for sequence
19. Index primers (Reverse Primers) (order custom DNA oligos, e.g. 25 nmol from IDT, standard desalting); see Appendix for table of sequences
20. Custom sequencing primer oIR300 (order custom DNA oligos, e.g. 25 nmol from IDT, standard desalting); see Appendix for sequence

Troubleshooting

Make AAV vectors containing sgRNA library

1 **Clone sgRNA library into CrAAVe-seq plasmid pAP215.** Once you have your library, clone it into the pAP215 plasmid backbone (available on **Addgene # 217635**). We suggest the protocol described in **Heo et al (2024), PMID: 38243310** for optimal results.

Optional: Before use, consider sequencing your library using a small number of reads to validate roughly similar representation between individual library elements, and to make sure little or no drop-out occurred during cloning. Aim for >100 reads per element.

2 **Design and order sgRNA library.** Determine which library you'd like to use for your screen. For example, pre-made libraries M1-M7 are available, which together target the whole mouse genome (**Horlbeck et al (2016), PMID: 27661255**).

For custom libraries, determine which genes you want to perturb and look up the associated protospacer sequences in Horlbeck et al. (2016): Supplementary File 4 (for use in mice). To each of these sequences, add the following adaptors to the top and bottom oligos to create the oligo pool (top oligo = protospacer sequence + adaptors; bottom oligo = reverse-complement of protospacer sequence + adaptors)

Top oligo: 5'- **TTG** + [20 bp sgRNA protospacer sequence (always starts with G)]
+ **GTTTAAGAGC** -3′
Bottom oligo: 5'- **TTAGCTCTTAAAC** + [20 bp reverse complement of protospacer]
+ **CAACAAG** -3′

To do this scalably, we recommend using our tool `*pyguide*` (https://github.com/pgrosjean/pyguide) which translates a list of genes into the associated protospacer sequences, attaches the above adapters, and generates a list of oligos to order. This list is formatted to be easily uploaded to companies that create oligo pools, such as IDT. We generally use 5 unique sgRNAs per gene. Remember to include non-targeting controls, generally 10-15% although we generally cap at ~250 sgRNAs for large libraries.

3 **Package library into AAV.** Once you have your sgRNA plasmid library, package it into AAV. This can be done using using many different protocols, as long as they produce a sufficient titer.

We have optimized a protocol for cost-effective, in-house AAV preparation for use with in vivo CRISPR screening applications. See: Rose, Ramani, Pan, and Kampmann (2025) *Protocols.io*, DOI: **10.17504/protocols.io.14egn6ezpl5d/v1**.

Once AAV to packaged, it can be stored for ~6 months at 4C. Do not freeze.

Transduce mouse brains with AAV

4 **Inject mice with AAV containing sgRNA library** (and Cre-encoding plasmid, if not using a Cre-expressing mouse line). For implementing neuron survival screens, we co-inject pAP215 containing a library of sgRNAs together with a Cre-containing plasmid (such as hSyn1-Cre), by intracerebroventricular (ICV) injection.

For ICV injection, we follow the protocol described in **Kim et al. (2014), PMID: 25286085**.

For ICV, we typically inject unilaterally in the left hemisphere using AAV diluted to 2 µL total volume in PBS. We suggest injecting in the range of: 1×10^{10} to 5×10^{11} viral particles per mouse. You can also achieve similar transduction efficiency by injecting retro-orbitally using higher titers, usually in the range of 1-5 $\times 10^{12}$ viral particles per mouse. Viral titer will need to be adjusted for optimal transduction MOI for your target area, which varies by application and will need to be empirically determined.

5 **Save sample of AAV** for sequencing, to compare against as a starting population distribution.

Dilute AAV 1:100:
- 99 µL ultrapure (DNase-/RNase-free) water
- 1 µL library-containing AAV, e.g. pAP215-M1 library PHP.eB AAV
- Use same aliquot used to inject mice
- Store diluted AAV at -80 °C until ready to start *CrAAVe-seq Part 2* (See go to step #45)

Collect & store mouse brains

6 For survival screens, dissect out whole mouse brain (or regions of interest) and snap-freeze in 2.0 ml tubes on dry ice. Store at -80 °C .

For screens on non-survival phenotypes, dissect out brain (or regions of interest) and dissociate cells or nuclei as desired. Then isolate into populations to compare. This can be done with FACS, MACS, etc.

CrAAVe-seq Part 1: Isolate episomal DNA from brains (in fume hood)

7 **Steps 8–38 can be completed in one afternoon plus one overnight (16 h) incubation.**

NOTE: These steps must be completed inside a fume hood or dedicated sample prep hood capable of (1) meeting the ventilation requirements for working with TRIzol reagent, and (2) helping to eliminate contaminating DNA, which dramatically interferes with the screen data. **NEVER bring PCR-amplified DNA into this space.**

Our workflow is to perform CrAAVe-seq Part 1 inside a normal chemical fume hood which we clean well, and then perform CrAAVe-seq Part 2 (pre PCR) inside a separate dedicated DNA Sample Prep Hood (generally, a Biosafety Cabinet Type II). CrAAVe-seq Part 3 (post PCR) **MUST** be completed outside of either of these two spaces, typically on a normal lab bench.

In principle, Parts 1 and 2 could be completed in the same hood, so long as this hood is (1) cleaned very well of contaminating DNA, (2) **never** exposed to post-PCR product, and (3) meets your institution's Environmental Health & Safety requirements for working with TRIzol and chloroform.

Clean fume hood and prepare necessary materials & reagents

8 **Clean DNA contamination from fume hood.**
Spray *excessively* and wipe down the hood and every surface inside hood with the following agents in the following order:

1. **0.5% SDS** & wipe down (to disrupt any biofilms)
2. **Water** & wipe down (to solubilize and wipe away ambient DNA - **MOST IMPORTANT**). Use spray bottle filled with MilliQ water.
3. **70% Ethanol** & wipe down dry (so water doesn't pool and rust metal surfaces -- can omit for non-metal surfaces in fume hood)

9 **Clean dounce homogenizers.**

- Bring dounces & Type A pestles to sink and scrub using brush with 0.5% SDS. Check for any particulates, especially at ends of dounces, and scrub clean.
- Copiously rinse off with DI water, then bring to fume hood.
- Dry dounce mortar upside-down so water doesn't pool inside. Dry pestles on paper towels.

10 **Clean following materials/reagents & bring into fume hood.** Before placing in hood, spray down each item with (1) 0.5% SDS & wipe down, then (2) MilliQ water and wipe down, then (3) 70% EtOH (only needed for metal surfaces)

1. Centrifuge and appropriate rotor (if not already inside hood). Must be capable of cooling to 4C, holding 5 ml tubes, and reaching 12,000 x g. If no centrifuge is available that holds 5 ml tubes, can split samples into multiple 2 ml tubes.
2. TRIzol bottle
3. Chloroform bottle
4. 2-Propanol bottle
5. Ethanol bottle (molecular biology grade)
6. Ultrapure water, DNase/RNase-free
7. PCR strip tubes, DNase/RNase-free

8. 5 ml Eppendorf tubes, DNase/RNase-free (or 2 ml tubes if you're using those instead)
9. 50 ml tubes, DNase/RNase-free
10. PCR strip tube rack
11. Stand for dounce homogenizers
12. Holder for 5 ml & 50 ml tubes
13. Holder for 2 ml tubes (for holding brains from freezer)
14. Liquid waste container for TRIzol/chloroform (use old bottle)
15. Solid waste container for TRIzol/chloroform-contaminated plastics (waste bag placed in beaker and folded out around edges so it's easy to close)
16. Pipettes: P1000, P200, and P20
17. Pipette tips: 1000 µL, 200 µL, and 20 µL tips
18. Pipette Aid

11 **Gather other materials nearby:**

1. Bucket with water ice
2. Paper towels (for wiping everything down)
3. Serological pipettes, a handful of 5 ml, 10 ml, and 25 ml

12 **Label tubes:** For each brain, label 1 × 5 ml tube. Our rotor fits 10 × 5 ml tubes total, so we typically prep up to 10 brains at a time.

13 **Retrieve brains from freezer:** Bring into fume hood, wiping down with 0.5% SDS, then water. No need to keep on dry ice. Melting slightly helps the TRIzol permeate.

Homogenize the brains in TRIzol

14 **Add TRIzol to dounces:**
Add 4 mL TRIzol to each dounce homogenizer (for prepping full brains; if prepping ½ a brain or less, scale down protocol to 2 ml TRIzol)

15 **Dislodge brains:**
Use P1000 to take 1 ml of that TRIzol and add to 2 ml tube containing brain. Use tip to dislodge frozen brain. Cap the tube and invert until the brain comes loose from the sides of the tube. This may take a minute and require some slight warming from hands.

