The Principle

The AdZ-CRISPR vectors contain the Ad5 vector genome, deleted for E1 & E3 regions (rendering the vector non-replicative) in a single copy vector. It contains the HCMV major immediate early promoter & PolyA signal sequence which drive expression of spCas9, and a U6 promoter that drives expression of a sgRNA, which incorporates the spacer sequencing your gene of interest, and the additional tracrRNA sequences needed for the system to function. The sgRNA contains a selectable cassette in the site of the spacer, that encoded ampicillin resistance, lacZα & SacB. SacB encodes for a gene giving sensitivity to sucrose, lacZα provides blue/white screening. This is replaced with your spacers of interest, enabling you to select the correct clones (see below). There are vectors with the original spCas9, or the more accurate SniperCas9[50]. All use an enhanced sgRNA sequence, which provides higher editing efficiency than regular sgRNA sequences[52].

The AdZ CMV promoter is repressible by tet repressors, thus if the vector is grown in 293TREx cells expression from the CMV promoter is prevented during growth. This allows the cloning of spacers targeting essential genes, or genes which would otherwise interfere with the Ad replication. Expression from the CMV promoter in cell lines not expressing the tet repressor (ie most other cell lines) is constitutively on.

SW102 bacteria contain a defective phage expressing the lambda red genes which mediate homologous recombination between DNA stretches as short as 50bp. The genes are under temperature sensitive control – they are turned off when grown at 32°C or can be induced by shifting the bacteria to 42°C for 15 minutes. Unfortunately the recombination genes do not survive freeze-thawing well, so bacteria must be made competent on the day that you use them.

Protocol outline

Design the spacers that target your gene of interest using whichever system you want. We like using ChopChop (https://chopchop.cbu.uib.no/) and e-crisp (http://www.e-crisp.org/E-CRISP/). Order a 100bp oligo that contains your 20nt spacer, as well as sequences either side that match the vector. Note that the U6 promoter requires the first base of the spacer to be a ‘g’. If the first base is not naturally a ‘g’, add an extra ‘g’ on the front – so it becomes a 21nt spacer.

SW102 bacteria containing the vector are made competent, the recombination genes induced and then the oligo electroporated into the bugs. If recombination occurs, the amp-lacZ-SacB cassette will be replaced by your gene. Bacteria are selected on plates containing sucrose so that any bacteria where recombination hasn’t occurred (and therefore still contain sacB) will die. Background bacteria where sacB has mutated (and therefore non-functional) will also appear. lacZα allows you to screen these out – blue colonies contain mutated sacB, white colonies will contain the correct insert.

AdZ vectors

These vectors are based on wildtype adenovirus type 5 virus kindly provided by Vivien Mautner from Birmingham University.

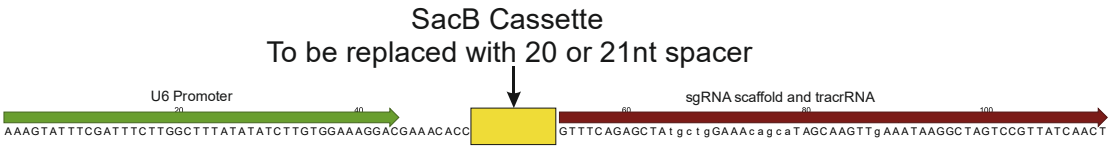
All vectors are Ad5 ΔE1 (461-3519bp), ΔE3 (28131-30,800bp) (deletion numbering based on the prototype Ad-5 sequence (AC000008)).

pAdZ-CRISPR encodes the wildtype spCas9, and enhanced sgRNA.
pAdZ-Sniper encodes the SniperCas9, and enhanced sgRNA.

Oligos

We order 100bp desalted oligos from Invitrogen. The size of the region of homology affects the efficiency of the recombination, so add as much of the sequences from either side of the spacer as you can. I.e. if ordering a 20nt spacer, you'd have 40bp on either side of it that matched the vector.

> **Note** Most companies recommend PAGE purifying primers of this length. With desalted primers of this length we have occasionally seen primers with bases missing. If you want to be guaranteed of the sequence first time, you can get the primer PAGE purified. However this costs quite a lot more and in our experience primer errors are rare.



As an example, this is the oligo you would order if you wanted to target B2M, using the 20nt spacer 'GGCCGAGATGTCTCGCTCCG'.

TTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACC**GGCCGAGATGTCTCGCTCCG**GT
TTCAGAGCTAtgctgGAAAcagcaTAGCAAGTTgAAAT

Making competent SW102's (see appendix 1 for media recipes)

1. Inoculate SW102's containing the desired plasmid into 5 ml LB + amp (50µg/ml) + chloramphenicol (12.5µg/ml). Incubate overnight at 32°C.

2. Inoculate 0.5ml of the overnight culture into 25ml LB + amp (50µg/ml). This will be enough for at least 8 inserts. Scale volumes up if necessary. Incubate at 32°C in a shaking incubator to an OD_{600} of 0.6 (approx 3 hours).

   During this incubation, put at least 150ml sterile ddH_2O into the fridge, turn on a waterbath to 42°C and turn a centrifuge that can take 50ml falcons down to 0°C.

3. Pour the culture into 50ml falcons (without skirts). Induce the lambda red proteins by incubating in a 42°C waterbath for 15 minutes. Invert the falcon twice over the 15 minutes to ensure good heat mixing.

   > **Note** If desired, grow 50ml of culture, then split the culture in half and only induce one of the samples – leave the other at 32°C. This can serve as a negative control for recombination. In practice there is little need for this however.

4. Transfer the falcon into ice and cool for 15-20 minutes. If possible put the ice bucket on a gentle shaker to increase the rate of cooling.

5. Centrifuge for 5 minutes @4000rpm, 0°C.

   > **Note** It's important to keep the bacteria as close to 0°C as possible throughout the protocol in order to get good competent cells.

6. Pour off supernatant and resuspend the pellet in 1 ml ice-cold ddH_2O by gently swirling the tube. When resuspended, add 25 ml ice-cold ddH_2O and centrifuge the sample again.

7. Repeat the washing step

8. Pour all supernatant off. Be careful – the cell pellet is very loose by now. Resuspend the pellet in the remaining water (if hardly any water is left, top up to ~400µl) by gently shaking it.

9. Transfer the competent SW102's into 0.5ml eppendorfs as 25µl aliquots. Add 1µoligo to a 25µl aliquot of induced bacteria.

   > **Note** our stock oligos are made up to 100pmol/ul.

10. Transfer aliquots +DNA to pre-cooled cuvettes and stand on ice for 5 minutes. We use 0.2cm cuvettes & electroporate at 2.50 kV, other people use 0.1cm cuvettes & electroporate at 1.8kV.

11. After electroporation recover bacteria in 5ml LB in a universal for 4 hours in a shaking incubator at 32°C. This extended recovery is needed to allow the loss of sacB DNA & proteins from the cells where recombination has occurred.

    Note. If you find yourself running out of time, recover for as long as you can (at least an hour), then fridge the culture and finish the incubation off the next day.

12. Plate 50µl bacteria onto LB + 5% sucrose + chloramphenicol (see appendix for recipe; note NO ampicillin, correct bacteria are now ampicillin sensitive!) and grow for 30-48 hours at 32°C.

    Note It is important to get well spaced colonies. The bacteria grow more slowly at 32°C than at 37 (hence leaving for a minimum of 30 hrs, not 24), and only have a single copy of lacZ per cell so it takes longer for the blue colour to develop. If they are too closely spaced, they don't grow well enough for the blue colour to be visible. Either plate out 50µl and examine after 24 hours, then replate a different volume if necessary or plate out 2 different dilutions to start with.

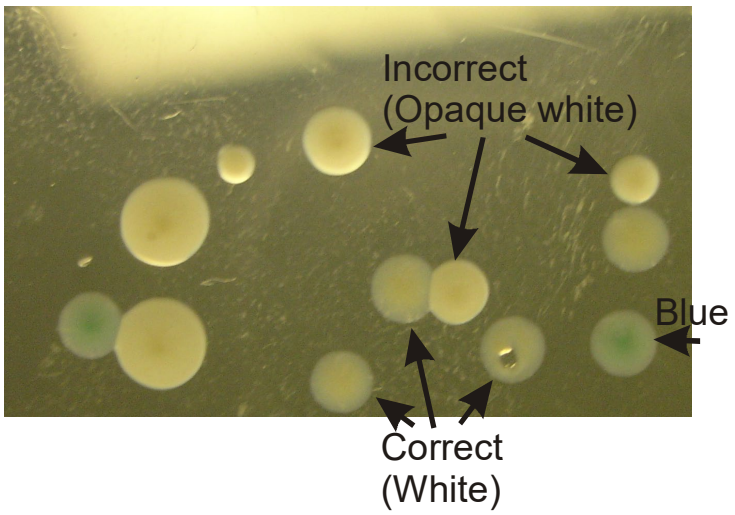
13. There should be plenty of white colonies among the blue ones.

