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Methods for recruiting endogenous and exogenous ADAR enzymes for site-specific RNA editing

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ABSTRACT

Adenosine deaminases acting on RNA (ADARs) can be repurposed to achieve site-specific A-to-I RNA editing by recruiting them to a target of interest via an ADAR-recruiting guide RNA (adRNA). In this chapter, we present details towards experimental methods to enable this via two orthogonal strategies: one, via recruitment of endogenous ADARs (i.e. ADARs already natively expressed in cells); and two, via recruitment of exogenous ADARs (i.e. ADARs delivered into cells). Towards the former, we describe the use of circular adRNAs to recruit endogenous ADARs to a desired mRNA target. This results in robust, persistent and highly transcript specific editing both *in vitro* and *in vivo*. Towards the latter, we describe the use of a split-ADAR2 system, which allows for overexpression of ADAR2 variants that can be utilized to edit adenosines with high specificity, including at challenging to edit adenosines in non-preferred motifs such as those flanked by a 5′ guanosine. We anticipate the described methods should facilitate RNA editing applications across research and biotechnology settings.

1. Introduction

Single nucleotide polymorphisms (SNPs) account for the majority of human genetic variants known to cause disease [1]. In this regard, DNA editing tools such as those based on the CRISPR-Cas systems have emerged as powerful tools for the correction of these mutations. However, their use for *in vivo* gene therapy poses major challenges including the risk of introducing permanent off-target mutations in the genome and a potential for inducing immunogenic responses due to their prokaryotic origin [2–5]. On the contrary RNA is targetable by short antisense oligonucleotides, and by engineering these to enable recruitment of endogenous RNA modifying enzymes one could potentially circumvent both the highlighted challenges of DNA editing [6]. Towards this, here we describe the use of adenosine deaminases acting on RNA (ADARs), enzymes that catalyze the conversion of adenosine to inosine (A-to-I), for programmable transcriptome engineering [7–9]. Specifically, these enzymes can be repurposed for site-specific RNA editing by recruiting them to target transcripts via engineered ADAR-recruiting guide RNAs (adRNAs) [10]. Due to their structural similarities, inosines are read as guanosines by the translation and splicing machinery, and hence ADARs can serve as powerful tools for altering protein sequences and repairing premature stop codons.

In particular, we recently developed two orthogonal methodologies to enable ADAR mediated programmable RNA editing. In the first approach, we showed that recruitment of endogenous ADARs to target transcripts can be achieved by utilizing simple long antisense adRNAs [6,11]. As this approach requires only the delivery of adRNAs, without needing co-delivery of any exogenous proteins, it also has potential for therapeutic applications [12]. However, the lack of persistence of RNA editing via these long antisense adRNAs remained a challenge. To solve the problem, we went on to engineer circularized versions of these adRNAs (which we call circular adRNA or cadRNAs) [6,11]. We do this by flanking the long antisense adRNAs with twister ribozymes, that in turn undergo autocatalytic cleavage leaving termini that are recognized and ligated by the endogenous RNA ligase RtcB, thereby resulting in the formation of a circular adRNA [7]. Here, we describe our protocols for generation and use of cadRNAs to enable robust and persistent RNA editing as compared to their linear counterparts.

While the approach highlighted above allows for highly transcript-specific editing, variability in enzyme activity across various cell types and 5′ neighbor preferences of the endogenous ADARs (A ≈ U > C = G) can potentially limit its utility [13,14]. Hyperactive enzyme variants can

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help to circumvent this issue, but overexpression of these mutants typically leads to significant transcriptome wide off-target editing [6,15]. Towards this, in our second approach, we recently showed that splitting the ADAR2 deaminase domain into two inactive fragments that come together to form a functional enzyme at the target of interest, allows for the expression of hyperactive variants while maintaining high transcriptomic specificity. Specifically, we outline our protocols for using a split-ADAR2 system which relies on the λN-BoxB and MCP-MS2 protein-RNA interactions to bring the catalytically inactive fragments together [6,16,17].

