An Optimised CRISPR/Cas9 Adenovirus vector (AdZ-CRISPR) for high throughput cloning of sgRNA, using enhanced sgRNA and Cas9 variants

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Abstract

Recombinant Adenovirus vectors enable highly efficient gene delivery *in vitro* and *in vivo.* As a result, they are widely used in gene therapy, vaccination, and anti-cancer applications. We have previously developed the AdZ vector system, which uses recombineering to permit high throughput cloning of transgenes into Adenovirus vectors, simplifies alteration of the vector backbone, and enables rapid recovery of infectious virus, even if a transgene is incompatible with vector replication. Here we adapt this vector system to enable high throughput cloning of sequences for CRISPR/Cas9 editing. Vectors were optimised to ensure efficient cloning, and high editing efficiency using spCas9 and sgRNA sequences in a single vector. Using a multiplicity of infection of 50, knockout efficiencies of up to 80% could be achieved with a single sgRNA. Vectors were further enhanced by altering the spCas9 sequence to match that of SniperCas9, which has reduced off-target activity but maintains on-target efficiency, and by applying modifications to the sgRNA sequence that significantly enhance editing efficiency. Thus, the AdZ-CRISPR vectors offer highly efficient knockout, even in hard to transfect cells, and enables large scale CRISPR/Cas9 projects to be undertaken easily and quickly.

Introduction

CRISPR/Cas9 enables rapid, simple, genome editing to achieve knockout or replacement of sequences both *in vivo* and *in vitro,* in a wide variety of species[1-5]. It is most widely used for knockout, which requires simply the Cas9 protein, and a single guide RNA (sgRNA) containing a targeting sequence for the gene of interest. Delivery of these *in vitro* can be done by plasmid transfection, protein transfection, mRNA, or Lentiviral delivery. However, plasmid and lentiviral use frequently relies on antibiotic resistance markers to enrich transduced/transfected cells – which is problematic if cells are already resistant due to prior transduction with other lentiviral constructs. Furthermore, plasmids, mRNA, and protein transfection require cells that transfect efficiently, while lentiviruses result in permanent expression of Cas9 which may lead to increased off-target effects over time. These issues also limit use of plasmids, proteins, and lentiviruses, for *in vivo* CRISPR/Cas9 delivery. As a result, Adeno-associated viruses (AAV) are often used for *in vivo* purposes, where their low immunogenicity is extremely advantageous in permitting long term expression of transgenes for gene therapy applications[6]. However long-term expression is unnecessary, and may be undesirable, in the case of CRISPR/Cas9, and the relatively small packaging limits of AAV can necessitate the use of novel Cas9 variants[7-9].

Adenovirus (Ad) vectors circumvent many of these issues[9]; they transduce a wide variety of cells *in vitro* and *in vivo* with extremely high efficiency; they do not integrate; they have higher packaging limits; they have an excellent *in vivo* safety profile; and they give transient expression. As a result, they are widely used for gene delivery, vaccination, and in oncolytic applications *in vivo*, and as highly efficient gene delivery agents in basic research. They also grow rapidly, to high titres, are extremely stable, readily manipulated, and comparatively cheap to grow at scale. Together these properties make them extremely useful for CRISPR/Cas9 delivery *in vitro* and *in vivo.* The inherent immunogenicity of Ad vectors does have the potential to reduce the efficacy of *in vivo* delivered CRISPR/Cas9 in comparison to AAV[6, 10]. Nevertheless, a number of studies have demonstrated that CRISPR/Cas9 expressing Ad vectors can successfully treat inherited disorders in animal models[9], and genetic or chemical manipulation of the vector can be used to reduce immunogenicity further[11].

The Adenovirus type 5 (Ad5) genome is the most widely used in vector applications. It comprises 36Kb linear dsDNA, bracketed by inverted repeats. Genes are grouped into four early transcription units (E1, E2, E3, E4), delayed early, as well as a multiple spliced major late transcript[12]. Most recombinant adenovirus (RAd) vectors are rendered replication deficient by deleting the essential E1 region, and must therefore be propagated on helper cells expressing E1 *in trans*[13, 14]. The E3 region is non-essential for replication *in vitro*, and removing E3 in addition to E1 permits insertion of coding sequences up to ~8Kb. Additional regions of the Ad genome can be removed to provide for larger inserts, however these vectors become more difficult to produce for routine applications.

Early RAd vector systems involved insertion of a transgene into a transfer plasmid using traditional cloning techniques, then recombination of that transfer plasmid with the Ad genome either in *E.coli*, yeast, or in mammalian cells[15-18]. These systems were widely used, however they were relatively labour-intensive and poorly suited to cloning multiple genes simultaneously due to the requirement for sequential sub-cloning steps to generate the transfer vector, followed by additional steps needed to recombine the vector with the backbone. Recombination could be problematic, and could suffer from low efficiency. Finally, if expression of the transgene was toxic, or incompatible with vector replication, the final vector could not be propagated.

To address these problems, we developed a novel RAd vector ('AdZ'; http://AdZ.cf.ac.uk) in which inserts could be inserted directly into the intact Ad genome through recombination mediated genetic

engineering (recombineering) in *E.coli*[19-21]. The vector is then recovered without needing linearisation of the genome, in a cell line that silences the transgene during growth (Figure 1). In this way, construction of the vector occurs in a single step, with no additional manipulation of DNA, and even toxic inserts can be cloned. As a result of these modifications, cloning of multiple genes into RAd vectors became much simpler and rapid. Indeed, we used the AdZ system to generate a bank of 170 RAd vectors expressing all canonical ORFs from human cytomegalovirus (HCMV), in order to enable systematic screening for gene function[22-26]. An additional advantage of generating a RAd vector compatible with recombineering is that this technique is not restricted to insertion of transgenes. It can also be used to modify any region of the Ad genome, in a completely scar-less manner. As a result, this vector system has been used by our lab and others, to generate oncolytic viruses[27, 28], to enhance their infection efficiency[29, 30], to expand the tropism of Adenovirus vectors[31], to alter tropism specificity of the virus to become tumour-selective[32-36], to shield virus from neutralising antibodies[37], to enhance immunogenicity[38], and to dissect adenovirus gene function[39].

In the current work we expand the use of the AdZ system to generate a CRISPR/Cas9 vector that is capable of performing CRISPR-Cas9 gene editing with high efficiency and accuracy, for use *in vivo*, or with hard to transfect cells *in vitro.*

Results

Generation of a AdZ vector encoding CRISPR/Cas9 sequences

To determine whether the AdZ vector system could be converted into a vector capable of mediating gene editing with CRISPR-Cas9, we inserted the complete coding sequence for a human optimised spCas9, along with two nuclear localisation signals, and a FLAG tag, downstream of the tetR-regulated HCMV major immediate early promoter (MIEP) in the AdZ vector. We then inserted an expression cassette encoding the U6 promoter, along with a sgRNA capable of targeting Cas9 to beta-2-microglobulin (*B2M*)[40]. Where two expression cassettes are incorporated into a single RAd vector, two sites are commonly used for the second cassette; either immediately following the first (in the E1 region), or in the E3 region. To determine whether there was any variation in expression efficiency between these sites, we inserted the sgRNA expression cassette either directly after the Cas9 expression cassette, or in E3, in both forward and reverse orientations (Fig 2A). Vectors were then grown, and knockout efficiency compared. The vector with the sgRNA in the reverse orientation within E3 grew to 20-fold lower titres than the other three vectors, and therefore was not tested further (Fig 2B). However, the remaining three vectors all grew to similar titres. To determine the relative efficiency of knockout, a cleavage detection assay can be used. However, cleavage does not necessarily correlate with loss of protein, due to reinitiation or exon skipping[41]. We therefore assessed knockout by flow cytometric staining for HLA-I, which is lost in the absence of functional β2m. No major differences were observed between the vectors when HLA-I knockout was tested by flow cytometry (Fig 2C). No cells were observed with an intermediate level of HLA-I expression. This implies that in the majority of cells where gene editing occurred, both alleles were altered. This effect also occurred when using spacer sequences targeting other genes, when using lentiviral or plasmid based vectors, and in other cell types (not shown), and is consistent with published data[42].