Note Occasionally colonies are present that appear to be white but which still contain the amp/sacB/lacZ cassette. These false positives are easily avoided. Hold the plate up at an angle to a fluorescent light (not directly in front of the light, or you won't be able to see the difference). The false positives look much more opaque, and consequently whiter, under these conditions than the real positives. Once you've seen the difference it's quite obvious & you should have no problems picking the real white ones. If you do an uninduced sample of bacteria you can compare the uninduced plate to the induced using this method & you should easily be able to see that there is one sort of colony which only appears on the induced plate.

As an alternative leave the cultures in the fridge for 24-48 hours after the extended recovery. When you plate them out, the false positives will be gone, or much reduced.

Note 2 PCR screens do not work very well – the low plasmid copy number means a lot of cycles are required to get product, which in turn can lead to false positives.

Here's what the 3 different sorts of colony look like:



Minipreps

The AdZ-5 vectors are in a single copy vector – you only get ~100ng total DNA from a miniprep. We use standard qiagen column miniprep kits, although any miniprep kit will work.

1. Pick white colonies into 5ml LB+chloramphenicol & grow 16-20hrs @32°C. 2-3 different colonies for each insert should be more than enough.

2. Pellet 4.5 mls, keep 0.5ml to make a glycerol stock later.

3. Follow the kit instructions to miniprep. Elute in 50ul.

Sequencing

There is too little DNA from the miniprep to sequence directly. We therefore PCR up the region where the spacer was inserted, and sequence the PCR product.

1. Using whichever Taq is your preferred, and 1ul of miniprep as template, PCR amplify using primers GAGGGCCTATTTCCCATGATT and CCGACTCGGTGCCACTTTT.

2. Run on a gel. This should give a product of ~350bp. Gel purify using whichever gel purification kit you usually use.

3. Send for sanger sequencing using just one of the PCR primers.

4. Note that when analysing the sequencing, you are not just checking the sequence of the spacer – because it has been inserted by recombination, occasionally that recombination results in a base being added or removed. If so, discard that colony.

Maxiprep

250ml bacterial culture should give ~30µg total DNA.

1. It's important to maximise bacterial growth. Inoculate a single colony into 5ml LB + chlor in the morning, allow it to grow during the day (32°C)

2. Dilute all 5mls in 250ml LB then grow overnight at 32°C in a large flask (volume >4× the volume of media).

3. Prepare DNA using whichever maxiprep kit you usually use. We use the BacMAX 100 kit (Machery Nagel). We follow the standard maxiprep instructions (i.e. resuspend in 12ml of buffer S1, lyse in 12ml buffer S2 etc), with 2 modification from the 'low copy DNA protocol':

   The instructions offer 2 ways of clearing the bacterial lysate. Filtration is kinder to the large BAC DNA, & quicker than centrifugation, however it helps if you give the lysate a quick spin (15 mins @6,000 rpm) before putting it onto the filter.

   Warm the elution buffer to 50°C before eluting DNA.

   At the end, resuspend DNA in 100µl 10mM Tris pH8.5. Concentration should be 200-400ng/µl.

   Note Culture volumes can be reduced if required, and midiprep can be used instead of maxiprep, as long as the DNA is transfection grade at the end. You only need enough DNA to do a single transfection (1-4µg depending on the transfection method).

Making virus

Unlike other Ad vectors, the genome does not need to be digested before transfecting. Just transfect circular maxiprepped DNA.

Any transfection method can be used. We transfect using Polyfect (Qiagen) as follows:

1. Seed 2×10^6 293TREx cells (these can be bought from Invitrogen) into a T25 the day before transfection.

   > Note. If you aren't using the tet-repression system, you can use any cells such as 911, 293 or PERC6 to grow virus.

2. Dilute 4µg vector DNA in 100µl DMEM (no FCS or antibiotics)

3. Add 40µl polyfect and incubate 10 mins at room temperature. During the incubation, change media on the cells & add 3mls DMEM (containing FCS/antibiotics).