2. Materials

2.1. Determination of target sequence

1. Access to NCBI RefSeq
2. Sequence editing and alignment software such as ApE, Benchling or Geneious Prime

2.2. Vector construction

1. PCR tubes
2. Thermocycler
3. Q5 Master Mix (NEB, M0492)
4. 80 % glycerol
5. Agarose gel for electrophoresis: 1.5 % (w/v) agarose
6. Agarose gel electrophoresis system
7. QIAquick PCR Purification Kit (Qiagen, 28104)
8. NanoDrop 2000 (ThermoFisher)
9. Nuclease-Free water
10. Gibson Assembly Master Mix (NEB, E3611L)
11. DNA ladder for electrophoresis
12. 6X Gel Loading Dye
13. AgeI-HF and NheI-HF restriction enzymes with CutSmart Buffer mix (NEB)
14. Guide RNA cloning vector for:
    i. cadRNA - Addgene #180184
    ii. Split-ADAR2 - Addgene #170136 OR Split-ADAR2(E488Q) - Addgene #170137
15. G-blocks or primers to create adRNAs:
    i. 100–200 bp antisense DNA with Gibson assembly overhangs for cloning into Addgene #180184
    ii. BoxB-MS2 adRNA with Gibson assembly overhangs for cloning into Addgene #170136 OR #170137

2.3. Plasmid synthesis

1. 1.5 mL microcentrifuge tubes
2. Stbl3 Competent Cells (ThermoFisher)
3. Water bath, 42 °C
4. S.O.C. medium (ThermoFisher)
5. Incubator shaker, 37 °C, 200–250 rpm
6. Sterilized bacteria spreader
7. LB broth: 1 % (w/v) Tryptone, 0.5 % (w/v) yeast extract, 0.5 % (w/v) sodium chloride
8. Kanamycin stock
9. 10 mL bacterial culture tubes
10. QIAprep Spin Miniprep Kit (Qiagen, 27104)
11. Ethanol
12. NanoDrop 2000 (ThermoFisher)
13. Sanger sequencing service
14. 1.5 mL microcentrifuge tubes
15. Tabletop centrifuge
16. Sequencing primers for confirming:
    a. adRNA sequence, U6 universal primer: 5′-GACTATCATATGCTTACCGT-3′
    b. Split-ADAR2 protein sequence, CMV-Forward universal primer: 5′-CGCAAATGGGCGGTAGGCGTG-3′ and TK_pA_seq_R primer: 5′-TCCTTCCGTGTTTCAGTTAGCC-3′

2.4. Tissue culture

1. HEK293FT cells (ThermoFisher)
2. 24 well tissue culture-treated plates
3. Cell culture incubator at 37 °C and 5 % CO2
4. Dulbecco's Modified Eagle Medium (DMEM), High Glucose (Invitrogen)
5. Fetal bovine serum (FBS) (Thermo Fisher)
6. 0.05 % Trypsin-EDTA (Gibco, 25300120)
7. 100X Antibiotic-Antimycotic (Gibco, 15240062)
8. Lipofectamine 20,000 (Invitrogen, 11668027)
9. Opti-MEM Medium (Invitrogen, 31985062)
10. 1.5 mL microcentrifuge tubes
11. PH 7.4 phosphate buffered saline (PBS) (ThermoFisher)

2.5. RNA extraction

1. RNeasy Mini Kit (Qiagen, 74104)
2. RNase-Free DNase Set (Qiagen, 79254)
3. Ethanol
4. Nuclease-free water
5. Tabletop centrifuge
6. RNaseZap (Invitrogen, AM9780)

2.6. Quantification of RNA editing

2.6.1. Quantification via Sanger sequencing:

1. Access to Sanger sequencing service
2. Q5 Master Mix (NEB, M0492)
3. Agarose gel for electrophoresis: 1.5 % (w/v) agarose
4. DNA ladder for electrophoresis
5. Agarose gel electrophoresis system
6. 6X Gel Loading Dye
7. PCR tubes
8. Thermocycler
9. NanoDrop 2000 (ThermoFisher).
10. Nuclease-Free water
11. ProtoScriptII First Strand cDNA Synthesis Kit (NEB, E6560L)
12. QIAquick PCR Purification Kit (Qiagen, 28104)
13. Ethanol
14. Forward and reverse sequencing primers binding between 50 and 300 bp from the target adenosine
15. HiScribe T7 Quick High Yield RNA Synthesis Kit (NEB, E2050S), *required only for cadRNA*
16. EDTA, *required only for cadRNA*

2.6.2. Quantification by RNAseq

1. AMPure XP Beads (Beckman Coulter, A63881)
2. Nuclease-free Water
3. PCR tubes
4. Magnetic rack for PCR tubes
5. Thermocycler
6. Nanodrop (ThermoFisher)
7. NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB, E7490S)
8. NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (NEB, E7420)
9. Access to Illumina NGS service
10. Access to github C library htslib (github.com/samtools/htslib)
11. Access to R functions for Fisher's exact test