AdZ vectors support rapid, high efficiency cloning of sgRNA sequences

We next replaced the 20bp spacer within the sgRNA that targets *B2M*, with a selectable cassette encoding *amp*^{r}, *lacZ*α, and *sacB*, in each vector (Fig. S1). This cassette enables the selection of correctly recombined colonies once spacers targeting genes of interest have been inserted. For any gene to be targeted, a 100bp oligo encoding the 20nt spacer that targets that gene is synthesized, along with 40bp on either side that has homology to the sgRNA cloning site. Recombineering is then used to replace the cassette with the oligo, followed by plating on selective media containing sucrose and X-gal, and selecting white colonies (Fig. S1). We then cloned 16 different spacer sequences targeting a variety of genes, into vectors containing sgRNA in either E1 or E3. Cloning efficiency was consistently higher in the vector containing the sgRNA in E3 (Fig. 3A); this vector was therefore used in all experiments moving forwards. When white colonies were sequenced, all contained the intended spacer insertion, however single base changes or deletions were occasionally observed either within the spacer insert, or in the region where recombination had occurred, presumably as a result of errors during recombination. When 39 different spacers were inserted, 32 colonies had the correct sequence after a single clone was picked, a further 6 after a second colony, and the final one after the third colony was sequenced (Fig 3B-C). Thus, cloning efficiency is extremely high, and sequencing a single colony from each plate is sufficient to obtain sequence-verified clones for the majority of inserts.

Higher MOI enables higher rates of editing

Having established a RAd vector that was compatible with high throughput cloning of sgRNA, we determined the parameters that affected knockout efficiency. HFFF-CAR (human foetal foreskin fibroblasts expressing the Coxsackie-Adenovirus Receptor (CAR) were transduced with the CRISPR RAd targeting *B2M*, at MOI=5. We did not observe strong knockout at 4-days post-transduction, however when cells were left to grow, a population containing lower surface levels of HLA-I was apparent by

day 9, and by day 13, HLA-I signals in this population were reduced to the level of negative control isotype staining (Fig 4A). Thus, in this system, it was important to wait 10-14 days to clearly define knockout populations.

Although the ability of the CRISPR RAd to give complete knockout in 50% of cells was impressive (Fig 2C), especially given that the low transfection efficiency of these cells meant that transfection of the same sgRNA in the popular PX459 plasmid vector did not result in detectable knockout (data not shown), we investigated whether it was possible to improve knockout efficiency by adding more vector. Using a GFP expressing RAd vector, ~70% of HFFF-CAR are transduced at MOI=5, but this can be increased to >95% by MOI=50 without observation of toxicity (Fig 4B). In accordance with this, knockout efficiency increased at higher MOI (Fig 4C). We also investigated whether knockout efficiency could be improved by increasing expression levels from the promoter that drives Cas9 expression, by the use of Forskolin[43]. The addition of forskolin increased knockout efficiency slightly, at lower MOIs, resulting in knockout of HLA-I in almost 80% of cells (Fig 4C).

HFFF-hCAR transduce relatively well with RAd, due to high levels of CAR expression[39]. To determine whether the same result occurred with cells that express lower amounts of CAR, we used the epithelial cell line RPE-1. These cells require MOI=200 to achieve >90% transduction (Fig 4D). Increasing the MOI to 200 gave a significant increase in knockout efficiency without obvious toxicity, however addition of further vector did not lead to increases in knockout (Fig 4E). Unlike in HFFF-CAR, the addition of forskolin did not increase knockout efficiency (Fig 4F).

Thus, optimum knockout efficiency is largely dependent on using an appropriate MOI for the cell type under investigation, and reached a maximum of ~80% knockout. Enhancing expression levels of Cas9 with forskolin can sometimes lead to small improvements in knockout efficiency at lower MOI. To provide direct evidence that the AdZ-CRISPR vector resulted in genome editing, DNA was extracted from transduced cells, a 151bp region surrounding the recognition site for the *B2M* spacer was PCR amplified, TOPO cloned, and individual clones sequenced. The proportion of clones demonstrating editing would not be expected to exactly match the proportion of cells demonstrating knockout of protein expression; edited clones can still express protein due to re-initiation or exon skipping[41], while cells in which Cas9 editing has resulted in larger deletions surrounding the target site (a well known phenomenon with CRISPR editing[44, 45]) will lack protein expression, but would not be detected due to loss of one or both PCR primer binding sites. Nevertheless, we observed clear evidence of genetic editing; following transduction of cells with a MOI sufficient to infect >90% of cells, 4/10 (HFFF-hCAR, expressing high CAR levels) or 9/10 (RPE-1, expressing low CAR levels) PCR clones demonstrated edits (Fig. 4G).

AdZ vectors carrying improved Cas9 and sgRNA sequences support optimised editing

One of the risks with CRISPR-Cas9 is that gene edits can be made at sites other than those intended. As a result, attempts have been made to increase the fidelity of Cas9 edits, through mutating the Cas9 protein[46-51]. To investigate whether higher fidelity spCas9 worked in the context of our RAd vector, we took advantage of the ability of recombineering to introduce seamless modifications anywhere in the genome; introducing N692A, M694A, Q695A and H698A mutations into Cas9 within the RAd, generated HypaCas9 (Figure S2)[49]. This has been reported to show high genome-wide specificity without compromising on-target activity in human cells. However, using the *B2M* sgRNA we consistently observed a lower knockout efficiency with HypaCas9, and this could not be enhanced with the addition of forskolin (Fig 5A).

As an alternative we therefore introduced the mutations F539S, M763I, and K890N, collectively termed SniperCas9 (Figure S2)[50]. These mutations have also been reported to reduce off-target effects

without affecting on-target editing by Cas9. They offer a further advantage, however, in that SniperCas9 is compatible with the addition of an extra 'G' base at the beginning of the sgRNA targeting sequence. I.e. the sgRNA must begin with a G for efficient expression from the U6 promoter; where a sgRNA does not naturally begin with a 'G', one can be added, but this modified sequence cannot normally be used with enhanced Cas9 variants. SniperCas9 is equally as effective when using such modified sgRNA sequences, giving greater flexibility in sgRNA selection. The introduction of SniperCas9 into our vector had a small impact on knockout efficiency (Fig 5B), however this was much less dramatic than seen with HypaCas.

Finally, we also introduced two enhancing modifications to the sgRNA scaffold. These mutations extend the sgRNA duplex by 10bp and remove a potential RNA pol III pause signal in the sgRNA, increasing the efficiency of CRISPR/Cas9 editing (Figure S2)[52]. The addition of this alteration had no effect on editing efficiency with the *B2M* sgRNA (Fig 5C), however it did enhance the activity of a vector carrying SniperCas9, such that it gave knockout efficiencies identical to Cas9 (Fig 5D). The *B2M* sgRNA already enabled very efficient knockout. To determine whether this modification enhanced the rate of knockout with lower efficiency sgRNA, we cloned multiple spacers targeting *PDGFRA* or *CD155* into the original vector, or the vector containing both SniperCas9 and the enhanced sgRNA scaffold. Using these optimised vectors, all 8 sgRNA showed enhanced activity, including three that showed no activity at all in the original vector, but clear activity in the enhanced vector (Fig 5E-F). Although these vectors contained two modifications (both sgRNA and Cas9 were modified), given that the SniperCas9 showed equal or slightly reduced activity to wildtype Cas9 (Fig 5B), it is likely that this enhancement was due to the sgRNA sequence. Thus, the 'SniperCas9/sgRNA+' vector offers both more accurate editing, and significantly improved editing efficiency.

Discussion

A number of systems have aimed to improve on adenovirus vectors that required traditional cloning into a transfer vector, followed by recombination with the adenovirus backbone. Systems exist to use gateway cloning from a 'entry' vector, directly into the Ad genome[53], or to use traditional cloning into a subgenomic fragment of the Ad vector, followed by re-assembly in a Gibson reaction[54]. These offer speed, ease-of-use, and throughput advantages over earlier systems. However, recombineering directly into the genome offers further advantages. There is no requirement for an 'entry' vector, and there is no requirement for re-assembly of the genome after construction. The first application of recombineering to Ad vectors was to allow manipulation of an Ad19-based vector[55], although this system requires an additional re-transformation to select colonies in which the desired modification has occurred. The AdZ system applied a simpler recombineering approach to Ad5 vectors deleted for E1 and E3[19], and the same technology has also been subsequently applied to gutless adenovirus vectors[56].

A number of CRISPR-based Adenovirus vectors have been generated in Adenovirus vector systems, however all require an initial cloning step for the gRNA, in a shuttle vector that is then recombined with the Ad backbone in subsequent steps[57-59]. The AdZ system therefore offers significant advantages in terms of time and effort for the insertion of gRNA sequences, especially if multiple different sgRNA expressing RAd are to be cloned simultaneously – there is not even a requirement to anneal oligonucleotides before recombineering, they are simply ordered, and electroporated directly into the competent bacteria containing the intact RAd genome. The ability to edit the vector backbone is another advantage of the AdZ system, exemplified by our modifications to the components of the CRISPR/Cas9 system, including (i) a Cas9 variant that offers lower off-target editing while keeping on-target efficiency, (ii) is tolerant of the addition of an initial 'G' for efficient transcription, (iii) a sgRNA scaffold structure that improves editing efficiency. As CRISPR/Cas9 technology improves further, it will be simple to adapt these vectors. Furthermore, the use of a promoter that is silenced in packaging cells means that vectors carrying sgRNA targeting genes that interfere with adenovirus replication can be grown to high titres, ensuring success when growing vectors[19]. Finally, the relatively high packaging limit of RAd vectors allows for the incorporation of homology-directed repair (HDR) templates if gene modifications, rather than gene knockouts, are required.