4. Add 1ml DMEM (with FCS/antibiotics) to DNA complex & transfer to cells.

5. After 24 hrs, change media. Keep changing it whenever media goes yellow.

There will be virus there by ~7 days, but it won't be obvious. To speed it up, we extract virus at 7d, and add to fresh cells.

6. Use a cell scraper to recover all cells into the media.

7. Transfer cells to a 15ml falcon, and pellet the cells (1500rpm, 3 min).

8. Discard the supernatant, and resuspend in 1ml PBS.

9. Add 1ml tetrachloroethylene, add the lid, and give it a good shake to lyse all cells.

10. Centrifuge 2000rpm 10 min. You should have 3 layers. Lowest layer is tetrachloroethylene, middle is debris, top layer is PBS with virus. Using a 1ml Gilson, remove the top layer and keep it.

11. Set up a T25 of fresh 293TREx, with ~5ml media. Add all 1ml of the extracted virus to the flask.

12. After ~4 days the cells should all round up due to virus infection. Bash the flask to knock the cells off, then repeat steps 7-10 to recover the virus. This can be aliquoted as 300ul aliquots at -80C.

13. Titrate the amount of virus (see separate protocol)

Performing knockout

The biggest thing that affects knockout efficiency is the MOI of virus you use. If you've never infected your cells with a Ad vector before, use a range of MOI with a RAd-GFP to work out which MOI gives you the best infection. For cells with high levels of the appropriate receptor, MOI=5 will give >90% infection. For cells with low levels of the receptor, you may need to go to MOI=100, or even 500.

For cells that infect well, infect with the CRISPR RAd at MOI=30-50. For cells that infect poorly, use MOI=200-500.

We do infections in a 6-well plate:

1. Set up adherent cells the day before, so that they are ~80% confluent.
2. Remove the media, add a minimal amount of media (0.5ml for a 6-well), along with appropriate amount of virus.
3. Place on a rocker for 1-2h.
4. Replace the inoculum with fresh media.
5. Change the media every 3 days.
6. If assaying at the protein level, or doing single cell cloning, wait 10-14 days, so that the cells have fully recovered from the Ad vector infection.

Appendix 1 - Selective plates & media

Sucrose plates:

1. When making plates containing sucrose + chloramphenicol do not use salt; the lack of salt makes the bacteria a lot more sensitive to the sucrose:

    10g/L tryptone
    5g/L yeast extract
    50g/L sucrose (i.e. 5%)
    15g/L agar

2. Dissolve some of the sucrose by warming to 37°C for 30 mins before autoclaving or it caramelises.

3. Autoclave, allow it to cool to hand-hot & add:

    Chloramphenicol (1:1000 of 12.5mg/ml stock)
    X-gal (1:500 of 40mg/ml stock)
    IPTG (1:500 of 100mM stock).

LB:
When growing bacteria in LB, we use low salt media:
    10g/L tryptone
    5g/L yeast extract
    5g/L salt

Autoclave, allow to cool & add antibiotics as required (remember, the bacteria carrying your gene after the recombineering are not amp resistant anymore):
    Chloramphenicol (1:1000 of 12.5mg/ml stock)
    Amp (1:1000 of 50mg/ml stick)

Appendix 2 – cloning efficiency

The following do effect cloning efficiency:

Competency of cells

More competent cells = more correct colonies.
The best way to ensure this is to be gentle to the bacteria, and keep them cold throughout the washing steps.

Media/antibiotics

We've had reports from collaborators that the following can cause recombineering to fail:
Growing the bacteria containing the BACs in high salt (10g/L) LB instead of low salt (5g/L).
Growing the bacteria in high concentrations of Amp (100ug/ml instead of 50ug/ml).

The following don't effect cloning efficiency

Amount of DNA

In comparisons of 500ng & 10ng of DNA, both gave equal numbers of colonies.

Concentration of electrocompetent bacteria

The volume of ddH_20 that the bacteria are resuspended in prior to electroporation does not make any difference to the percentage of blue/white colonies. In comparisons bacteria prepared from 25ml competent culture were resuspended in either 50µl or 400µl ddH_2O, and equal percentages of white/blue colonies were obtained. The advantage of diluting the bacteria more is that you can do more inserts with the same volume of bacteria.