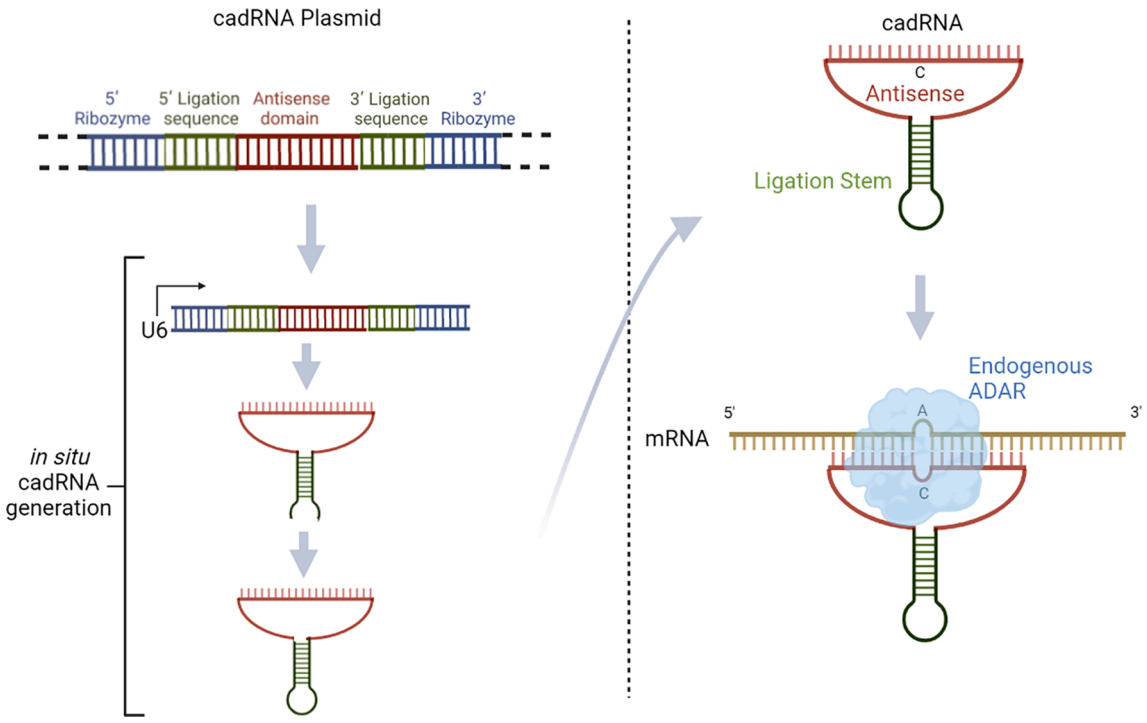

Fig. 1. Schematics of plasmid-delivered *in situ* cadRNA generation and structure of cadRNA to recruit endogenous ADARs.

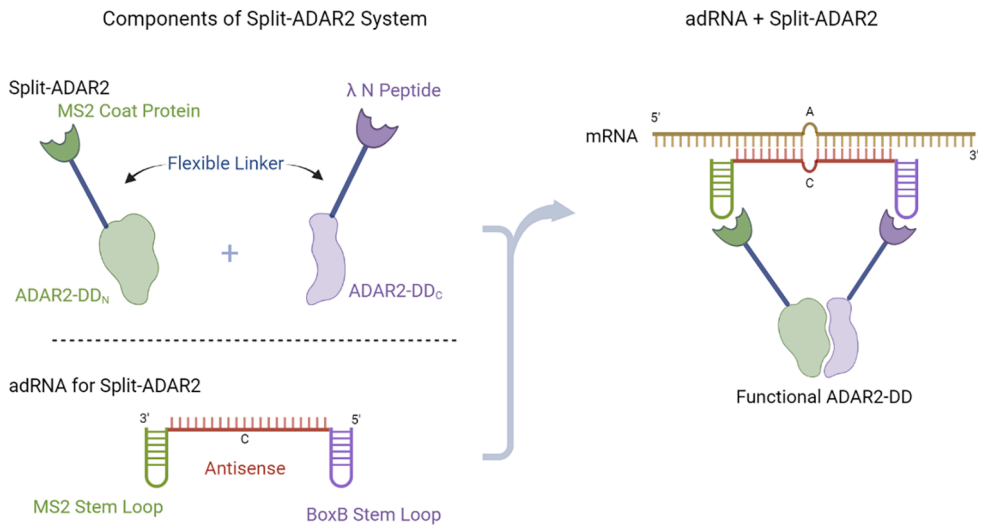

Fig. 2. Schematics of the Split-ADAR2 system and structure of adRNA to recruit exogenous Split-ADAR2.