16 **Transfer brains to dounces:**
Pour out the brain and TRIzol solution into the top of the dounce homogenizer, such that the homogenizer now contains the brain and the full 4 ml of TRIzol solution.

17 **Insert pestles and homogenize:**
Try not to remove the pestle above the top of the liquid so as to prevent bubbles from forming, although minimal bubble formation is acceptable. Homogenize until the solution appears an almost uniform and homogenous cloudy pink color and all large brain particulates are gone. This may take 20-30+ strokes. Overdouncing is not a huge worry.

18 **Transfer homogenate to tubes:**
Remove pestle (place on paper towel) and transfer the **brain/TRIzol homogenate** into 1 × 5 mL tube per brain. Can use bounce to pour into tube, or transfer with 5 ml serological pipette. TRIzol-contaminated serological pipettes need to be put into dedicated TRIzol solid waste container.
If using a whole brain, there should be almost exactly ~4.2 mL total volume after homogenizing.

Add chloroform

19 **Mix chloroform and homogenate:**
Add 800 µL chloroform to each tube already containing TRIzol/brain homogenate.
Mix by inverting tubes 3-4 times.
Solution should turn a brilliant "milkshake pink".

20 **Centrifuge 12,000 x g, 15 mins, 4°C.**
While tubes are spinning, retrieve and label new 5 ml tubes, 1 per brain.

15m

Precipitate with 2-propanol

21 **Fill fresh 5 ml tubes with** 2 mL 2-propanol **per sample**

22 **Observe TRIzol phases:**
Retrieve tubes from centrifuge.
2 distinct phases should be visible, along with a white interphase.
The top phase should be clear and aqueous, it should be a little over 2 mL (this phase contains RNA as well as the episomal DNA we want to collect in this protocol).
The bottom phase should be dark pink/purple and be viscous and contains gDNA.

23 **Transfer aqueous phase:**
Remove **2 mL** of the aqueous (top, clear) phase, and add it to the new 5 ml tube containing the 2-propanol.
DO NOT disrupt the interphase!
Try to remove most of aqueous phase, but it's ok to leave some behind so as not to risk touching the interphase. If you do touch the interphase, put back all the solution and spin again for a second try.

24 **Mix solution:**
Mix quickly after adding TRIzol/homogenate solution by inverting tubes 3-4 times.

25 **Incubate on ice for 10 mins** to let solution precipitate

10m

26 **Centrifuge 12,000 x g, 10 mins, 4°C**

10m

Wash episome-containing pellet with ethanol

27 **While tubes are spinning, prepare 75% EtOH in DNase/RNase-free water:**
Mix 100% Ethanol (mol bio grade) and DNase/RNase-free water (inside hood)
Put in a new 50 ml conical tube.
You will need 4 ml per brain.

28 **Discard supernatant:**
Retrieve tubes from centrifuge. Discard supernatant by pipetting it up into a non-hazardous waste container. Pellet should be visible: large, white, and whispy.
Try to remove all the solution without disturbing the pellet, but a small amount remaining is acceptable

29 **Add 75% ethanol to tubes:**
Add 4 mL 75% EtOH to each tube.
Voretx briefly so that pellet detaches from bottom of tube to wash it, but the pellet will not re-solubilize. Can also use P1000 instead of vortex to mix.

30 **Centrifuge 7,500 x g, 5 mins, 4°C**

5m

31 **Remove as much ethanol as possible (while leaving pellet intact):**
Remove most ethanol with a serological pipette, then a P1000.
Return tubes to centrifuge to briefly spin them down so traces of ethanol on sides of tube pool at the bottom.
Then use a P20 pipette to remove all remaining liquid.

32 **Open tubes caps and let air-dry for ~10 mins:**
Verify that all solution is gone, but do not let tubes over-dry (they get crackly, and then turn from white opaque to clear, making it seem like your pellet has disappeared).
Keep tubes upright in tube rack during this process.
This might take longer, depending on how well you pipetted out the last little remaining buffer from each tube.

Digest pellet with RNase

16h

33 ***[Optional]* Warm water for elution buffer:**

Warm up DNase/RNase-free water on 37 °C heatblock for 5+ mins

This helps the pellet dissolve quicker, but isn't necessary

34 **Add RNase A to water to create elution buffer:**

Add 1 µL RNase A per 100 µL of DNase/RNase-free water

You will need 100 µL per brain.
If you don't treat with RNase A, then you won't get amplified bands out post-PCR; we tried this.

35 **Dissolve RNA/episomal DNA pellet with elution buffer:**

Add 100 µL Elution buffer to each tube.

Pellet should solubilize in a few minutes. Wait for pellet to fully solubilize before moving to the next step. You can help it along by vortexing and briefly spinning down.

36 **Transfer sample to PCR strip tubes:**

May need to split sample into 2 × 50 µL depending on the capacity of your thermocycler.

37 **RNase samples:** 16h

Incubate tubes at 37 °C for 16 hours in a thermocycler.

Set a thermocycler program for 37°C for 16 hours, then 4-12°C hold.
This removes RNA from your sample, leaving it enriched in episomal DNA (which contains sgRNAs for amplification).

38 **Store samples:**

Freeze DNA at -20 °C for storage, or move on directly to amplification

CrAAVe-seq Part 2: Prepare PCR Amplification (in DNA Prep Hood)

39 **The following steps (Steps 40–47) MUST be completed inside of a dedicated DNA preparation space (such as a dedicaded DNA Sample Prep Hood) where sources of DNA plasmid contamination can be controlled.** *Not doing this is an easy way to mess up your screen!*

In principle, CrAAVe-seq Parts 1 and 2 could be completed in the same hood, so long as this hood is (1) cleaned well of potential contaminating DNA, (2) **never** exposed to post-PCR product, and (3) meets your institution's safety requirements for working with TRIzol and chloroform (usually a ducted fume hood). However, CrAAVe-seq Part 2 can also be performed in a separate standard Class II Biological Safety Cabinet since it does not use reagents requiring a ducted fume hood, so long as it meets criteria (1) and (2) above.

Rules for working in the DNA Sample Prep Hood:

1. Use DNA Sample Prep Hood, **DO NOT** use RNA Prep Hood or Cell Culture Hoods.
2. Clean DNA Sample Prep Hood and its contents before and after use. Spray once with (1) 0.5% SDS, wipe down, (2) MilliQ water, wipe down, and (3) 70% ethanol, wipe down dry. Wipe down surfaces inside the hood, including inside the hood centrifuge. DNA is soluble in water, so the DNA washing step is of *paramount importance* to keeping your samples safe from plasmid contamination that may poison your screen data. The ethanol prevents water from pooling to avoid rust. If your hood is made of composite material instead of metal, ethanol is not necessary. For metal hoods, **DO NOT** leave the hood wet - the surface starts to rust.
3. Use fresh gloves and wear disposable sleeves when doing sample prep. Wipe them down with 0.5% SDS, then water, then 70% ethanol.
4. The pipettes stay inside the hood. **DO NOT** bring the tube racks/pipets from inside the hood out to the molecular bench. It will be a source of plasmid contamination.
5. Use only filtered tips inside the hood.
6. Use only fresh dedicated reagents inside the hood. Before bringing them into the hood, wipe off with 0.5% SDS, then water, then 70% ethanol.
7. Post-PCR samples or other concentrated sgRNA samples/libraries should **NEVER** be taken inside the hood.
8. It's incredibly important to keep pre-PCR product isolated from any contaminating DNA especially post-PCR DNA as it is amplified. Therefore all steps before the samples are put into the PCR machines are carried out with equipment only used for pre-PCR steps, in the hood. Don't use pipets that are used for cloning and rinse questionable surfaces with water before performing protocol.
9. **DO NOT** leave Q5 polymerase out in the hood for a long time or forget it in the hood - it is expensive!
10. **NEVER** use primers directly from the primers stock tubes! Instead, use aliquots to set up PCR to avoid cross-screen contamination of sgRNAs.
11. When running below 2 aliquots for a given primer, make new aliquots (in the hood! avoid contamination).
12. When the stock tube has less the 50 µL in it, reorder (see Table of Primers at end of protocol).
13. Leave the hood clean for the next person: put boxes/pipets away, close pipet boxes, bag tubes, etc.
14. **DO NOT bring AAV into this space under any circumstances.** For PCR reactions to amplify AAV, prepare everything but the AAV inside the hood and then add the AAV once you've removed the PCR tubes from the hood.