A major advantage of Adenovirus vectors is their ability to transiently deliver genes to a high proportion of target cells, without any requirement for drug selection. Where a cell type does not express high levels of CAR, it is still possible to transduce many cells by simply using higher MOI, although this needs to be empirically tested to ensure there is no particle-associated toxicity. Alternatively, a variety of genetic approaches can be used to retarget RAds to use novel receptors[60], or Factor X can be added to the virus to enable entry via heparan-sulfate proteoglycans[61, 62]. The transduction efficiency is demonstrated here by our ability to knock genes out in a cell line (HFFF) where plasmid transfection failed, with knockout in over 80% of cells achievable. It is also possible to co-deliver vectors targeting multiple sites within the same gene. If each sgRNA works efficiently, this can result in knockout of a gene in essentially all cells, with no requirement to sort or single-cell clone[57]. The biggest issue with CRISPR/Cas9 systems remains the selection of an efficient sgRNA sequence, as demonstrated by the disparate knockout efficiency seen with sgRNA targeting *B2M* as opposed to those targeting *PDGFRA* and *CD155*. A large number of computational tools are now available to design efficient sgRNA with minimal off-target effects, which can reduce this problem[63, 64]. The main limitation of our system, in comparison to other vector types, is that the RAd vector must be grown up over the course of ~2 weeks, and titrated prior to use.

In summary, the combination of a vector system (i) carrying an enhanced sgRNA sequence, (ii) with a more accurate Cas9, (iii) that supports rapid and efficient cloning of sgRNA sequences from oligonucleotides directly into an intact Ad vector genome, (iv) that is automatically excised from the prokaryotic vector in mammalian cells, (v) that suppresses Cas9 expression during vector propagation, (vi) that enables high efficiency editing even of hard to transfect cells, (vii) without any need for drug selection, and (viii) uses transient Cas9 expression, means that CRISPR/Cas9 editing projects can now be approached with confidence in almost any cell type.

Materials and Methods

Cells and Viruses

293TREx cells were purchased from Invitrogen, RPE-1 cells were purchased from Clontech. HFFF-hCAR are human fetal foreskin fibroblasts that have been immortalised with human telomerase, and express the coxsackie-adenovirus receptor (CAR), and have been described before[39]. All cells were grown in DMEM containing 10% fetal calf serum, at 37°C, in 5% CO_2. The AdZ vector has been described before[19]. Vector was recovered from AdZ constructs by midiprepping DNA (Nucleobond Xtra Midi, Machery-Nagel), and transfecting 293TREx cells using effectene, according to manufacturer's instructions. Vector was passaged in 293TREx cells, and titrated by infecting 293TREx cells with serial dilutions of the preparation, followed by staining for adenovirus proteins as previously described[65]. In all cases, MOI was calculated on the basis of spot forming units, which are equivalent to plaque forming units (PFU)[65].

Recombineering

All recombineering steps were carried out as previous described[19]. In brief, SW102 bacteria containing the AdZ BAC of interest were grown at 32°C until OD_{600}=0.55, then incubated at 42°C for 15 minutes to induce expression of recombineering genes. Bacteria were washed twice with ice cold water, resuspended in a small volume, and DNA constructs to be recombined with the vector were added. Mixtures were electroporated using the 'EC3' program (Micropulser, Biorad), then 1ml LB media added. After recovering for 1h at 32°C, bacteria were plated onto selective media. For modifications to the vector backbone, an initial positive selection step using a cassette encoding ampicillin resistance, *lacZ*α, and *sacB* was performed. This cassette was PCR amplified using primers containing 80bp homology to the target insertion site in the 5' region of both primers, gel purified, recombineered into the target site, and bacteria were plated on media containing X-gal, IPTG, chloramphenicol (12.5μg/ml), and ampicillin (50μg/ml) to select for recombinants. In a second, negative selection step, sequences to be inserted were PCR amplified using 80bp arms of homology, and gel purified, before being inserted in place of the selection cassette, again using recombineering. This time, bacteria were recovered for 3h in 5ml of LB after electroporation, and plated on LB Agar lacking NaCl, but containing 5% sucrose. This efficiently selects against colonies in which the original selection cassette remains. Full protocols are available on our website, AdZ.cf.ac.uk.

Primers used in recombineering are listed in Table 1, all constructs were verified by Sanger sequencing. To insert spCas9 under the control of the HCMV MIEP, the entire ORF was gene synthesized and inserted into the AdZ vector pAdZ5-CV5[19]. To insert the sgRNA sequence, the *amp^r/lacZ/sacB* cassette was inserted either immediately after the polyadenylation sequence for spCas9, or in the E3 region. The cassette was then replaced with a sgRNA sequence that was PCR amplified from pX459 (Addgene), using primers gRNAF-1/gRNAR-1 (after the polyadenylation sequence, forward orientation), gRNAF-2/gRNAR-2 (after the polyadenylation sequence, reverse orientation), gRNAF-3/gRNAR-3 (E3, forward orientation), gRNAF-4/gRNAR-4 (E3, forward orientation). To enable insertion of spacers targeting different genes into the sgRNA constructs, the *amp^r/lacZ/sacB* cassette was inserted in place of the original gene-specific spacer sequence, using primers sacbF gRNA and sacbR gRNA.

To modify spCas9 to carry HypaCas9 modifications, the *amp^r/lacZ/sacB* cassette was amplified using primers SB-HypaCasF and SVB-HypaCasR, then the cassette removed and replaced with HypaCas modifications using oligo HypaCas. To modify spCas9 to carry the SniperCas sequences, the *amp^r/lacZ/sacB* cassette was amplified using primers Sniper-SacBF and Sniper-SacBR, before being replaced with a gene synthesized DNA fragment carrying the appropriate modifications. To introduce enhancing modifications into the sgRNA sequence, the *amp^r/lacZ/sacB* cassette was amplified using

primers gRNA_optSacBF and gRNA_optSacBR, then replaced using the paired oligos gRNA_optF and gRNA_optR.

gRNAF-1	GCAGCGCCCCTCTTAACAAGCCGACCCCCACCAGCGTCGCGGTTACTAACACTCCTCT CCCCGACCTGCAGCCCAAGCTTGAGGGCCTATTTCCCATGAT
gRNAR-1	CTGCTGCAAAACAGATACAAAACTACATAAGACCCCCACCTTATATATTCTTTCCCACC CTTAAGCCACGCCCACACAAAAAAAAGCACCGACTCGGTGC
gRNAF-2	GGCTGCTGCAAAACAGATACAAAACTACATAAGACCCCCACCTTATATATTCTTTCCC ACCCTTAAGCCACGCCCACACAGAGGGCCTATTTCCCATGAT
gRNAR-2	AGCGCCCCTCTTAACAAGCCGACCCCCACCAGCGTCGCGGTTACTAACACTCCTCTCC CCGACCTGCAGCCCAAGCTTAAAAAAAGCACCGACTCGGTGC
gRNAF-3	AGCGCCCCCTGCTAGTTGAGCGGGACAGGGGACCCTGTGTTCTCACTGTGATTTGCA ACTGTCCTAACCTTGGATTACATGAGGGCCTATTTCCCATGAT
gRNAR-3	ACTGATTTTAAGTAAGTGATGCTTTATTATTTTTTTTTATTAGTTAAAGGGAATAAGAT CTTTGAGACCGCACAGGGTAAAAAAAGCACCGACTCGGTGC
gRNAF-4	TAACTGATTTTAAGTAAGTGATGCTTTATTATTTTTTTTTATTAGTTAAAGGGAATAAG ATCTTTGAGACCGCACAGGGTGAGGGCCTATTTCCCATGAT
gRNAR-4	CGCCCCCTGCTAGTTGAGCGGGACAGGGGACCCTGTGTTCTCACTGTGATTTGCAAC TGTCCTAACCTTGGATTACATAAAAAAAGCACCGACTCGGTGC
sacbF grna	GACTATCATATGCTTACCGTAACTTGAAAGTATTTCGATTTCTTGGCTTTATATATCTT GTGGAAAGGACGAAACACCCCTGTGACGGAAGATCACTTCG
sacbR grna	AAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTT GCTATTTCTAGCTCTAAAACCTGAGGTTCTTATGGCTCTTG
SB-HypaCasF	CTGATCAACGGCATCCGGGATAAGCAGTCCGGCAAGACCATCCTGGACTTTCTGAAG TCCGACGGCTTCGCCAATCGGCCTGTGACGGAAGATCACTTCG
SVB-HypaCasR	CAATGTGCTCATGCAGAGAATCGCCCTGGCCGGACACCTGGGCTTTCTGGATATCCTC TTTAAAGGTCAGGCTGTCGTCCTGAGGTTCTTATGGCTCTTG
HypaCas	CGGCAAGACCATCCTGGACTTTCTGAAGTCCGACGGCTTCGCCAATCGGGCCTTCGC GGCGCTGATCGCCGACGACAGCCTGACCTTTAAAGAGGATATCCAGAAAGCCCAGGT GTCCGG
Sniper-SacBF	CTGCTGTACGAGTACTTCACCGTGTACAACGAGCTGACCAAAGTGAAATACGTGACC GAGGGAATGAGAAAGCCCGCCCCTGTGACGGAAGATCACTTCG
Sniper-SacBR	TGAAGCCGGCCTTATCCAGTTCAGACAGGCCGCCTCTCTCGGCCTTGGTCAGGTTATC GAACTTCCGCTGGGTAATCAGCTGAGGTTCTTATGGCTCTTG
gRNA_optSacBF	TGAAAGTATTTCGATTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGGCC GAGATGTCTCGCTCCGGTTTCCTGTGACGGAAGATCACTTCG
gRNA_optSacBR	GATCTTTGAGACCGCACAGGGTAAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGT TGATAACGGACTAGCCTTATTTCTGAGGTTCTTATGGCTCTTG
gRNA_optF	GGCTTTATATATCTTGTGGAAAGGACGAAACACCGGCCGAGATGTCTCGCTCCGGTT TCAGAGCTAtgctgGAAAcagcaTAGCAAGTTgAAATAAGGCT
gRNA_optR	TAGAAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTAT TTcAACTTGCTAtgctgTTTCcagcaTAGCTCTGAAACCGGA