12. Access to STAR version 2.7.3a

3. Methods

Here, we present a general protocol for site-specific RNA editing by recruiting 1) endogenous ADAR1/2 via cadRNA (Fig. 1) and 2) exogenous split-ADAR2 via BoxB-MS2 adRNA (Fig. 2). Our plasmids are available via Addgene and carry a human U6 promoter for expression of cadRNAs and BoxB-MS2 adRNAs. In the cadRNA vectors, a CMV promoter drives expression of GFP while in the split-ADAR2 vectors a CMV promoter drives expression of two ADAR2 fragments linked by a "self-cleaving" peptide. The steps involved in setting up a RNA editing experiment utilizing ADARs are as follows.

3.1. Determination of target sequence

1. Acquire transcript sequence in the region of interest from NCBI RefSeq.
2. Using sequence editing software, identify your intended genomic target as 5′-NAN-3′.
3. Decide on which approach to utilize: 1) endogenous ADAR1/2 via cadRNA or 2) exogenous split-ADAR2 via BoxB-MS2 adRNA:

Conditions	Suggested Systems
Target cells have robust activity of ADARs Target site is 5'-TAN-3', 5'-CAN-3', 5'-AAN-3'	Endogenous ADAR1/2 via cadRNA
Target cells have low activity of ADARs Target gene show suppression by long antisense RNA Target site is 5'-GAN-3'	Exogenous split-ADAR2 via BoxB-MS2 adRNA

3.2. Vector construction

3.2.1. Plasmid synthesis from bacterial stab obtained from Addgene

1. Obtain the desired plasmid from Addgene:
a. cadRNA - Addgene #180184.
b. split-ADAR2 - Addgene #170136 OR split-ADAR2(E488Q) - Addgene #170137.
2. Prepare 10 cm LB agar plates (50 μg/mL kanamycin) and LB medium (50 μg/mL kanamycin).
3. Streak out bacterial stab obtained from Addgene onto the LB agar plate and incubate at 37 °C for 12–16 h, face down. Store at 4 °C when bacteria colonies grow to desired size.
4. Pick 3 colonies. Culture each colony in 5 mL LB medium at 37 °C with shaking at 225 rpm for 12–16 h. Prepare glycerol stock in a 1.5 mL tube for each culture with a 4:1 (v/v) medium:glycerol ratio, and store it at −80 °C. Use the QIAprep Spin Miniprep Kit to extract plasmid DNA from each culture as per the manufacturer's protocol.
5. Obtain the concentration of each plasmid using the NanoDrop and prepare samples for Sanger sequencing with:
   a. U6 universal primer for cadRNA.
   b. U6 universal primer, CMV-Forward and TK_pA_seq_R primers for split-ADAR2
6. Using sequence alignment software, confirm plasmid sequencing matches Addgene's NGS result.
7. If needed, repeat steps 4–6.

3.2.2. Cloning of the desired adRNA

1. Dilute 2 μg of correct plasmid DNA (cadRNA or split-ADAR2) to a total volume of 43 μL with nuclease free water in a PCR tube. Thaw the CutSmart buffer and keep the restriction enzymes AgeI-HF and NheI-HF on ice. Add 5 μL of CutSmart buffer with the plasmid followed by 1 μL of each restriction enzyme. Mix by pipetting and incubate at 37 °C for 3 h.
2. Set up a 1.5 % Agarose gel for electrophoresis, load the DNA ladder, 6 μL digested plasmid mix contain 1X DNA loading dye and 2–4 μL digestion mix, 6 μL undigested plasmid mix containing 100 ng plasmid and 1X DNA loading dye, side by side. Complete digestion will result in one clean, straight band that is slightly above the crest-shaped undigested band.
3. Purify the digestion mix using QIAquick PCR Purification Kit. This should yield a digested plasmid concentration of 20–30 ng/μL when eluted in a 50 μL volume.
4. Design gBlocks and/or primers to clone the adRNA plasmid DNA construct as follows:
   a. For cadRNA plasmid DNA construct:
      i. It is recommended to include +/−100 bp around the targeted adenosine as the region of antisense binding. Design the plasmid DNA for guide RNA antisense construct as the follows by changing the T complementary to the target A to a C: e.g. to target a 5′-TAG-3′ on the RAB7A transcript:

Target:	5′-N-TTATGT**A**GGCCT-N-3′
cadRNA antisense:	3′-N-AATACA**C**CCGGA-N-5′

      i. Note, long antisense domains of 200 bp in length result in significant bystander editing of flanking adenosines in the dsRNA stretch formed between the guide and the target. To minimize this, see Section 4.2 and Fig. 6 [11].

      ii. Order gblock + primers to create the following in the order mentioned:

1. 5' overhang and linker + antisense region from 4.a.i or 4.a.ii + linker + 3' overhang
2. A sample insert sequence targeting RAB7A for cloning:
   5′-
   GCCGACTGATGGCAGAAAAAAAAAAGACAGTTGTCCCCCTGGA
   GAGATGAAAAGCTTGTTGGCTCTTAAGTCTTTGATAAAAGGCG
   TACATAATTCTTGTGTCTACTGTACAGAATACTGCCGC**C**AGCT
   GGATTTCCCAATTCTGAGTAACACTCTGCAATCCAAACAGGGT
   TCAACCCTCCACCTTACAGGCCTGCATTACAGGACTTAAACAC
   ATAATCCAAAAAAAAAAA**CTGCCATCAGTCGGCGT**-3′
3. Sample Forward Primer sequence: 5′-
   GCCGACTGATGGCAGAAAAAAAAAAGACAGTTGTCCCCCTGGA
   GA-3′
   Sample gblock sequence:
   5′-
   GACAGTTGTCCCCCTGGAGAGATGAAAAGCTTGTTGGCTCTTA
   AGTCTTTGATAAAAGGCGTACATAATTCTTGTGTCTACTGTAC
   AGAATACTGCCGC**C**AGCTGGATTTCCCAATTCTGAGTAACACT
   CTGCAATCCAAACAGGGTTCAACCCTCCACCTTACAGGCCTGC
   ATTACAGGACTTAAACACATAATCCAA-3′
   Sample Reverse Primer sequence:
   5′-
   ACGCCGACTGATGGCAGTTTTTTTTTTTTGGATTATGTGTTTA
   AGTCCT-3′

*In case of cadRNAs antisense insert construction, due to its complementary overhangs and poly A linkers, we suggest ordering primers + gblock combinations to achieve insert construction via PCR amplification.

      iii. Dilute the gblock to 10 ng/μL and primers to 10 pmol/μL. Prepare a PCR reaction mix in a PCR tube using the following formula:

Substance	Quantity
gblock	1 μL
Forward Primer	1 μL
Reverse Primer	1 μL
Q5 Master Mix	10 μL
Nuclease-free water	7 μL
Total Volume	20 μL

      iv. Run PCR reaction in thermocycler using the following program:

Temperature	Time	Cycles
95 °C	180 s	1
95 °C	20 s	20
60–72 °C	15 s	
72 °C	30 s/kb	
72 °C	180 s	1
4 °C	∞	

      v. Set up a 1.5 % Agarose gel for electrophoresis, load the DNA ladder, 6 μL PCR reaction mix containing 1X DNA loading dye, 3 μL water and 2 μL PCR product. Check the PCR product size (~260 bp for cadRNA and ~ 120 bp for BoxB-MS2 adRNA including overhangs).

vi. Purify the remaining PCR product using QIAquick PCR Purification Kit. This should yield a DNA concentration of 20–30 ng/μL when eluted in 50 μL EB Buffer.
   a. For split-ADAR2 plasmid DNA construct:
i. It is recommended to include +/−15 bp around the targeted adenosine as the region of antisense binding. Design the plasmid DNA for the guide RNA antisense construct as the follows by changing the T complementary to the target A to a C: e.g. to target a 5′-TAG-3′ on the RAB7A transcript:

Target:	5′-N-TTATGTAGGCCT-N-3′
adRNA antisense:	3′-N-AATACACCCGGA-N-5′

ii. Order a gblock to create the following in the order mentioned:

1. 5' overhang + BoxB stem loop + 30 bp antisense DNA sequence from 4.b.i + MS2 loop stem + 3' overhang
2. A sample adRNA guide sequence targeting RAB7A:

5′-
CTTGTGGAAAGGACGAAACACCACGGCCCTGAAAAAGGGCCAC
AGAATACTGCCGCCAGCTGGATTTCCCAAACATGAGGATCACC
CATGTCTTTTTCGTACTGAGTCGCCCAG-3′

5. Dilute the gblock or purified DNA to 10 ng/μL using nuclease free water, this is our insert. Thaw Gibson Assembly Master Mix on ice. Add 1 μL of insert and 40 ng of digested cloning vector (cadRNA or split-ADAR2 as prepared in step 3) in the PCR tube, dilute the volume to 5 μL. Add 5 μL Gibson Assembly Master Mix to PCR tube and run the thermocycler with the following program:

Temperature	Time
50 °C	60 mins
4 °C	∞

6. Thaw Stbl3 competent cells on ice (50 μL aliquots). Add 5 μL of the Gibson product to Stbl3 cells, incubate on ice for 15–30 min. Heat shock the mix in a 42 °C water bath for 45 s, then immediately return to ice for 2 min. Add 250 μL of S.O.C. medium (room temperature) and incubate in a 37 °C shaker for 1 h.
7. Perform plasmid selection following step 4–7 in Subheading 3.2.1.