Set up PCR for sgRNA amplification

40 **Clean hood and reagents:**

Clean inside of hood with (1) 0.5% SDS, wipe down, (2) MilliQ water, wipe down, and (3) 70% ethanol, wipe down dry. Do the same with the following materials and reagents:

1. Ultrapure water

2. PCR strip tubes
3. PCR strip tube holders (refreezable, to maintain cool temperature) (from -20°C freezer)
4. 1.5 ml tubes
5. 1.5 ml tube holders (standard, room-temp holders)
6. 1.5 ml tube holder (refreezable, to maintain cool temperature for Q5) (from -20°C freezer)
7. Q5 2X High-Fidelity Mixer Mix (from -20°C freezer): thaw, briefly spin down, and keep on iced holder to keep cool.
8. Pipettes and filtered pipette tips
9. Episomal DNA samples (from Part 1) in PCR strip tubes (thaw and briefly spin down before moving into DNA hood)

41 **Thaw primers and bring into hood:**

In addition, thaw necessary primers and bring into hood after cleaning and briefly spinning down as above:

1. oMK732 (common forward primer)
2. Necessary number of reverse (index) primers: one inverted (e.g. "Ms_i1" for index 1) primer per brain sample and one uninverted (e.g. "i1" for index 1) per AAV replicate you have in your experiment. (See Appendix)

If you use e.g. inverted index 1 (Ms_i1) for your first sample, you cannot use the uninverted (i1) index for an AAV sample in the same experiment!!! Even though they are different full sequences, the index portion of the sequence is the same for each sample name and they cannot be differentiated during sequencing. This is the same for Ms_i2 and i2, Ms_i3 and i3 etc.

Preventing primer contamination is of paramount importance. **Here are some tips:**

- Primers should be initially resuspended to 100 µM and some should be moved to a new stock aliquot. Then, some of this aliquot should be diluted to 10 µM stocks in a fresh tube. These working stocks should be kept in a separate box from the 100 µM stock aliquots, which should themselves be in a separate box from the original 100 µM tubes. If contamination happens, it's then easy to contain.
- All primer resuspension should happen inside the DNA hood, using Ultrapure, DNase-/RNase-free water.

42 **Transfer episomal DNA samples to 1.5 ml tubes:**

May need to pool together the same sample back into one tube if you split them up during go to step #36.

Samples should be 100 µL total.

It's *very important* to use a large fraction (or ideally all) of your sample for PCR amplification, as failure to do this can artificially introduce bottlenecking into your screen data. If you're concerned about whether the PCR will work, you can take a small sample (e.g. 5%) of the episomal DNA sample and run a scaled down test PCR to verify you see the presence of the PCR band before proceeding. However, this is usually unnecessary once you get a hang of the protocol.

43 **Create PCR master mix & dispense into 1.5 ml tubes:**
Create master mix of 2X Q5 polymerase and oMK732 (common forward primer, 10 µM stock):

	A	B	C	D
		Vol for 1 rxn (µL)	**Vol for X rxns (µL)**	**Vol for X rxns + 15% (µL)**
	2X Q5 High-Fidelity Master Mix	110 µL		
	Common Fwd Primer oMK732 (10 µM stock)	5.5 µL		
	Total:	115.5 µL		

Sequence of oMK732 is listed in the Appendix.

Dispense **115.5 µL of Q5/oMK732 master mix** into the 1.5 mL tubes containing the 100 µL episomal DNA sample. Also add some to fresh 1.5 mL tubes for amplification of reference AAVs, typically 2-3 technical replicates.

44 **Add Index primers, water, & distribute into PCR strip tubes:**
Add **5.5 µL of 10 µM Index Primer** stocks (reverse primers) to 1.5 mL tubes.
Use a unique index for each sample you have. Make sure to use inverted (mouse) primers (e.g. Ms_i1, Ms_i2, etc.) for episomal DNA isolated from mouse brains and the uninverted primers (e.g. i10, i11, etc.) for AAV samples. Again, **ensure no index overlap** (i.e. don't use Ms_i1 and i1 in the same experiment).

Add 99 µL RNase-/DNase-free water to each of the tubes that will be used to amplify AAV instead of episomal DNA (see table below). ***DO NOT bring AAV into the DNA Hood.***

Distribute PCR reactants into PCR strip tubes. You may need to split across multiple PCR strip tubes depending on the volume capacity of your thermocycler.

Example spreadsheet for setting up the PCR reactions:

	A	B	C	D	E	F	G	H	I	J	K	L	M
	Sample Name	Index Primer Long Name	Index Primer Short Name	Index Sequence	Product size (bp)	2X Q5 High-Fidelity (µL)	10 µM Fwd Primer (oMK732) (µL)	Master mix to add to each PCR tube (µL)	10 uM Rev Primer (index) (µL)	Nuclease-free Water (µL)	Sample Template (µL)	Notes	Post-SPRI Qubit Conc (ng/µL)
	Example Brain 1	oIR201_mirror_Ms_i1	Ms_i1	ATCACG	354	110	5.5	115.5	5.5	0	100		
	Example Brain 2	oIR202_mirror_Ms_i2	Ms_i2	CGATGT	354	110	5.5	115.5	5.5	0	100		
	Example Brain 3	oIR203_mirror_Ms_i3	Ms_i3	TTAGGC	354	110	5.5	115.5	5.5	0	100		
	Example Brain 4	oIR204_mirror_Ms_i4	Ms_i4	TGACCA	354	110	5.5	115.5	5.5	0	100		
	Example Brain 5	oIR205_mirror_Ms_i5	Ms_i5	ACAGTG	354	110	5.5	115.5	5.5	0	100		
	Example Brain 6	oIR206_mirror_Ms_i6	Ms_i6	GCCAAT	354	110	5.5	115.5	5.5	0	100		
	Example	oIR207_mirr	Ms_i7	CAGATC	354	110	5.5	115.5	5.5	0	100		

	A	B	C	D	E	F	G	H	I	J	K	L	M
	Brain 7	or_Ms_i7											
	Example Brain 8	oIR208_mirror_Ms_i8	Ms_i8	ACTTGA	354	110	5.5	115.5	5.5	0	100		
	Example AAV Replicate 1	oMK730_HS4Kmirror_CRISPR_index10	i10	TAGCTT	276	110	5.5	115.5	5.5	99	1	AAV is already 1:100 diluted. This step further dilutes.	
	Example AAV Replicate 2	oMK754_HS4Kmirror_CRISPR_index11	i11	GGCTAC	276	110	5.5	115.5	5.5	99	1	AAV is already 1:100 diluted. This step further dilutes.	
	Example AAV Replicate 3	oSEQ12_mirror_i12	i12	CTTGTA	276	110	5.5	115.5	5.5	99	1	AAV is already 1:100 diluted. This step further dilutes.	
					Totals:	1100	55						
					Mix to	1210	60.5						

	A	B	C	D	E	F	G	H	I	J	K	L	M
					make MM (above +10%):								

45 **Add diluted AAV into AAV reactions:**

Thaw previously frozen 1:100 diluted AAV (see: go to step #5), vortex and briefly spin down.

Remove PCR strip tubes from DNA hood and bring to bench. Directly add 1 µL of 1:100 (further) diluted AAV into each reaction (or proportionally less if you split the reactions into multiple tubes).

[Optional]: AAV reactions can also be proportionally scaled down to 110.5 µL total (½ volume), to fit into one PCR strip tube.

46 **Run PCR:**

Thermocycler settings

	A	B	C	D	E	F	G
	Step 1	***Step 2***	***Step 3***	***Step 4***	***Step 5***	***Step 6***	***Step 7***
	98°C	98°C	60°C	72°C	GO TO Step 2, 22X	72°C	7°C
	30 sec	30 sec	15 sec	15 sec	(23 times total)	10 min	Hold

23 cycles generally produces enough material without introducing too much PCR bias. However, if you're not starting with much input material, you may want to increase up to 25 total cycles. However, this needs to be consistent across all your samples, including your AAV samples.

47 **Store samples at** -20 °C

CrAAVe-seq Part 3: Post-PCR cleanup, QC, and sequencing

48 **The following steps (Steps 49–71) MUST be completed OUTSIDE the previously used DNA preparation space, as this now becomes a major source of potential cross-experiment DNA contamination. *This is usually a standard lab benchtop space.***

49 ***[Optional]*: Run sample on gel pre-SPRI to verify correct band size**
This step can be helpful before SPRI-selection if you have many samples, as it can narrow down which samples you decide to do SPRI-selection on.

Remove 3 µL of POST-PCR sample and mix with 3 µL of 6X Loading Dye.
Run on a 2% agarose gel, 120V, 40 mins.
Verify inverted (brain) samples create a **354 bp amplicon** and AAV samples create a **276 bp amplicon**.

Alternatively, can use TapeStation D5000 kit, looking for the same band size information. In addition to the sgRNA amplicon bands, expect to see trace primer dimer and a smear of un-amplified very large DNA species. We suspect this smear is gDNA carryover from incomplete isolation during the TRIzol steps; however, if doesn't pose a problem for the experiment besides minor inference with concentration).