Table 1

To introduce spacers targeting additional genes by CRISPR/Cas9, into vectors carrying the original sgRNA, the primers listed in Table 2 were used. To introduce the same spacers into vectors carrying the enhanced sgRNA, the primers listed in Table 3 were used.

CD155-2	TTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGCGCAGGGGACGTCGTCGTGCG TTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGT

CD155-3	TTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGCCTGTTCGTCACGTTCCCGCGTT TTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTC
CD155-4	TTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGCGGGATGCCCAATACGAGCCGT TTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTC
CD155-5	TTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGCGTTTGGACTCCGAATAGCTGTT TTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTC
PDGFRa-1	TCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGAAAGCCCTGTCTGCTGTCGTGTTT TAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCC
PDGFRa-2	TCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGCTTCCAAGACCGTCACAAAAGTTT TAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCC
PDGFRa-3	TTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGTCGGGATCAGTTGTGCGACAGT TTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTC
PDGFRa-4	TTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGTAAGACCAGGAACGCCGGATGT TTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTC

Table 2. Oligos encoding spacer sequences targeting different genes. The gene targeted is noted in the oligo name.

CD155-1	TTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGCGGGATGCCCAATACGAGCCG TTTCAGAGCTAtgctgGAAAcagcaTAGCAAGTTgAAA
CD155-2	TTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGCGCAGGGGACGTCGTCGTGCG TTTCAGAGCTAtgctgGAAAcagcaTAGCAAGTTgAAA
CD155-3	TTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGCCTGTTCGTCACGTTCCCGCGT TTCAGAGCTAtgctgGAAAcagcaTAGCAAGTTgAAA
CD155-4	TTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGCGGGATGCCCAATACGAGCCG TTTCAGAGCTAtgctgGAAAcagcaTAGCAAGTTgAAA
CD155-5	TTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGCGTTTGGACTCCGAATAGCTG TTTCAGAGCTAtgctgGAAAcagcaTAGCAAGTTgAAA
PDGFRA-1	TTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGAAAGCCCTGTCTGCTGTCGTGT TTCAGAGCTAtgctgGAAAcagcaTAGCAAGTTgAAA
PDGFRA-2	TTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGCTTCCAAGACCGTCACAAAAG TTTCAGAGCTAtgctgGAAAcagcaTAGCAAGTTgAAA
PDGFRA-3	TTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGTCGGGATCAGTTGTGCGACAG TTTCAGAGCTAtgctgGAAAcagcaTAGCAAGTTgAAA
PDGFRA-4	TTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGTAAGACCAGGAACGCCGGATG TTTCAGAGCTAtgctgGAAAcagcaTAGCAAGTTgAAA

Table 3. Oligos encoding spacer sequences targeting different genes. The gene targeted is noted in the oligo name.

Plasmid Transfection

gRNA were cloned into the pX459 vector according to the guide at addgene (https://www.addgene.org/crispr/zhang/). The vector was midiprepped using the Nucleobond Xtra Midi kit (Machery Nagel), and transfected into HFFF-hCAR using an Amaxa Nucleofector, program T16, and the Basic Fibroblast kit, according to manufacturer's instructions. This protocol was previously found to offer high transfection efficiency in this cell line[66].

Flow Cytometry

Cells were dissociated with TrypLE, stained with antibodies targeting HLA (W632), PDGFRα (BD Pharmingen) or CD155 (D171; Invitrogen) followed by anti-mouse AF647 (Thermo), before being washed and fixed in 4% PFA, then run on a Accuri C6 cytometer (BD) and analysed in FlowJo. 'All gates were set using a non-binding isotype matched control antibody (for the negative gate), and cells that had been transduced with a control RAd vector lacking expression of Cas9 (for the positive gate).

Analysis of genetic editing by sequencing

DNA was extracted from cells using the DNeasy Blood & Tissue Kit (Qiagen) according to manufacturers instructions, then A 151bp region surrounding the target site for the *B2M* gRNA was PCR amplified using primers AGAGACTCACGCTGGATAG and CTGGGCACGCGTTTAAT, and the Expand HiFi PCR kit (Roche). Amplified DNA was gel purified, TOPO cloned into pCR4.1-TOPO according to manufacturer's instructions (Thermo), then sequenced by Sanger sequencing (Eurofins Genomics).

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References

1. Adli M. The CRISPR tool kit for genome editing and beyond. Nature communications 2018;9:1911.
2. Jinek M, Chylinski K, Fonfara I et al. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 2012;337:816-821.
3. Jinek M, East A, Cheng A et al. RNA-programmed genome editing in human cells. Elife 2013;2:e00471.
4. Cong L, Ran FA, Cox D et al. Multiplex genome engineering using CRISPR/Cas systems. Science 2013;339:819-823.
5. Mali P, Yang L, Esvelt KM et al. RNA-guided human genome engineering via Cas9. Science 2013;339:823-826.
6. Shirley JL, de Jong YP, Terhorst C et al. Immune Responses to Viral Gene Therapy Vectors. Mol Ther 2020;28:709-722.
7. Schmidt MJ, Gupta A, Bednarski C et al. Improved CRISPR genome editing using small highly active and specific engineered RNA-guided nucleases. Nature communications 2021;12:4219.
8. Ibraheim R, Tai PWL, Mir A et al. Self-inactivating, all-in-one AAV vectors for precision Cas9 genome editing via homology-directed repair in vivo. Nature communications 2021;12:6267.
9. Boucher P, Cui X, Curiel DT. Adenoviral vectors for in vivo delivery of CRISPR-Cas gene editors. Journal of Controlled Release 2020;327:788-800.
10. Wang D, Mou H, Li S et al. Adenovirus-Mediated Somatic Genome Editing of Pten by CRISPR/Cas9 in Mouse Liver in Spite of Cas9-Specific Immune Responses. Hum Gene Ther 2015;26:432-442.
11. Kreppel F, Hagedorn C. Capsid and Genome Modification Strategies to Reduce the Immunogenicity of Adenoviral Vectors. International Journal of Molecular Sciences 2021;22:2417.
12. Davison AJ, Benko M, Harrach B. Genetic content and evolution of adenoviruses. J Gen Virol 2003;84:2895-2908.
13. Graham FL, Smiley J, Russell WC et al. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J Gen Virol 1977;36:59-74.
14. Fallaux FJ, Bout A, van der Velde I et al. New helper cells and matched early region 1-deleted adenovirus vectors prevent generation of replication-competent adenoviruses. Hum Gene Ther 1998;9:1909-1917.
15. McGrory WJ, Bautista DS, Graham FL. A simple technique for the rescue of early region I mutations into infectious human adenovirus type 5. Virology 1988;163:614-617.
16. Bett AJ, Haddara W, Prevec L et al. An efficient and flexible system for construction of adenovirus vectors with insertions or deletions in early regions 1 and 3. Proc Natl Acad Sci U S A 1994;91:8802-8806.
17. Ng P, Cummings DT, Evelegh CM et al. Yeast recombinase FLP functions effectively in human cells for construction of adenovirus vectors. BioTechniques 2000;29:524-526, 528.
18. Ng P, Parks RJ, Cummings DT et al. An enhanced system for construction of adenoviral vectors by the two-plasmid rescue method. Hum Gene Ther 2000;11:693-699.
19. Stanton RJ, McSharry BP, Armstrong M et al. Re-engineering adenovirus vector systems to enable high-throughput analyses of gene function. BioTechniques 2008;45:659-662, 664-658.
20. Yu D, Ellis HM, Lee EC et al. An efficient recombination system for chromosome engineering in Escherichia coli. Proc Natl Acad Sci U S A 2000;97:5978-5983.
21. Warming S, Costantino N, Court DL et al. Simple and highly efficient BAC recombineering using galK selection. Nucleic Acids Res 2005;33:e36.
22. Fielding CA, Aicheler R, Stanton RJ et al. Two novel human cytomegalovirus NK cell evasion functions target MICA for lysosomal degradation. PLoS Pathog 2014;10:e1004058.
23. Stanton RJ, Prod'homme V, Purbhoo MA et al. HCMV pUL135 remodels the actin cytoskeleton to impair immune recognition of infected cells. Cell Host Microbe 2014;16:201-214.