3.3. Plasmid synthesis from glycerol stocks

1. Thaw the correct bacterial plasmid glycerol stocks.
2. Prepare LB medium (50 μg/mL kanamycin), aliquot 5 mL for each stock in a 10 mL bacterial culture tube.
3. Add 5 μL glycerol stock to the tube. Incubate in a shaker at 37 °C for 12–16 h.
4. Use QIAprep Spin Miniprep Kit and follow its protocol to extract plasmid DNA as per the manufacturer's protocol. Elute in 1.5 mL microcentrifuge tubes. This should yield no less than 100 ng/μL in a 50 μL elution.
5. Confirm the plasmid sequence by Sanger sequencing.

3.4. Tissue culture

3.4.1. Day 0: Plating HEK293FT cells

1. Add 1 mL 0.05 % Trypsin (room temperature) to one well of a confluent 6-well tissue culture dish. Incubate in a cell culture incubator for 3 min.

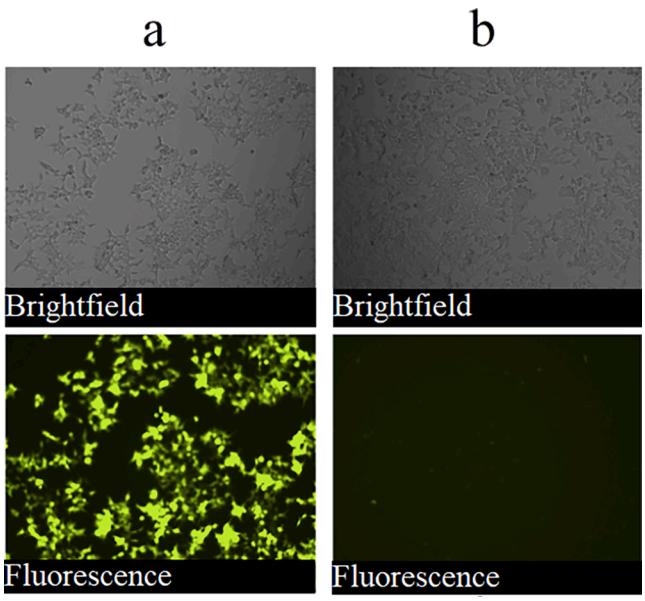

Fig. 3. (a) Example of a successful transfection. (b) Example of an unsuccessful transfection.

2. Neutralize the Trypsin with 1 mL of complete DMEM media (10 % FBS, 1X Antibiotic-Antimycotic) at room temperature. Gently pipette up and down to evenly suspend all cells in the media.
3. In a 15 mL tube, add 12 mL of complete DMEM media and all 2 mL of neutralized cells to the tube. Mix gently by inverting the tube several times.
4. Seed a 24 well tissue culture plate by adding 500 μL of the resuspended cells to each well.

3.4.2. Day 1: Transfection of HEK293FT cells

1. Confirm that the cells are at 25–30% confluency and look healthy.
2. Re-measure the concentration of the cadRNA or split-ADAR2 plasmids prepared in Subheading 3.3 before transfections.
3. For our cell confluency, 1.5 μL per well Lipofectamine 2000 provides good cell survival and transfection efficiency. For N wells, prepare a master mix containing 1.5*(N + 1) μL Lipofectamine 2000 and 25*(N + 1) μL Opti-MEM in a 1.5 mL microcentrifuge tube, invert tube several times. Incubate at room temperature for 5 min.
4. Prepare the diluted DNA mix containing 1 μg plasmid DNA and 25 μL Opti-MEM per well in 1.5 mL microcentrifuge tubes. It is recommended to have 3 replicates for each construct, prepared as independent mixes.
5. Add 25 μL Lipofectamine mastermix to every 25 μL DNA plasmid master mix. Mix well by pipetting. Incubate for 15 min at room temperature.
6. Add 50 μL mixture dropwise to each well of cells. Very gently, shake the plates a couple of times.