Sample purification by SPRI selection

50 **Make or obtain SPRI Beads:**
We home-brew our SPRI breads using the following protocol: **Boswell (2020) *Protocols.io*, DOI: 10.17504/protocols.io.bkppkvmn**, but they can also be purchased commercially: e.g. Beckman Coulter, B23317).

51 **Set up reagents/materials:**
- Warm SPRI beads to room temperature
- Retrieve magnetic tube holder for 1.5 ml tubes
- Label 3 sets of 1.5 ml tubes (1 for transfering from PCR stirp tubes initially, 1 for intermediate step, and 1 with full info for final labeling of tubes)
- Remove post-PCR samples from freezer, thaw, vortex, and briefly spin down
- Make 80% EtOH: Use molecular biology grade ethanol and RNase-/DNase-free Ultrapure water, make >1 ml for each 100 µL sample

52 Move **100 µL of PCR product** for each sample to a 1.5 ml tube.
This leaves behind remaining 100+ µL of PCR product in case SPRI cleanup fails

53 Mix SPRI beads by vortexing

54 Add **65 µL of SPRI beads** (0.65X) directly to 100 µL reaction. Mix well.
100 µL * 0.65 = 65 µL SPRIselect. At this ratio, fragments >360 will bind to the beads
This will create a SPRI to sample ratio of 0.65:1 (0.65X)

Mix well. You want a homogenous mixture. If you splash any of the mixture onto the sides of your tubes or cap spin down briefly.

55 Incubate **5-10 min at RT** (until clear)

56 Place tubes on magnetic stand for 5 minutes or until clear

57 Transfer **supernatant** to new 1.5 ml tubes (Keep supernatant)
This should be 165 µL of volume.
It's ok if there is very slightly less.

58 Add **45 µL of SPRI beads** (1.1X) to this supernatant and MIX WELL (vortex or pipetting)
Can even add SPRI beads to new tubes first and then transfer in supernatant
This will create a SPRI to sample ratio of 1.1:1 (1.1X)
Tube will now contain 210 µL
This concentration will bind fragments smaller than your amplicon and larger, excluding very small fragments
((1.1X-0.65X)*100uL = 45 µL- THIS IS THE CORRECT AMOUNT!)

59 Incubate **5-10 min at RT** (until clear)

60 Place tubes on magnetic stand for **5 min** or until clear.
Remove supernatant (~200 µL), leaving ~10 µL behind.

61 Wash with **500 µL fresh 80% EtOH**. Incubate for **30 sec.**
Pipette away from the beads to avoid distrubing them.

62 Remove EtOH. **Repeat wash once**.

63 Dry beads until they are no longer shiny.
Tip: to dry beads faster, spin tubes down. Put tubes on magnet and wait until liquid is clear. Remove residual EtOH w/20 µL pipette tip

64 Elute with **30 µL Elution Buffer** (5 mM Tris-HCl, pH 8.5) at **RT for 2 mins**

65 Save **25 µL supernatant** by transfering it to a fresh 1.5 ml tube
Don’t carry over any beads!

66 **Quantify sample concentration using Qubit**

Use Qubit dsDNA HS Assay Kit following manufacturer's instructions

DO NOT use NanoDrop for quantification; it is not of sufficient quality for accurate library pooling.

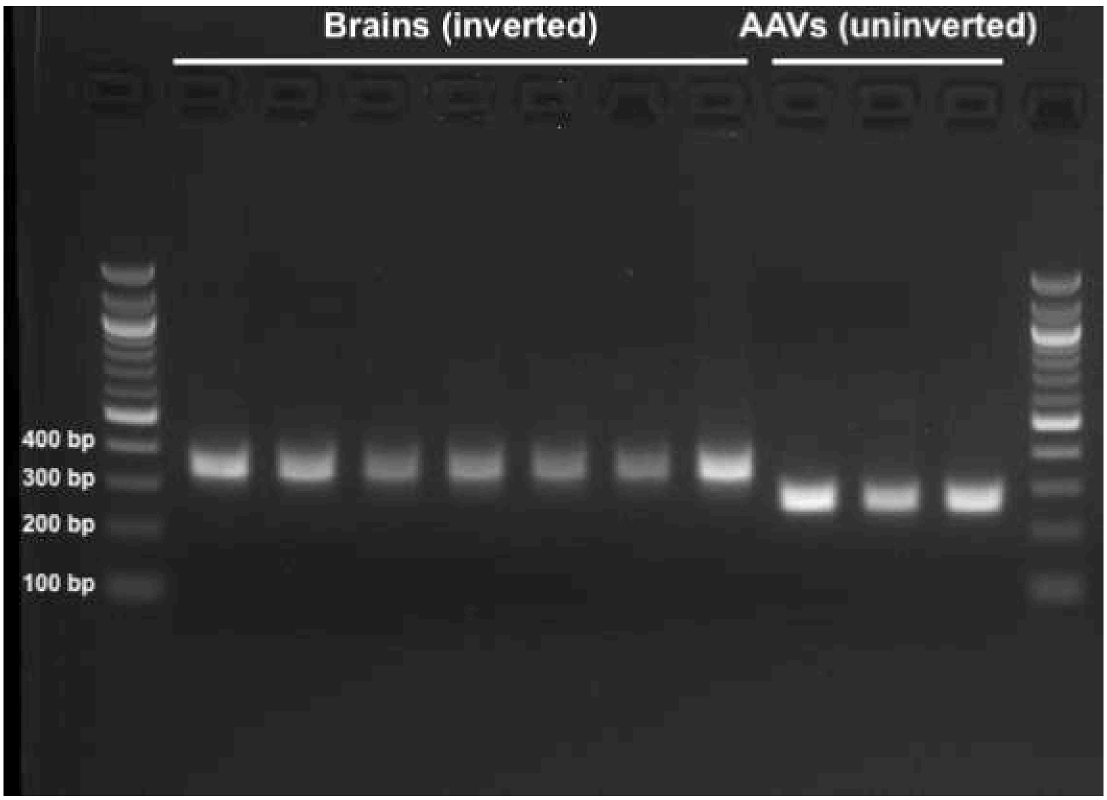
Can record on same spreadsheet in go to step #44

67 **Run samples on gel to check for bands:**

Remove 3 µL of POST-SPRI sample and mix with 3 µL of 6X Loading Dye.

Run on a 2% agarose gel, 120V, 40 mins.

Verify inverted (brain) samples create a **354 bp amplicon** and uninverted (AAV reference) samples create a **276 bp amplicon**.



Post-SPRI amplicons with successful cleanup of primer dimer showing the expected sizes for n=7 brains and n=3 AAV samples.

Can also use TapeStation D5000 High-Sensitivity Kit for scalable quantification of amplicon size distribution.

68 Store post-SPRI samples at -20 °C for pooling and sequencing, or move directly to pooling.

Sample pooling and sequencing

69 **Pool samples at equimolor ratio:**
Mix samples to create a 5 nM solution, ideally at 20+ µL to have plenty of sample for sequencing.

70 **Submit sample to Sequencing Core (or sequence yourself):**
If using a core, follow their procedures for sequencing.
Use custom sequencing primer oIR300 (gactagccttatttaaacttgctatgctgtttccagcttagctcttaaac), or design another primer that will amplify out your sgRNA protospacer sequence.

71 **In house sequencing:**
If sequencing yourself, dilute pool to 2 nM with Ultrapure water.
Further dilute 12 µL of 2 nM pool with 12 µL of RSB Buffer (from Illumina) to create 1,000 pM pool.
Dilute 1.8 µL of 100 µM oIR300 custom sequencing primer with 600 µL HT1 Buffer (from Illumina) to create 0.3 µM solution.
We sequence using a P1, P2, P3, or P4 cartridge (depending on scale and using the smallest possible cycle number size, i.e. 50 cycles for P4 or 100 cycles for P1-P3) using an Illumina NextSeq 2000, set for **21 cycles for Read1** and **6 cycles for Index1**. Read2 and Index2 are set to 0 cycles.
Further details for our method of in house sequencing available with this attachment:
NextSeq2000 CRISPR sequencing i... 119KB

Data Analysis

72 **Analysis guidelines:**

- Analysis will vary depending on your application, here we'll describe a general case as applied to neuronal survival screens using the M1 library (see main paper for details).
- For CRISPR screen analysis, we developed a highly efficient analysis toolkit called `sgcount` for sgRNA mapping and `crispr_screen` for differential gene abundance testing. The sgRNA mapping utility (`sgcount`, version 0.1.32) is available on GitHub (https://github.com/noamteyssier/sgcount) and Zenodo (https://zenodo.org/doi/10.5281/zenodo.12774352).
- The differential gene abundance tool (`crispr_screen`, version 0.2.8) is available on GitHub (**https://github.com/noamteyssier/crispr_screen/**) and Zenodo (https://zenodo.org/doi/10.5281/zenodo.12774208).
- Bootstrapping analyses can be performed using a custom python package (`rescreener`, version 0.1.0) available on GitHub (https://github.com/noamteyssier/bootstrap_analysis_invivo_crispr_screen).