24. Hsu JL, van den Boomen DJ, Tomasec P et al. Plasma membrane profiling defines an expanded class of cell surface proteins selectively targeted for degradation by HCMV US2 in cooperation with UL141. PLoS Pathog 2015;11:e1004811.
25. Wang ECY, Pjechova M, Nightingale K et al. Suppression of costimulation by human cytomegalovirus promotes evasion of cellular immune defenses. Proc Natl Acad Sci U S A 2018;115:4998-5003.
26. Vlahava VM, Murrell I, Zhuang L et al. Monoclonal antibodies targeting nonstructural viral antigens can activate ADCC against human cytomegalovirus. J Clin Invest 2021;131.
27. Puig-Saus C, Rojas LA, Laborda E et al. iRGD tumor-penetrating peptide-modified oncolytic adenovirus shows enhanced tumor transduction, intratumoral dissemination and antitumor efficacy. Gene Ther 2014;21:767-774.
28. Al-Zaher AA, Moreno R, Fajardo CA et al. Evidence of Anti-tumoral Efficacy in an Immune Competent Setting with an iRGD-Modified Hyaluronidase-Armed Oncolytic Adenovirus. Mol Ther Oncolytics 2018;8:62-70.
29. Yu D, Jin C, Leja J et al. Adenovirus with hexon Tat-protein transduction domain modification exhibits increased therapeutic effect in experimental neuroblastoma and neuroendocrine tumors. J Virol 2011;85:13114-13123.
30. Puig-Saus C, Laborda E, Rodriguez-Garcia A et al. The combination of i-leader truncation and gemcitabine improves oncolytic adenovirus efficacy in an immunocompetent model. Cancer Gene Ther 2014;21:68-73.
31. Yu D, Jin C, Ramachandran M et al. Adenovirus serotype 5 vectors with Tat-PTD modified hexon and serotype 35 fiber show greatly enhanced transduction capacity of primary cell cultures. PLoS One 2013;8:e54952.
32. Uusi-Kerttula H, Legut M, Davies J et al. Incorporation of Peptides Targeting EGFR and FGFR1 into the Adenoviral Fiber Knob Domain and Their Evaluation as Targeted Cancer Therapies. Hum Gene Ther 2015;26:320-329.
33. Uusi-Kerttula H, Davies JA, Thompson JM et al. Ad5NULL-A20: A Tropism-Modified, alphavbeta6 Integrin-Selective Oncolytic Adenovirus for Epithelial Ovarian Cancer Therapies. Clin Cancer Res 2018;24:4215-4224.
34. Uusi-Kerttula H, Davies J, Coughlan L et al. Pseudotyped alphavbeta6 integrin-targeted adenovirus vectors for ovarian cancer therapies. Oncotarget 2016;7:27926-27937.
35. Man YKS, Davies JA, Coughlan L et al. The Novel Oncolytic Adenoviral Mutant Ad5-3Delta-A20T Retargeted to alphavbeta6 Integrins Efficiently Eliminates Pancreatic Cancer Cells. Molecular cancer therapeutics 2018;17:575-587.
36. Hulin-Curtis SL, Uusi-Kerttula H, Jones R et al. Evaluation of CD46 re-targeted adenoviral vectors for clinical ovarian cancer intraperitoneal therapy. Cancer Gene Ther 2016;23:229-234.
37. Rojas LA, Condezo GN, Moreno R et al. Albumin-binding adenoviruses circumvent pre-existing neutralizing antibodies upon systemic delivery. J Control Release 2016;237:78-88.
38. Rodriguez-Garcia A, Svensson E, Gil-Hoyos R et al. Insertion of exogenous epitopes in the E3-19K of oncolytic adenoviruses to enhance TAP-independent presentation and immunogenicity. Gene Ther 2015;22:596-601.
39. McSharry BP, Burgert HG, Owen DP et al. Adenovirus E3/19K promotes evasion of NK cell recognition by intracellular sequestration of the NKG2D ligands major histocompatibility complex class I chain-related proteins A and B. J Virol 2008;82:4585-4594.
40. van der Ploeg K, Chang C, Ivarsson MA et al. Modulation of Human Leukocyte Antigen-C by Human Cytomegalovirus Stimulates KIR2DS1 Recognition by Natural Killer Cells. Frontiers in immunology 2017;8:298.
41. Smits AH, Ziebell F, Joberty G et al. Biological plasticity rescues target activity in CRISPR knock outs. Nat Methods 2019;16:1087-1093.

42. Yuen G, Khan FJ, Gao S et al. CRISPR/Cas9-mediated gene knockout is insensitive to target copy number but is dependent on guide RNA potency and Cas9/sgRNA threshold expression level. Nucleic Acids Research 2017;45:12039-12053.
43. Wilkinson GW, Akrigg A. Constitutive and enhanced expression from the CMV major IE promoter in a defective adenovirus vector. Nucleic Acids Res 1992;20:2233-2239.
44. Adikusuma F, Piltz S, Corbett MA et al. Large deletions induced by Cas9 cleavage. Nature 2018;560:E8-E9.
45. Wen W, Quan Z-J, Li S-A et al. Effective control of large deletions after double-strand breaks by homology-directed repair and dsODN insertion. Genome Biology 2021;22:236.
46. Vakulskas CA, Dever DP, Rettig GR et al. A high-fidelity Cas9 mutant delivered as a ribonucleoprotein complex enables efficient gene editing in human hematopoietic stem and progenitor cells. Nat Med 2018;24:1216-1224.
47. Kleinstiver BP, Pattanayak V, Prew MS et al. High-fidelity CRISPR-Cas9 nucleases with no detectable genome-wide off-target effects. Nature 2016;529:490-495.
48. Slaymaker IM, Gao L, Zetsche B et al. Rationally engineered Cas9 nucleases with improved specificity. Science 2016;351:84-88.
49. Chen JS, Dagdas YS, Kleinstiver BP et al. Enhanced proofreading governs CRISPR-Cas9 targeting accuracy. Nature 2017;550:407-410.
50. Lee JK, Jeong E, Lee J et al. Directed evolution of CRISPR-Cas9 to increase its specificity. Nature communications 2018;9:3048.
51. Casini A, Olivieri M, Petris G et al. A highly specific SpCas9 variant is identified by in vivo screening in yeast. Nat Biotechnol 2018;36:265-271.
52. Dang Y, Jia G, Choi J et al. Optimizing sgRNA structure to improve CRISPR-Cas9 knockout efficiency. Genome Biol 2015;16:280.
53. Choi EW, Seen DS, Song YB et al. AdHTS: a high-throughput system for generating recombinant adenoviruses. J Biotechnol 2012;162:246-252.
54. Miciak JJ, Hirshberg J, Bunz F. Seamless assembly of recombinant adenoviral genomes from high-copy plasmids. PLoS One 2018;13:e0199563.
55. Ruzsics Z, Wagner M, Osterlehner A et al. Transposon-assisted cloning and traceless mutagenesis of adenoviruses: Development of a novel vector based on species D. J Virol 2006;80:8100-8113.
56. Muck-Hausl M, Solanki M, Zhang W et al. Ad 2.0: a novel recombineering platform for high-throughput generation of tailored adenoviruses. Nucleic Acids Res 2015;43:e50.
57. Jin YH, Joo H, Lee K et al. Streamlined procedure for gene knockouts using all-in-one adenoviral CRISPR-Cas9. Scientific reports 2019;9:277.
58. Ehrke-Schulz E, Schiwon M, Leitner T et al. CRISPR/Cas9 delivery with one single adenoviral vector devoid of all viral genes. Scientific reports 2017;7:17113.
59. Nakanishi T, Maekawa A, Suzuki M et al. Construction of adenovirus vectors simultaneously expressing four multiplex, double-nicking guide RNAs of CRISPR/Cas9 and in vivo genome editing. Scientific reports 2021;11:3961.
60. Cunliffe TG, Bates EA, Parker AL. Hitting the Target but Missing the Point: Recent Progress towards Adenovirus-Based Precision Virotherapies. Cancers 2020;12:3327.
61. Bradshaw AC, Parker AL, Duffy MR et al. Requirements for receptor engagement during infection by adenovirus complexed with blood coagulation factor X. PLoS Pathog 2010;6:e1001142.
62. Waddington SN, McVey JH, Bhella D et al. Adenovirus serotype 5 hexon mediates liver gene transfer. Cell 2008;132:397-409.
63. Zhang Y, Zhao G, Ahmed FYH et al. In silico Method in CRISPR/Cas System: An Expedite and Powerful Booster. Front Oncol 2020;10:584404.
64. Sledzinski P, Nowaczyk M, Olejniczak M. Computational Tools and Resources Supporting CRISPR-Cas Experiments. Cells 2020;9.
65. Bewig B, Schmidt WE. Accelerated titering of adenoviruses. BioTechniques 2000;28:870-873.