3.4.3. Day 2: Harvesting HEK293FT cells

1. Check transfection efficiency 48 h post transfections. cadRNA plasmids also express GFP. For split-ADAR2 plasmids, include a well for a transfection control such as a GFP containing plasmid (Fig. 3).
2. Harvest cells 48 h post transfection. Use a PBS wash if cell death is prevalent. Collect cells in each well in a 1.5 mL microcentrifuge tube and spin at 400 rcf for 5 min. Aspirate media. Freeze cell pellets at −80 °C if not immediately proceeding to Section 3.5.

3.5. RNA extraction

1. Spray RNase Zap on related bench surfaces, gloves and tube racks prior to extraction to eliminate RNases prior to RNA extraction.
2. Proceed to RNA extraction using RNeasy Mini Kit as per the manufacturer's instructions, following all the optional steps including DNA clean up using the RNase-Free DNase Set.
3. Keep the extracted RNA on ice. Obtain RNA concentration on the Nanodrop. Expect a concentration between 100 and 300 ng/μL in a 50 μL elution volume.
4. Store extracted RNA at −80 °C.

3.6. Quantification of RNA editing

3.6.1. Capture of cadRNAs

1. This step applies only to cadRNA samples. For the split-ADAR2 approach, proceed to Section 3.6.2. For cadRNAs, the long antisense RNA can hinder cDNA synthesis from the bound mRNA, thus it is essential to separate the bound guide RNA from the target mRNA using a sense IVT oligo RNA [1].
2. Design primers to amplify the target region from cDNA prepared from an untransfected control sample. A T7 promoter sequence `5′-TAATACGACTCACTATAGGG-3′` is added to the 5′ end of the amplification primer. The length of the sense strand is kept at 200 bp (such that it binds the entire antisense on cadRNAs).
3. To obtain a sense PCR product, set up a reaction as below:

Substance	Quantity
cDNA*	2 μL
Forward Primer	1 μL
Reverse Primer	1 μL
Q5 Master Mix	10 μL
Nuclease-free water	6 μL
Total Volume	20 μL

* We used 2 μL of the WT HEK293FT cDNA, synthesized using the methods in Subheading 3.6.1, cDNA is diluted 1:4 in water.

The thermocycler settings are:

Temperature	Time	Cycles
95 °C	180 s	1
95 °C	20 s	20
60–72 °C	15 s	
72 °C	30 s/kb	
72 °C	180 s	1
4 °C	∞	

4. Purify the PCR product using the QIAquick PCR Purification Kit. Send it out for Sanger sequencing, using the T7 Primer: `5′-TAATACGACTCACTATAGGG-3′`. Verify the DNA oligo sequence.
5. Set up an *in vitro* transcription reaction using the HiScribe T7 Quick High Yield RNA Synthesis Kit, as per the manufacturer's instructions. For a 300 bp oligo, use the following program:

Temperature	Time
37 °C	180 mins
4 °C	∞

6. In a 1.5 mL microcentrifuge tube, perform the DNA clean up step using the supplied DNase as per the manufacturer's protocol and proceed to LiCl purification of the oligo RNA.
7. Prepare 0.1 mM EDTA buffer from 0.5 M EDTA stock, resuspend IVT sense oligo RNA in 50 μL 0.1 mM EDTA. Nanodrop for concentration and adjust concentration to ~ 500 ng/μL. Store in −80 °C.
8. Prior to cDNA synthesis (20 μL reaction volume) for cadRNA editing analysis, in PCR tubes, add the following:

a. 1 μL IVT sense oligo RNA
b. Up to 1 μg RNA
c. Water to a total volume of 6 μL

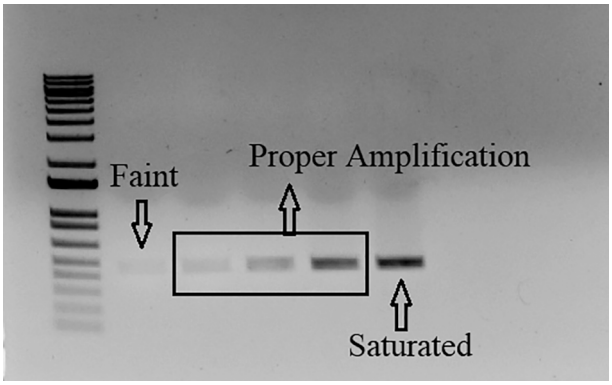

Fig. 4. Band intensity guideline for PCR optimization using our protocol.