73 **Alignment and sgRNA counting using `sgcount`:**

This tool segments out sgRNA protospacer sequences, aligns them to list of sgRNA sequences, and aligns them to a targeting gene.
Install `sgcount` according to instructions on Github: https://github.com/noamteyssier/sgcount.
You will need to install Rust as well if you don't already have it.
You will need a file containing your library, as well as a file that describes the mapping between your sgRNAs and the genes they target. This is described in detail on the Github.
Here is an example of those two files using the M1 library:

m1.uniq.var.fa 548KB

m1_lib.g2s.txt 387KB

You'll need these files in addition to your FASTQ files (after demultiplexing).

Here is an example of a generic alignment and sgRNA counting command using `sgcount`:

```
sgcount \
-l m1.uniq.var.fa \
-i AAV1.fastq.gz AAV2.fastq.gz AAV3.fastq.gz Brain1.fastq.gz
Brain2.fastq.gz Brain3.fastq.gz Brain4.fastq.gz \
-n  AAV1 AAV2 AAV3 Brain1 Brain2 Brain3 Brain4 \
-t 4 \
-g m1_lib.g2s.txt \
-o output_mapping.tab
```

Here is an example output that looks great in terms of mapping percentage (93-95% mapped):

```
✓   [00:00:00] Calculated Offsets: [Reverse(2), Reverse(2),
Reverse(2), Reverse(2), Reverse(2), Reverse(2), Reverse(2),
Reverse(2)]
✓   [00:00:00] Finished Mismatch Library
✓   [00:00:17] Finished: AAV1; Fraction mapped: 0.945 [15987734 /
16924499]
✓   [00:00:17] Finished: AAV2; Fraction mapped: 0.944 [15960711 /
16899431]
✓   [00:00:17] Finished: AAV3; Fraction mapped: 0.944 [15824146 /
16759777]
✓   [00:00:07] Finished: Brain1; Fraction mapped: 0.934 [6583635 /
7049013]
✓   [00:00:16] Finished: Brain2; Fraction mapped: 0.936 [8183618 /
8740398]
✓   [00:00:25] Finished: Brain3; Fraction mapped: 0.938 [7727089 /
8240609]
✓   [00:00:25] Finished: Brain4; Fraction mapped: 0.938 [7230034 /
7704155]
```

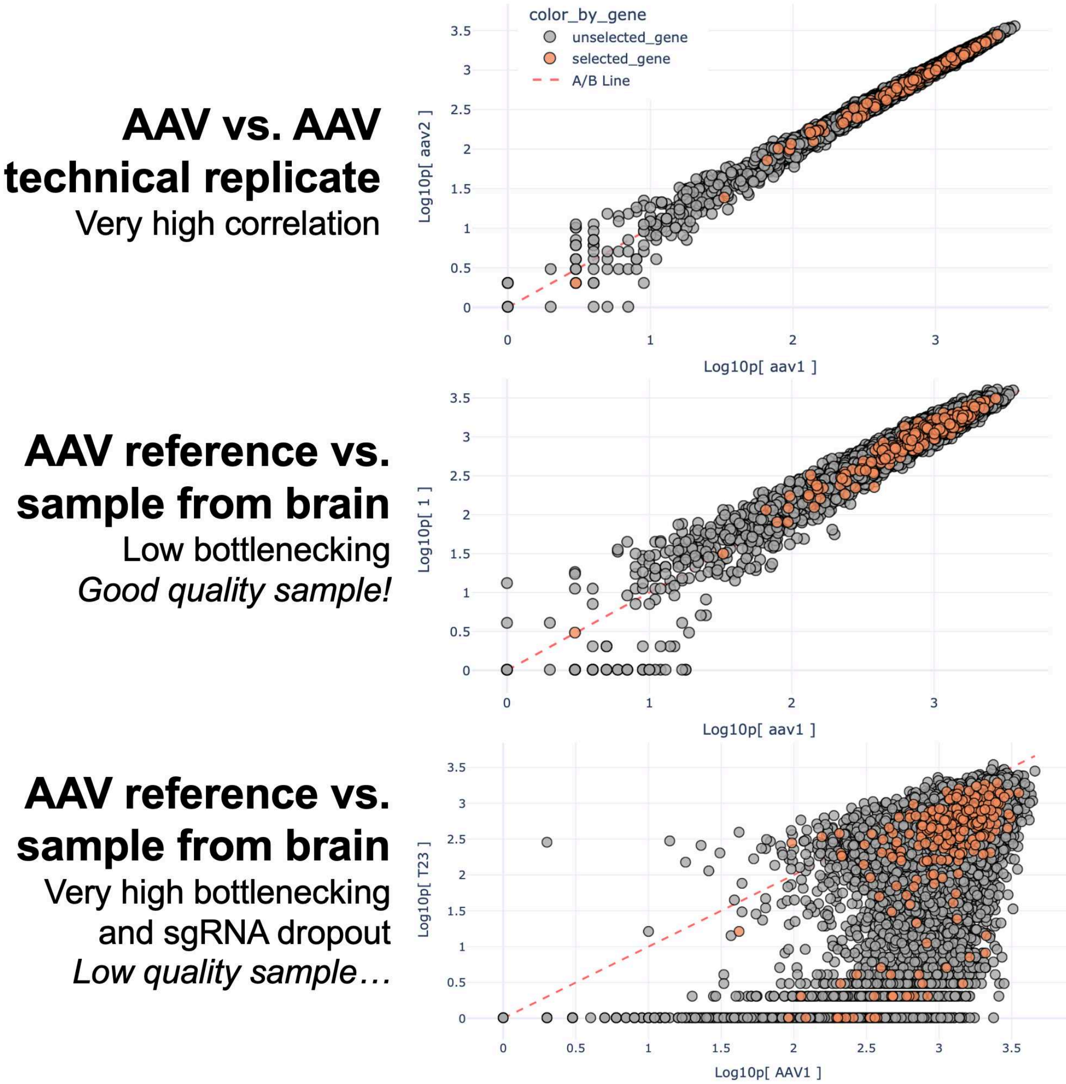
74 ***[Optional]:* Check mapping using `screenviz`:**
Install `screenviz` according to instructions on Github:
https://github.com/noamteyssier/screenviz.
You will also need Python for this step, as described on the Github.
You can use the QC function in `screenviz` to generate a html dashboard describing the quality metrics of your CRISPR screen:

```
screenviz qc -i output_mapping.tab
```

This will generate a link which you copy and paste into a web browser to open the QC utility. It will show you:

- Correlation plots for each of your samples against any other sample, with the ability to highlight specific sgRNAs
- Total read counts per sample
- A sample correlation matrix
- Distribution of gene membership size
- Distribution of log10 sgRNA counts

Using sample correlation plots, you can easily check for bottlenecking (which can come from low sequencing read count, or poor sgRNA recovery from brain):



Example showing bottlenecking from poor sgRNA recovery from brain (last plot). Orange dots indicate non-targeting controls (NTCs) and gray dots indicate targeting sgRNAs. The presence of many NTCs dropping out indicates poor sample quality due to bottlenecking. Since this sample was sequenced with >1000X read coverage per sgRNA element, we can infer this is due to biological bottlenecking and not poor sequencing.

75 **sgRNA Aggregation using `crispr_screen`:**

This tool aggregates the multiple sgRNAs targeting each gene and performs statistical tests to determine if a gene in a hit or not.

Install `crispr_screen` according to instructions on Github: https://github.com/noamteyssier/crispr_screen.

`crispr_screen` supports 3 different algorithms for calling hits: RRA, INC, and GeoPAGG (for details, see Github or main paper).

Here is an example sgRNA aggregation command using the `test` function using the GeoPAGG algorithm:

```
crispr_screen test \
-i output_mapping.tab \
-c AAV1 AAV2 AAV3 \
-t Brain1 Brain2 Brain3 Brain4 \
-o Brains_vs_AAVs.txt \
-g geopagg \
--use-product \
-M 0
```

This uses the `--use-product` flags, and also does not set a read level cutoff using `-M 0`. This is just an example and may or may not be appropriate given your experiment. See GitHub documentation for details.