66. Stanton RJ, Baluchova K, Dargan DJ et al. Reconstruction of the complete human cytomegalovirus genome in a BAC reveals RL13 to be a potent inhibitor of replication. J Clin Invest 2010;120:3191-3208.

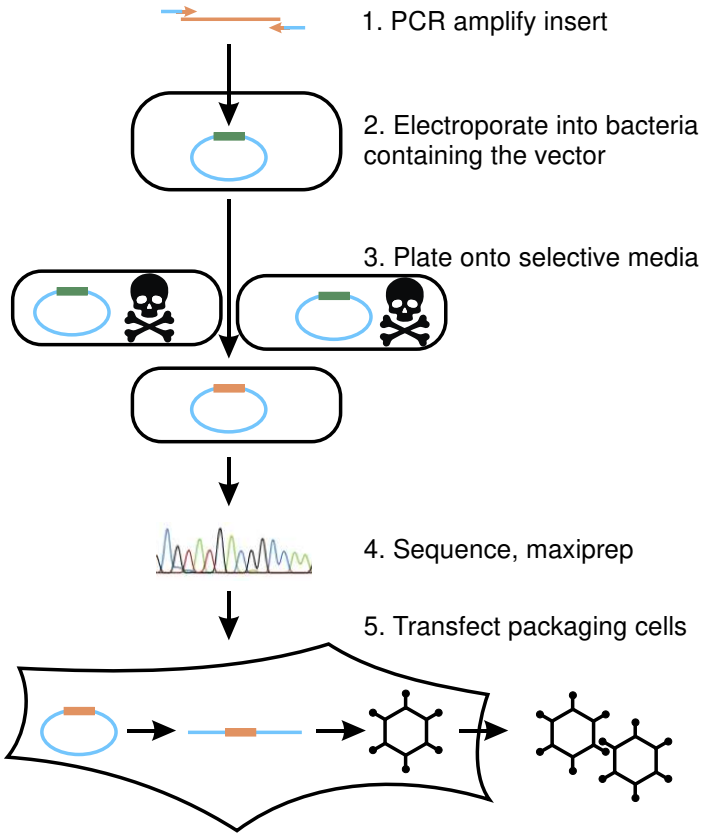


Figure 1. Schematic of transgene insertion in the AdZ system. Inserts are PCR amplified with sequences in the primer that are homologous to the target insertion site. E.coli containing the RAd vector as a stable bacterial artificial chromosome (BAC) are grown to mid-log phase, heat-shocked to induce expression of genes for recombineering, and electroporated with the PCR product. After recovery, bacteria are plated on selective media. Colonies containing the insert are identified based on blue-white screening, miniprepped, and modifications verified by sanger sequencing. Vectors are maxiprepped, and transfected into 293TREx cells. The RAd genome must be linearised to be infectious; this occurs through I-SceI sites engineered into the genome termini, which are cut by I-SceI expressed from the vector backbone when in mammalian cells. In addition, in 293TREx cells, expression of the cloned transgene is silenced by expression of TetR, but is constitutively on in the absence of TetR (i.e. most other cell types).

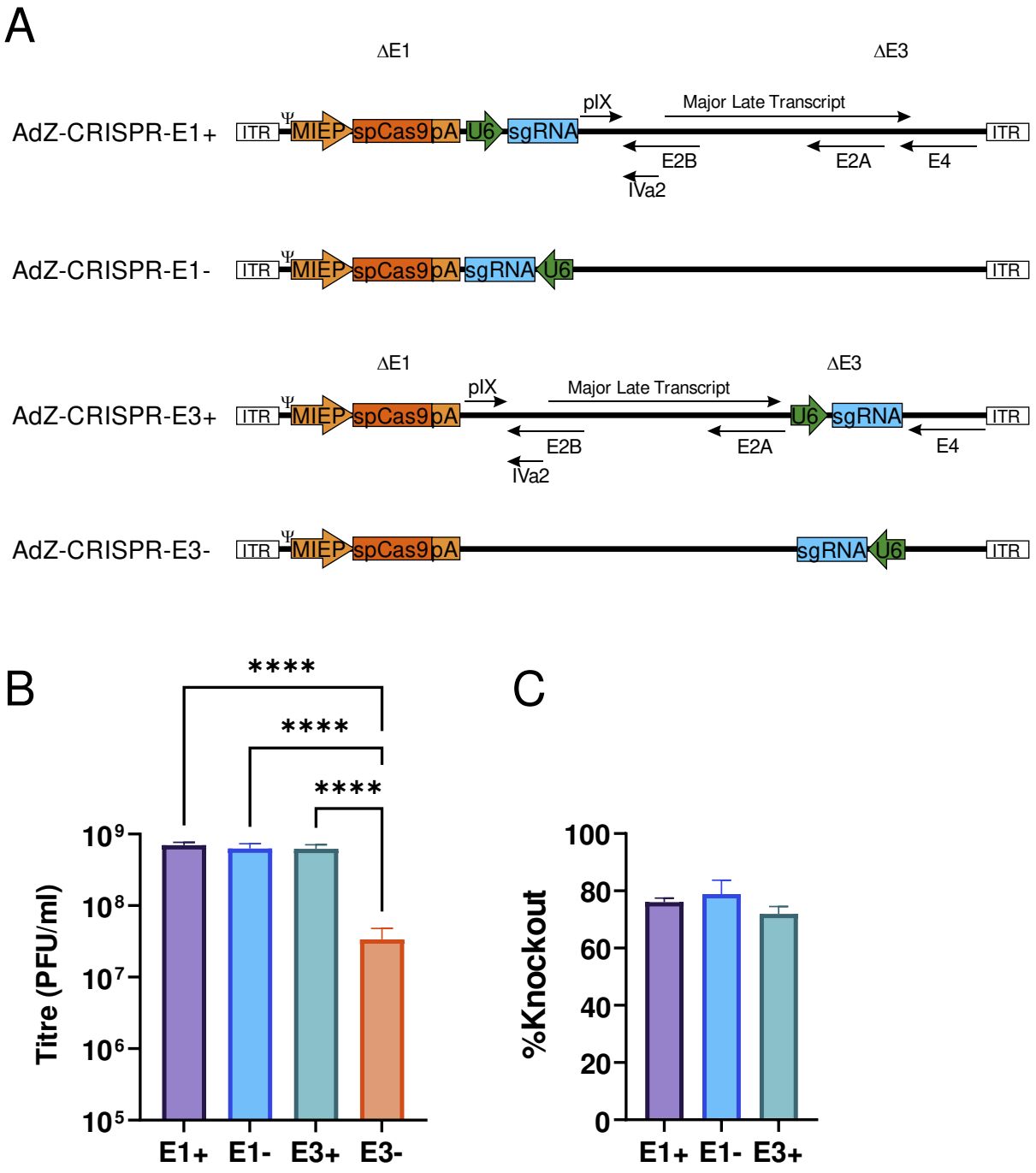


Figure 2. Construction of a CRISPR/Cas9 RAd vector in the AdZ system. (A) Schematic showing Ad vectors constructed. (B) Titres of vectors recovered from constructs in (A). (C) HFFF-hCAR were transduced with vectors from (B) at MOI=5, then 14 days later were stained with anti-HLA antibody, and analysed by flow cytometry for loss of staining. Error bars show mean ±SD, n=3. 1-way ANOVA ****<0.0001