Here is an example output:

```
Run Configuration
>> Control Group              :  ["AAV1", "AAV2", "AAV3"]
>> Treatment Group            :  ["Brain1", "Brain2", "Brain3",
"Brain4"]
>> Number of sgRNAs           :  12313
>> Number of Genes            :  2421
>> Normalization Method       :  MedianRatio
>> Aggregation Method         :  GeoPAGG { token: Some("non-
targeting"), weight_config: DropFirst { alpha: 0.50000 }, fdr:
0.10000, use_product: true, zscore_threshold: None }
>> P-Value Correction Method  :  BenjaminiHochberg

Filtering Low Count sgRNAs
>> Minimum Base Mean          :  0.00000
>> Number of Filtered sgRNAs  :  0

Modeling Mean Variance
>> Removed Outlier sgRNAs     :  5
>> Removed Undervaried sgRNAs :  6578
>> Linear Model Type          :  Wols
>> Fit Parameter; K           :  0.16455
>> Fit Parameter; B           :  1.23204

Performing Differential Abundance
>> Sample Aggregation Strategy:  CountMedian

Performing Gene Aggregation
>> Removed Zero sgRNAs        :  4
>> NTC Token                  :  "non-targeting"
>> FDR                        :  0.10000
>> Weight Configuration       :  DropFirst { alpha: 0.50000 }
>> Seed                       :  42

Hits
>> Number of Hits             :  209
>> Number Upregulated Hits    :  0
>> Number Downregulated Hits  :  209
```

This example screen produced 209 hit genes (all with a negative phenotype) using a 0.1 FDR cutoff. It will also write files which detail the hit genes, as well as the phenotype and statistics for all genes and all sgRNAs.

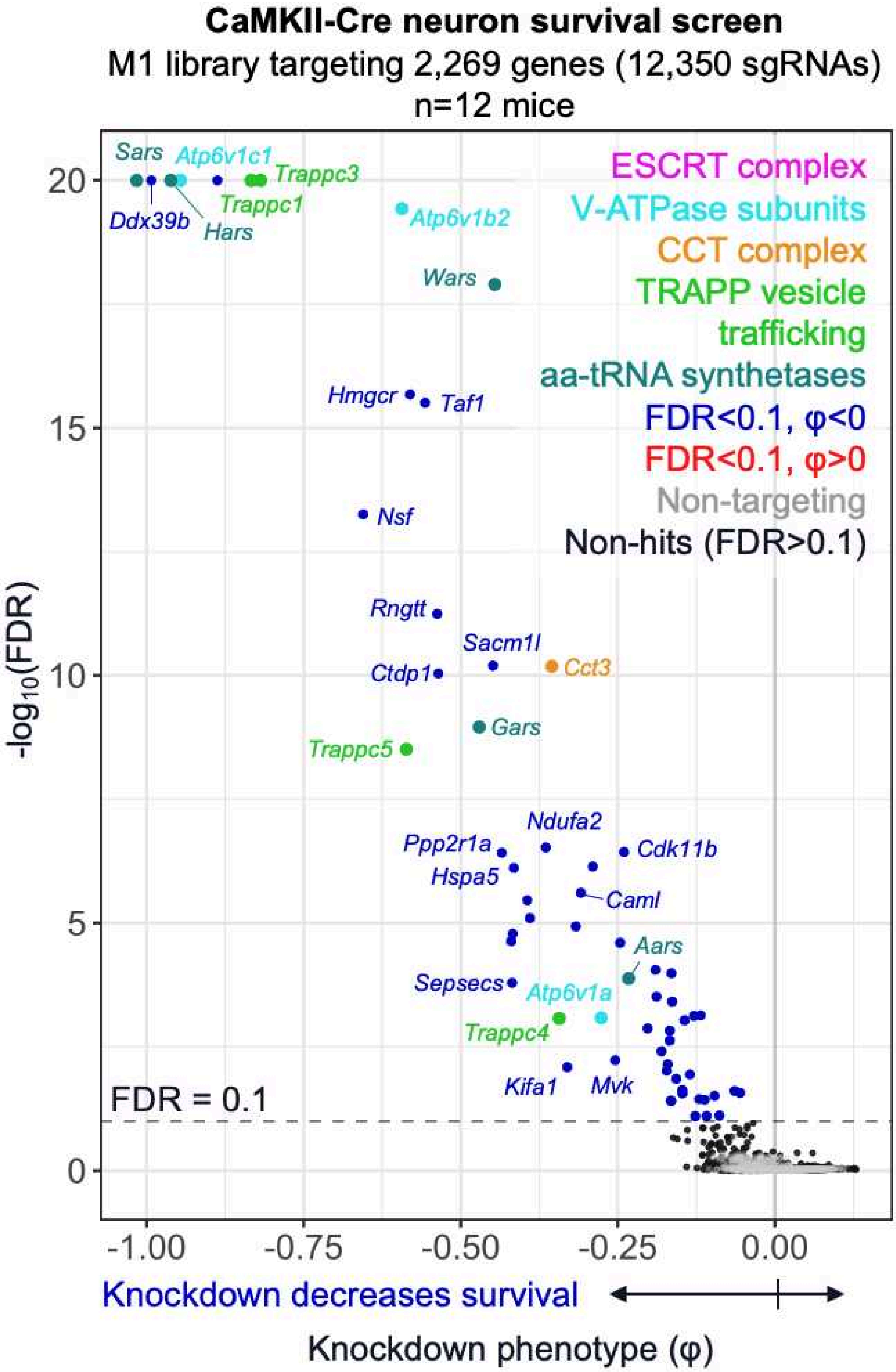
76 **Generate volcano plots and/or scatterplots using `screenviz` or packages of choice:**

`screen_viz` lets you quickly and easily generate volcano plots for your screen data, at both the sgRNA and gene level. This is useful for getting a quick peak at your data, but other plotting tools can be used to create more sophisticated plots.
If not done before, install `screenviz` according to instructions on Github: https://github.com/noamteyssier/screenviz.

Example command to open interactive volcano plot generating tool:

```
screenviz results -n YourExperiment_Brains_vs_AAVs.txt
```

Example volcano plot showing the results of a survival screen in CaMKII-Cre+ neurons made with R:



CaMKII-Cre neuron survival screen
M1 library targeting 2,269 genes (12,350 sgRNAs)
n=12 mice
ESCRT complex
V-ATPase subunits
CCT complex
TRAPP vesicle trafficking
aa-tRNA synthetases
FDR<0.1, φ<0
FDR<0.1, φ>0
Non-targeting
Non-hits (FDR>0.1)
Sars
Atp6v1c1
Trappc3
Trappc1
Ddx39b
Hars
Atp6v1b2
Wars
Hmgcr
Taf1
Nsf
Rngtt
Sacm1l
Cct3
Ctdp1
Gars
Trappc5
Ndufa2
Ppp2r1a
Cdk11b
Hspa5
Caml
Aars
Sepsecs
Atp6v1a
Trappc4
Kifa1
Mvk
FDR = 0.1
-log10(FDR)
20
15
10
5
0
-1.00
-0.75
-0.50
-0.25
0.00
Knockdown decreases survival
Knockdown phenotype (φ)

Appendix: Sequences for primers and indicies

77 **Custom sequencing primer oIR300:**

	A	B
	Primer Name	Sequence (5'- to 3'-)
	oIR300_mirror_SeqPrimer	GACTAGCCTTATTTAAACTTGCTATGCTGTTTCCAGCTTAGCTCTTAAAC

78 **List of primers used for PCR amplication of sgRNAs:**
We use one common forward primer (oMK732) and a variety of reverse (index) primers, which add a unique sample index for each sample.

78.1 **Common PCR forward primer** (used for all samples), does not contain index

	A	B
	Primer Name	**Sequence (5'- to 3'-)**
	oMK732_HS4Kmirror_CRISPR_rev	caagcagaagacggcatacgaGATgcacaaaggaaactcacct

78.2 **Indexing (reverse) primers: Inverted** (Use for most mouse screening samples, e.g. episomes from brains injected with pAP215, pIR110, pIR112, etc. and where Cre recombinase was present)

	A	B	C	D
	Index Long Name	**Index Short Name**	**Index Sequence**	**Full Sequence (5'- to 3'-)**
	oIR201_mirror_Ms_i1	Ms_i1	ATCACG	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACATCACGTGACTGGTACTGACACGTCG
	oIR202_mirror_Ms_i2	Ms_i2	CGATGT	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACCGATGTTGACTGGTACTGACACGTCG
	oIR203_mirror_Ms_i3	Ms_i3	TTAGGC	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACTTAGGCTGACTGGTACTGACACGTCG
	oIR204_mirror_Ms_i4	Ms_i4	TGACCA	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACTGACCATGACTGGTACTGACACGTCG

	A	B	C	D
	oIR205_mirror_Ms_i5	Ms_i5	ACAGTG	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACACAGTGTGACTGGTACTGACACGTCG
	oIR206_mirror_Ms_i6	Ms_i6	GCCAAT	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACGCCAATTGACTGGTACTGACACGTCG
	oIR207_mirror_Ms_i7	Ms_i7	CAGATC	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACCAGATCTGACTGGTACTGACACGTCG
	oIR208_mirror_Ms_i8	Ms_i8	ACTTGA	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACACTTGATGACTGGTACTGACACGTCG
	oIR209_mirror_Ms_i9	Ms_i9	GATCAG	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACGATCAGTGACTGGTACTGACACGTCG
	oIR210_mirror_Ms_i10	Ms_i10	TAGCTT	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACTAGCTTTGACTGGTACTGACACGTCG
	oIR211_mirror_Ms_i11	Ms_i11	GGCTAC	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACGGCTACTGACTGGTACTGACACGTCG
	oIR212_mirror_Ms_i12	Ms_i12	CTTGTA	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACCTTGTATGACTGGTACTGACACGTCG
	oIR213_mirror_Ms_i13	Ms_i13	AGTCAA	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACAGTCAATGACTGGTACTGACACGTCG
	oIR214_mirror_Ms_i14	Ms_i14	AGTTCC	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACAGTTCCTGACTGGTACTGACACGTCG
	oIR215_mirror_Ms_i15	Ms_i15	ATGTCA	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACATGTCATGACTGGTACTGACACGTCG
	oIR216_mirror_Ms_i16	Ms_i16	CCGTCC	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACCCGTCCTGACTGGTACTGACACGTCG
	Not in use	-	-	
	oIR217_mirror_Ms_i18	Ms_i18	GTCCGC	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACGTCCGCTGACTGGTACTGACACGTCG
	oIR218_mirror_Ms_i19	Ms_i19	GTGAAA	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACGTGAAATGACTGGTACTGACACGTCG

	A	B	C	D
	oIR219_mirror_Ms_i20	Ms_i20	GTGGCC	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACGTGGCCTGACTGGTACTGACACGTCG
	oIR220_mirror_Ms_i21	Ms_i21	GTTTCG	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACGTTTCGTGACTGGTACTGACACGTCG
	oIR221_mirror_Ms_i22	Ms_i22	CGTACG	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACCGTACGTGACTGGTACTGACACGTCG
	oIR222_mirror_Ms_i23	Ms_i23	GAGTGG	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACGAGTGGTGACTGGTACTGACACGTCG
	Not in use	-	-	
	oIR223_mirror_Ms_i25	Ms_i25	ACTGAT	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACACTGATTGACTGGTACTGACACGTCG
	Not in use	-	-	
	oIR224_mirror_Ms_i27	Ms_i27	ATTCCT	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACATTCCTTGACTGGTACTGACACGTCG
	Not in use	-	-	
	Not in use	-	-	
	Not in use	-	-	
	Not in use	-	-	
	Not in use	-	-	
	oIR225_mirror_Ms_i33	Ms_i33	ATGTGG	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACATGTGGTGACTGGTACTGACACGTCG
	oIR226_mirror_Ms_i34	Ms_i34	CTCAAC	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACCTCAACTGACTGGTACTGACACGTCG
	oIR227_mirror_Ms_i35	Ms_i35	TCTCCT	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACTCTCCTTGACTGGTACTGACACGTCG
	oIR228_mirror_Ms_i36	Ms_i36	CGGCTG	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACCGGCTGTGACTGGTACTGACACGTCG

	A	B	C	D
	oIR229_mirror_Ms_i37	Ms_i37	GCCTTT	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACGCCTTTTGACTGGTACTGACACGTCG
	oIR230_mirror_Ms_i38	Ms_i38	TACGCA	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACTACGCATGACTGGTACTGACACGTCG
	oIR231_mirror_Ms_i39	Ms_i39	GATATA	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACGATATATGACTGGTACTGACACGTCG
	oIR232_mirror_Ms_i40	Ms_i40	AGAAGC	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACAGAAGCTGACTGGTACTGACACGTCG
	oIR233_mirror_Ms_i41	Ms_i41	TCATAA	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACTCATAATGACTGGTACTGACACGTCG
	oIR234_mirror_Ms_i42	Ms_i42	CGAGGG	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACCGAGGGTGACTGGTACTGACACGTCG
	oIR235_mirror_Ms_i43	Ms_i43	ATCGAG	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACATCGAGTGACTGGTACTGACACGTCG
	oIR236_mirror_Ms_i44	Ms_i44	GACACT	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACGACACTTGACTGGTACTGACACGTCG
	oIR237_mirror_Ms_i45	Ms_i45	CGTATC	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACCGTATCTGACTGGTACTGACACGTCG
	oIR238_mirror_Ms_i46	Ms_i46	TAACTC	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACTAACTCTGACTGGTACTGACACGTCG
	oIR239_mirror_Ms_i47	Ms_i47	CTAACT	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACCTAACTTGACTGGTACTGACACGTCG
	oIR240_mirror_Ms_i48	Ms_i48	GCGCGA	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACGCGCGATGACTGGTACTGACACGTCG
	oIR241_mirror_Ms_i49	Ms_i49	TCAGCC	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACTCAGCCTGACTGGTACTGACACGTCG
	oIR242_mirror_Ms_i50	Ms_i50	GAAAAC	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACGAAAACTGACTGGTACTGACACGTCG

	A	B	C	D
	oIR243_mirror_Ms_i51	Ms_i51	TACCGT	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACTACCGTTGACTGGTACTGACACGTCG
	oIR244_mirror_Ms_i52	Ms_i52	AGTGTG	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACAGTGTGTGACTGGTACTGACACGTCG
	oIR245_mirror_Ms_i53	Ms_i53	CTTTCC	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACCTTTCCTGACTGGTACTGACACGTCG
	oIR246_mirror_Ms_i54	Ms_i54	AGGCCT	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACAGGCCTTGACTGGTACTGACACGTCG
	oIR247_mirror_Ms_i55	Ms_i55	CTGGCG	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACCTGGCGTGACTGGTACTGACACGTCG
	oIR248_mirror_Ms_i56	Ms_i56	GCCAAA	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACGCCAAATGACTGGTACTGACACGTCG
	oIR249_mirror_Ms_i57	Ms_i57	ATCAGA	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACATCAGATGACTGGTACTGACACGTCG
	oIR250_mirror_Ms_i58	Ms_i58	TCGGTG	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACTCGGTGTGACTGGTACTGACACGTCG
	oIR251_mirror_Ms_i59	Ms_i59	CGCTGC	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACCGCTGCTGACTGGTACTGACACGTCG
	oIR252_mirror_Ms_i60	Ms_i60	TCAATT	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACTCAATTTGACTGGTACTGACACGTCG
	oIR253_mirror_Ms_i61	Ms_i61	GAACCA	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACGAACCATGACTGGTACTGACACGTCG
	oIR254_mirror_Ms_i62	Ms_i62	CGATTT	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACCGATTTTGACTGGTACTGACACGTCG
	Not in use	-	-	
	Not in use	-	-	
	Not in use	-	-	
	Not in use	-	-	
	Not in use	-	-	

	A	B	C	D
	Not in use	-	-	
	Not in use	-	-	
	Not in use	-	-	
	oIR255_mirror_Ms_i71	Ms_i71	AGCTAA	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACAGCTAATGACTGGTACTGACACGTCG
	oIR256_mirror_Ms_i72	Ms_i72	TACATG	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACTACATGTGACTGGTACTGACACGTCG
	oIR257_mirror_Ms_i73	Ms_i73	GCTCTG	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACGCTCTGTGACTGGTACTGACACGTCG
	oIR258_mirror_Ms_i74	Ms_i74	TAATCT	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACTAATCTTGACTGGTACTGACACGTCG
	oIR259_mirror_Ms_i75	Ms_i75	TAGTTA	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACTAGTTATGACTGGTACTGACACGTCG
	oIR260_mirror_Ms_i76	Ms_i76	CTCCCA	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACCTCCCATGACTGGTACTGACACGTCG
	oIR261_mirror_Ms_i77	Ms_i77	AGCCGG	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACAGCCGGTGACTGGTACTGACACGTCG
	oIR262_mirror_Ms_i78	Ms_i78	CTACAG	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACCTACAGTGACTGGTACTGACACGTCG
	oIR263_mirror_Ms_i79	Ms_i79	GAAGGT	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACGAAGGTTGACTGGTACTGACACGTCG
	oIR264_mirror_Ms_i80	Ms_i80	ATGGTT	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACATGGTTTGACTGGTACTGACACGTCG
	oIR265_mirror_Ms_i81	Ms_i81	TATCAA	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACTATCAATGACTGGTACTGACACGTCG
	oIR266_mirror_Ms_i82	Ms_i82	CGTGCT	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACCGTGCTTGACTGGTACTGACACGTCG
	oIR267_mirror_Ms_i83	Ms_i83	GCGATC	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACGCGATCTGACTGGTACTGACACGTCG