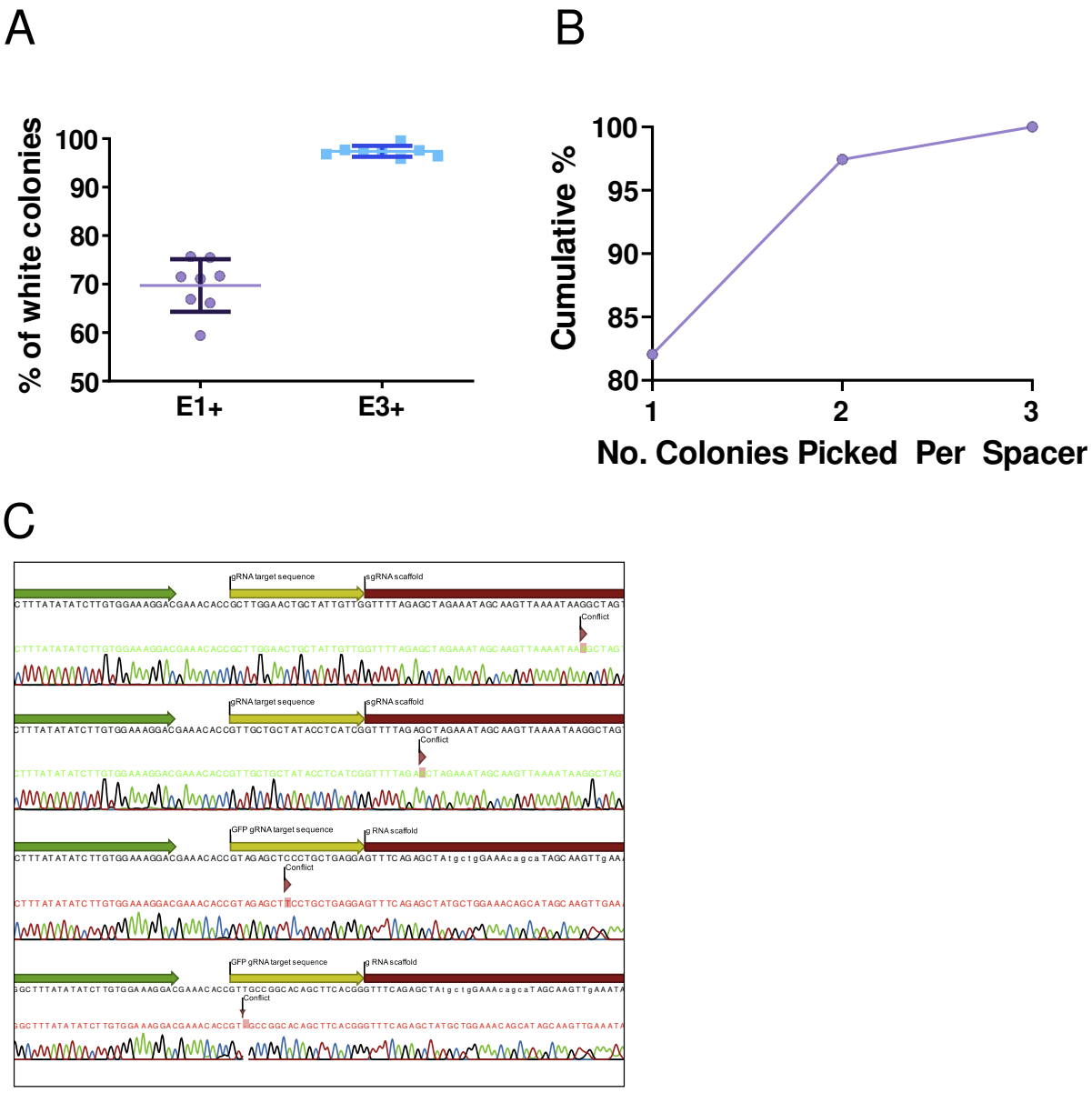


Figure 3. Cloning efficiency of different spacers into sgRNA within the AdZ-CRISPR Vector. (A) 16 different spacer sequences were recombineered into the AdZ-CRISPR-E3+ vector, then the number of white sucrose resistant colonies present after cloning counted, as a measure of cloning efficiency. (B) Clones from (A) were sequenced by Sanger sequencing. The number of colonies that had to be picked for each spacer sequence, to find one with the correct sequence, was calculated. (C) Examples of clones containing errors following insertion of spacers into the sgRNA.

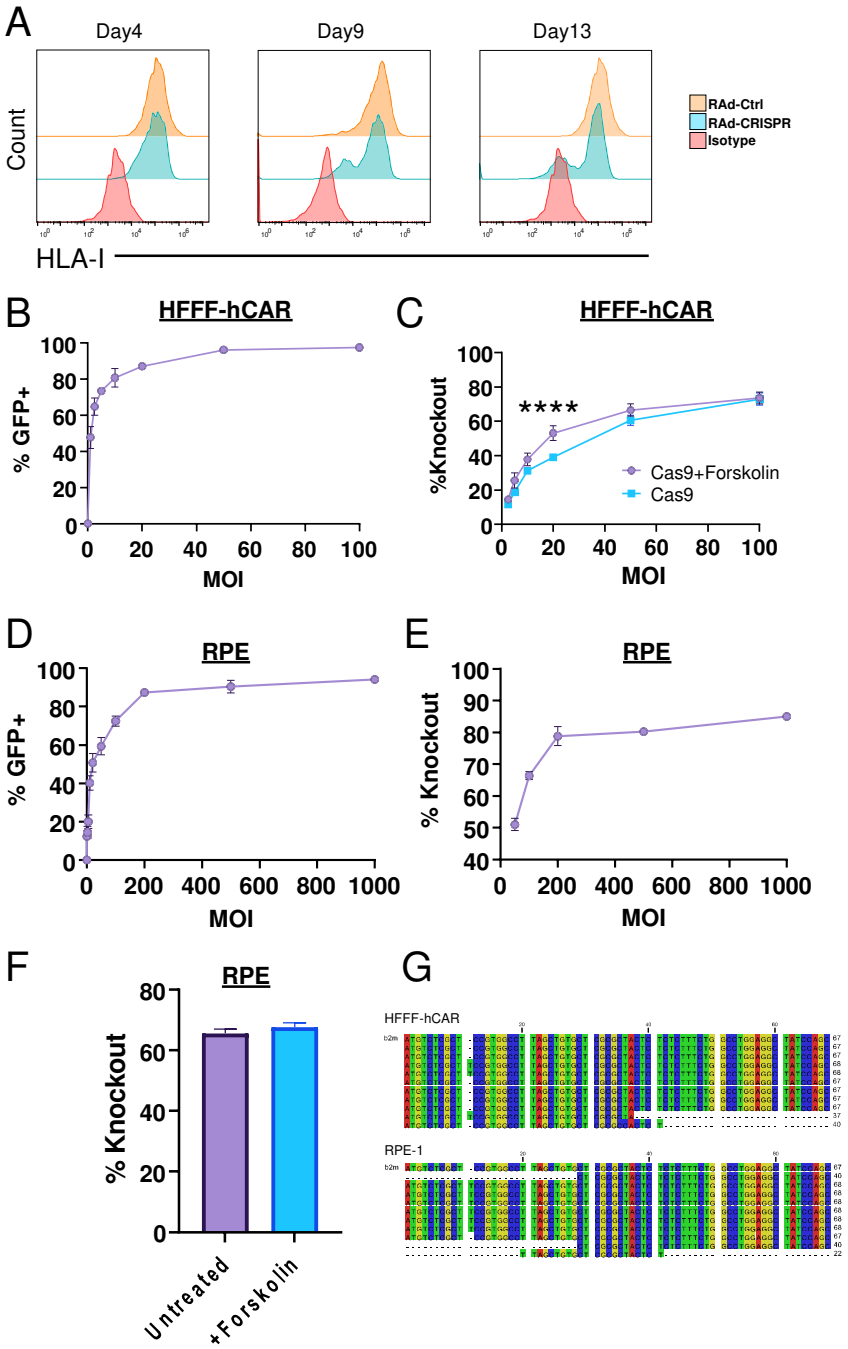


Figure 4. CRISPR/Cas9 editing efficiency. (A) HFFF-hCAR were transduced with AdZ-CRISPR-E3+ carrying a spacer targeting *B2M*, or a vector lacking a Cas9 coding sequence (RAd-Ctrl), at MOI=5. At the indicated times, cells were dissociated, stained with anti-HLA antibody, or with an isotype control antibody that does not bind, and analysed by flow cytometry. (B-F) HFFF-hCAR (B/C) or RPE-1 (D-F) were transduced with AdZ-CRISPR-E3+ carrying a spacer targeting *B2M* (C/E/F), or RAd-GFP (B/D) at the indicated MOI (B-E) or MOI=200 (F). In some cases, forskolin was added 24h after transduction. After 14 days, cells were dissociated, stained with anti-HLA antibody, and analysed by flow cytometry for loss of HLA staining (C/E/F). Alternatively, after 48h, cells were dissociated and analysed by flow cytometry for GFP expression (B/D). (G) HFFF-hCAR or RPE-1 transduced with AdZ-CRISPR vector at MOI=50 or 500 respectively, then DNA extracted 14 days later. The region surrounding the sgRNA target site was PCR amplified, TOPO cloned, individual clones sequenced, and aligned to the reference sequence (top line). Error bars show mean ±SD, n=3. 2-way ANOVA ****<0.0001

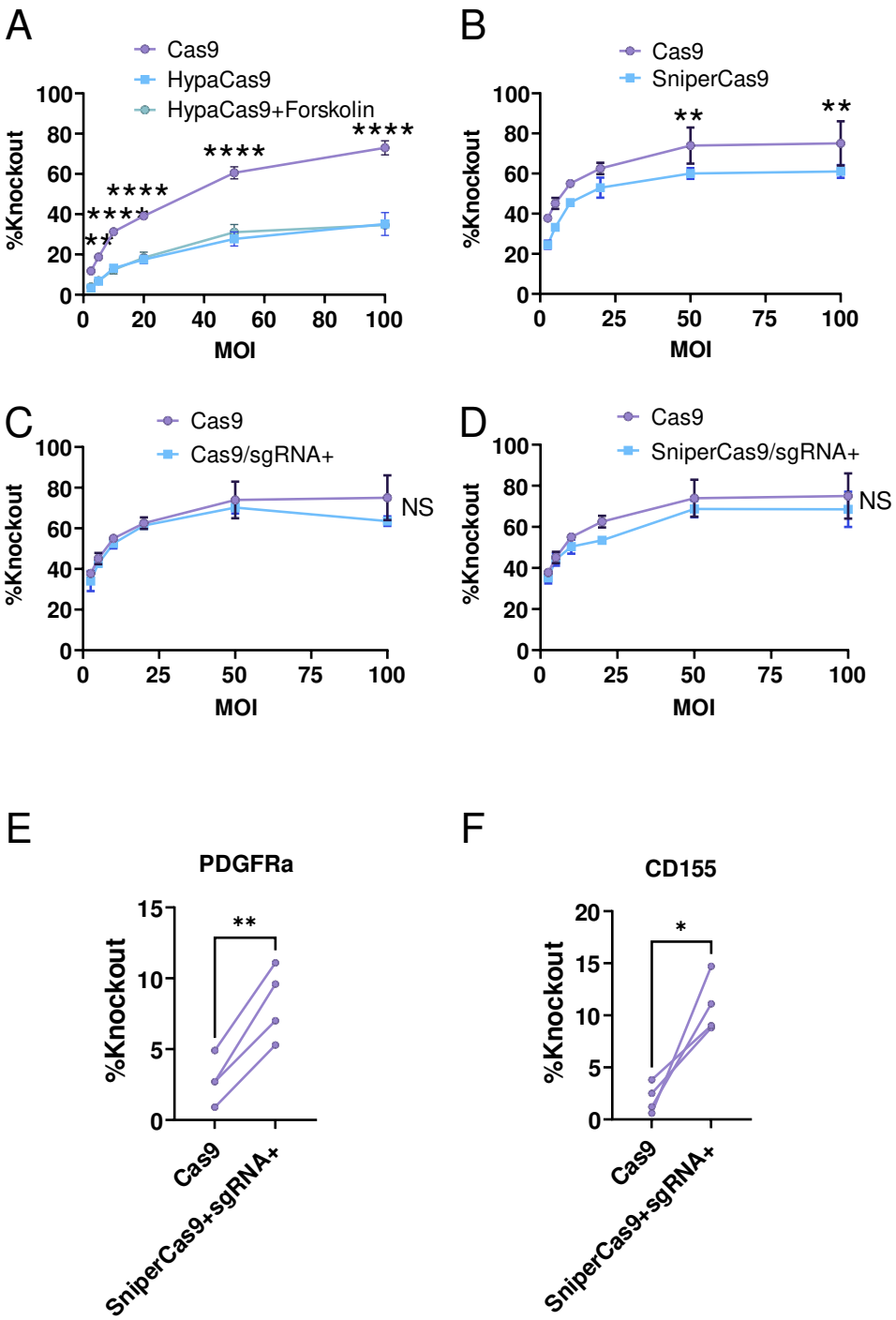


Figure 5. Optimising CRISPR/Cas9 expressing AdZ Vectors. (A-D) HFFF-hCAR were transduced at the indicated MOI with RAds carrying spCas9, HypaCas9, or SniperCas9 as indicated. In some vectors, and enhanced sgRNA sequence (sgRNA+) was used. In all cases, the sgRNA targeted *B2M*. After 14 days, cells were dissociated, stained with anti-HLA antibody, and analysed by flow cytometry for loss of HLA staining. (E-F) HFFF-CAR were transduced with RAds containing spCas9, or SniperCas9 and an enhanced sgRNA, using multiple different spacer sequences targeting *PDGFRA* (D) or *CD155* (E). After 14 days, cells were dissociated, stained with an appropriate antibody, and analysed by flow cytometry for loss of staining. All experiments were repeated at least 3 times. Error bars show mean ±SD, n=3. 2-way ANOVA with Tukey post-tests (A-D), paired t-test (E-F), *p<0.05, **p<0.01, ****p<0.0001

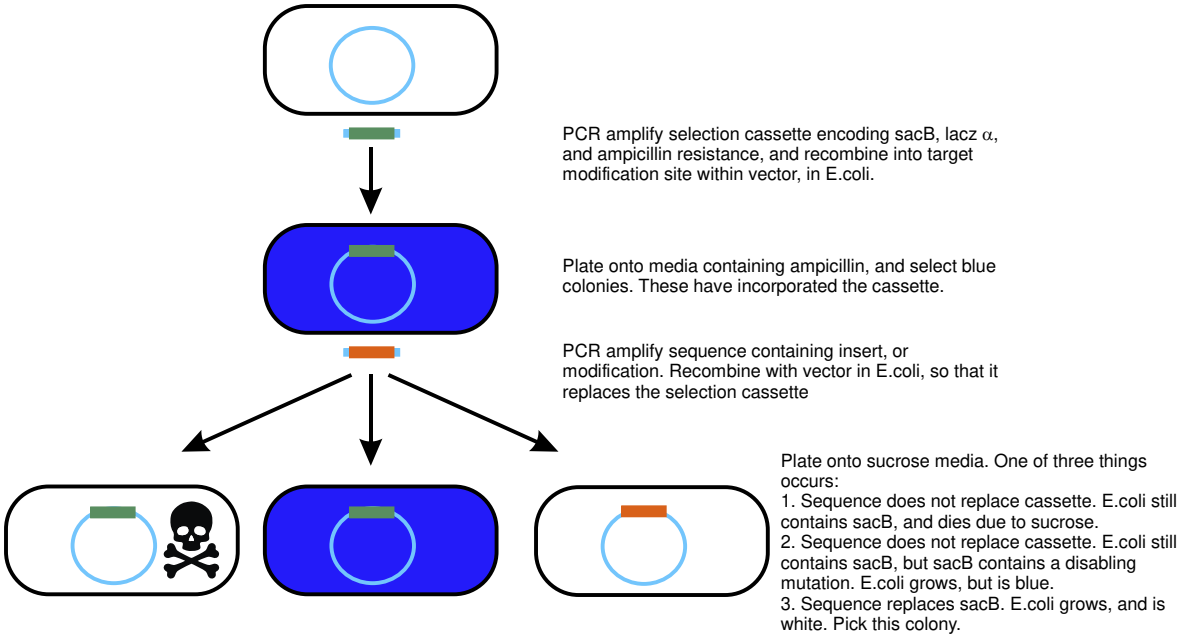
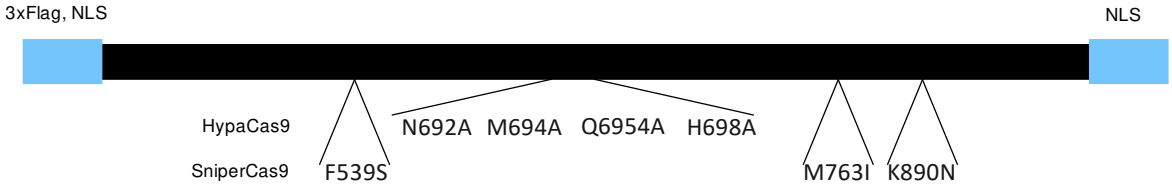


Figure S1. Schematic for making modifications to the AdZ BAC.

A



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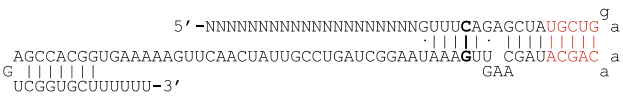

Figure S2. Schematic of the mutations introduced into Cas9 (A), and the sgRNA (B). Figure in (B) reproduced from[52].