	A	B	C	D
	oIR268_mirror_Ms_i84	Ms_i84	GCATGC	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACGCATGCTGACTGGTACTGACACGTCG
	oIR269_mirror_Ms_i85	Ms_i85	GAGGAA	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACGAGGAATGACTGGTACTGACACGTCG
	oIR270_mirror_Ms_i86	Ms_i86	ATATAC	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACATATACTGACTGGTACTGACACGTCG
	oIR271_mirror_Ms_i87	Ms_i87	TCGACA	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACTCGACATGACTGGTACTGACACGTCG
	oIR272_mirror_Ms_i88	Ms_i88	AGGTTC	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACAGGTTCTGACTGGTACTGACACGTCG
	oIR273_mirror_Ms_i89	Ms_i89	CGGAGT	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACCGGAGTTGACTGGTACTGACACGTCG
	oIR274_mirror_Ms_i90	Ms_i90	TCCAGC	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACTCCAGCTGACTGGTACTGACACGTCG
	oIR275_mirror_Ms_i91	Ms_i91	GGGTAG	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACGGGTAGTGACTGGTACTGACACGTCG
	oIR276_mirror_Ms_i92	Ms_i92	AGATCG	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACAGATCGTGACTGGTACTGACACGTCG
	oIR277_mirror_Ms_i93	Ms_i93	GCAGTA	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACGCAGTATGACTGGTACTGACACGTCG
	oIR278_mirror_Ms_i94	Ms_i94	GCCGGG	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACGCCGGGTGACTGGTACTGACACGTCG
	oIR279_mirror_Ms_i95	Ms_i95	AGTCAC	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACAGTCACTGACTGGTACTGACACGTCG
	oIR280_mirror_Ms_i96	Ms_i96	CTGTAT	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACCTGTATTGACTGGTACTGACACGTCG

We used the index prefix "Ms_" before these index indicators to refer to mouse. However, these are Cre inversion-specific index primers so they can theoretically be used in other species as long as Cre is present to invert the handle sequence in pAP215 or other plasmids using the same targeting strategy.

78.3 **Indexing Primers: UN-inverted** (Use **ONLY** with AAV reference samples)

	A	B	C	D
	Index Long Name	**Index Short Name**	**Index Sequence**	**Full Sequence (5'- to 3'-)**
	oMK731_HS4Kmirror_CRISPR_index1	i1	ATCACG	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACATCACGCGACTCGGTGCCACTTTTTC
	oSEQ2_mirror_i2	i2	CGATGT	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACCGATGTCGACTCGGTGCCACTTTTTC
	oSEQ3_mirror_i3	i3	TTAGGC	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACTTAGGCCGACTCGGTGCCACTTTTTC
	oMK752_HS4Kmirror_CRISPR_index4	i4	TGACCA	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACTGACCACGACTCGGTGCCACTTTTTC
	oSEQ5_mirror_i5	i5	ACAGTG	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACACAGTGCGACTCGGTGCCACTTTTTC
	oMK729_HS4Kmirror_CRISPR_index6	i6	GCCAAT	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACGCCAATCGACTCGGTGCCACTTTTTC
	oSEQ7_mirror_i7	i7	CAGATC	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACCAGATCCGACTCGGTGCCACTTTTTC
	oMK766_HS4Kmirror_CRISPR_index8	i8	ACTTGA	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACACTTGACGACTCGGTGCCACTTTTTC
		Not in use	-	
	oMK730_HS4Kmirror_CRISPR_index10	i10	TAGCTT	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACTAGCTTCGACTCGGTGCCACTTTTTC
	oMK754_HS4Kmirror_CRISPR_index11	i11	GGCTAC	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGT

	A	B	C	D
				CACGGCTACCGACTCGGTGCCACTTTT TC
	oSEQ12_mirror_i12	i12	CTTGTA	aatgatacggcgaccaccgaGATCTACACGAT CGGAAGAGCACACGTCTGAACTCCAGT CACCTTGTACGACTCGGTGCCACTTTTT C
	oMK750_HS4Kmirror_CRISPR_index13	i13	AGTCAA	aatgatacggcgaccaccgaGATCTACACGAT CGGAAGAGCACACGTCTGAACTCCAGT CACAGTCAACGACTCGGTGCCACTTTTT C
	oSEQ14_mirror_i14	i14	AGTTCC	aatgatacggcgaccaccgaGATCTACACGAT CGGAAGAGCACACGTCTGAACTCCAGT CACAGTTCCCGACTCGGTGCCACTTTT TC
	oSEQ15_mirror_i15	i15	ATGTCA	aatgatacggcgaccaccgaGATCTACACGAT CGGAAGAGCACACGTCTGAACTCCAGT CACATGTCACGACTCGGTGCCACTTTTT C
	oSEQ16_mirror_i16	i16	CCGTCC	aatgatacggcgaccaccgaGATCTACACGAT CGGAAGAGCACACGTCTGAACTCCAGT CACCCGTCCCGACTCGGTGCCACTTTT TC
	-	Not in use	-	
	oSEQ18_mirror_i18	i18	GTCCGC	aatgatacggcgaccaccgaGATCTACACGAT CGGAAGAGCACACGTCTGAACTCCAGT CACGTCCGCCGACTCGGTGCCACTTTT TC
	oSEQ19_mirror_i19	i19	GTGAAA	aatgatacggcgaccaccgaGATCTACACGAT CGGAAGAGCACACGTCTGAACTCCAGT CACGTGAAACGACTCGGTGCCACTTTT TC
	oSEQ20_mirror_i20	i20	GTGGCC	aatgatacggcgaccaccgaGATCTACACGAT CGGAAGAGCACACGTCTGAACTCCAGT CACGTGGCCCGACTCGGTGCCACTTTT TC
	oSEQ21_mirror_i21	i21	GTTTCG	aatgatacggcgaccaccgaGATCTACACGAT CGGAAGAGCACACGTCTGAACTCCAGT CACGTTTCGCGACTCGGTGCCACTTTT TC
	oSEQ22_mirror_i22	i22	CGTACG	aatgatacggcgaccaccgaGATCTACACGAT CGGAAGAGCACACGTCTGAACTCCAGT CACCGTACGCGACTCGGTGCCACTTTT TC
	oSEQ23_mirror_i23	i23	GAGTGG	aatgatacggcgaccaccgaGATCTACACGAT CGGAAGAGCACACGTCTGAACTCCAGT

	A	B	C	D
				CACGAGTGGCGACTCGGTGCCACTTTTTC
	-	Not in use	-	
	oSEQ25_mirror_i25	i25	ACTGAT	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACACTGATCGACTCGGTGCCACTTTTTC
	-	Not in use	-	
	oSEQ27_mirror_i27	i27	ATTCCT	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACATTCCTCGACTCGGTGCCACTTTTTC
	oQSEQ7096_mirror_i28	i28	ACACGC	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACACACGCCGACTCGGTGCCACTTTTTC
	oQSEQ7088_mirror_i29	i29	AGACCA	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACAGACCACGACTCGGTGCCACTTTTTC
	oQSEQ7080_mirror_i30	i30	AACAAG	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACAACAAGCGACTCGGTGCCACTTTTTC
	oQSEQ7072_mirror_i31	i31	GTAGAA	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACGTAGAACGACTCGGTGCCACTTTTTC
	oQSEQ7064_mirror_i32	i32	CAGGAC	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACCAGGACCGACTCGGTGCCACTTTTTC
	Supplementary higher indicies:			
	oSEQ63_mirror_i63	i63	TCGCAC	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACTCGCACCGACTCGGTGCCACTTTTTC
	oSEQ64_mirror_i64	i64	GATGCG	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACGATGCGCGACTCGGTGCCACTTTTTC

	A	B	C	D
	oSEQ65_mirror_i65	i65	CTTCTT	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACCTTCTTCGACTCGGTGCCACTTTTTC
	oSEQ66_mirror_i66	i66	GACTGA	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACGACTGACGACTCGGTGCCACTTTTTC
	oSEQ67_mirror_i67	i67	ATAGCA	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACATAGCACGACTCGGTGCCACTTTTTC
	oSEQ68_mirror_i68	i68	CGGGAC	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACCGGGACCGACTCGGTGCCACTTTTTC
	oSEQ69_mirror_i69	i69	TGTAAG	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACTGTAAGCGACTCGGTGCCACTTTTTC
	oSEQ70_mirror_i70	i70	GCTAGT	aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACGCTAGTCGACTCGGTGCCACTTTTTC

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