and boil in a thermocycler using the following program:

Temperature	Time
95 °C	180 s
4 °C	∞

9. Return to ice immediately. Proceed to Section 3.6.2.

3.6.2. Quantification via Sanger sequencing:

1. In a PCR tube, synthesize cDNA using the ProtoScriptII First Strand cDNA Synthesis Kit following the manufacturer's protocol. For cadRNA samples, add the remaining components to the tubes from Section 3.6.1 and proceed as per the manufacturer's instructions.
2. In PCR tubes, set up a PCR reaction as follows:

a. 3 μL of diluted cDNA (1:4)
b. 0.6 μL each of 1:10 diluted sequencing primers, Forward and Reverse.
c. 5.8 μL nuclease free water
d. 10 μL Q5 master mix

Thermocycler program:

Temperature	Time	Cycles
95 °C	180 s	1
95 °C	20 s	15–34
60–72 °C	15 s	
72 °C	30 s/kb	
72 °C	180 s	1
4 °C	∞	

3. Typically, a PCR protocol optimization is recommended for every target. Set up reactions at different annealing temperatures and cycle numbers to optimize the PCR reaction when testing with new primers or targets. Make sure that the primers to amplify the target are designed outside of the antisense binding region.
4. Set up a 1.5 % Agarose gel for electrophoresis, load the DNA ladder, 6 μL diluted PCR mix contain (1X DNA loading dye, 3 μL water, 2 μL PCR mix). Check the band saturation. Saturation is reached when the band intensity stops increasing with higher cycle numbers for the same reaction. The band should be clearly visible and not saturated (Fig. 4). Depending on the prevalence of primer dimer, adjustment to the thermocycler program should be made and alternate PCR purification methods such as magnetic beads purification or gel extraction should be considered. Generally, an unclear band results in bad sequencing quality.
5. Purify the digestion mix using QIAquick PCR Purification Kit. Elute in 30 μL EB.
6. Nanodrop for concentration and prepare for Sanger sequencing
7. Sequence samples with primers binding between 50 and 300 bp from the target to maintain good sequencing quality. Make sure that the primers are outside the antisense binding region.

8. Typically the Sanger sequencing facility should provide ab1 trace files for sequencing results. Tools such as ab1 Peak Reporter, 4PEaks can automate the peak measurements. Else, one can always open the ab1 trace files using ApE and measure the peaks using a ruler tool, or an actual ruler. Quantify the percentage editing at the target by computing the ratio of peak heights G*100/(A + G). For example, in Fig. 5a below, the editing efficiency is 5/(5 + 25) = 16.7%. If trace quality is bad for all samples with one particular set of primers, we recommend designing new primers binding to a different region of the transcript. Oftentimes a binding site further away from the target provides cleaner trace. Typically, we design primer sets binding at around +/−250 bp from the target.

clip3pAdapterSeq: AGATCGGAAGAGCACACGTCTGAACTCCAGTCA
AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT

3.6.3. Quantification by RNAseq

1. Thaw RNA extracted in Subheading 3.7 on ice. Aliquot 250 ng of total RNA, use the NEBNext Poly(A) mRNA Magnetic Isolation Module (E7490S) to remove rRNA and obtain mRNA following the protocol for mRNA isolation in the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina kit.
2. Use NEBNext Ultra II Directional RNA Library Prep Kit for Illumina to prepare samples for RNAseq. Use the Illumina Novaseq 6000 System at 100 bp paired-end run to obtain 40–45 million reads per sample.
3. Map sequencing reads to the reference human genome hg38 using STAR aligner version 2.7.3a with the following command line options:

a.
b. quantMode GeneCounts
c. alignSJDBoverhangMin 1
d. overlap bassMan = 10
e. peOverlapMMp = 0.05
f. outSAMmultNmax 1
g. alignEndsType EndToEnd
h. outFilterMismatchNmax −1
i. outFilterMismatchNoverReadLmax 0.2
j. outFilterMultimapNmax 1

4. Download the BAM file and use the C library htslib (github.com/samtools/htslib) to generate a list of base counts. Modify the list to follow the following guidelines:

a. Consider sites where at least one sample had a non-zero count of G (C) at reference A-sites (T-sites)
b. Ignore reference sites covered by less than ten reads
c. Ignore base deletions and insertions relative to the reference
d. Ignore reads with Phred quality score less than 13

5. Using Fisher's exact test in R to generate a comparison of base proportion between control samples and test samples.
6. For detailed methods refer to [7,11].

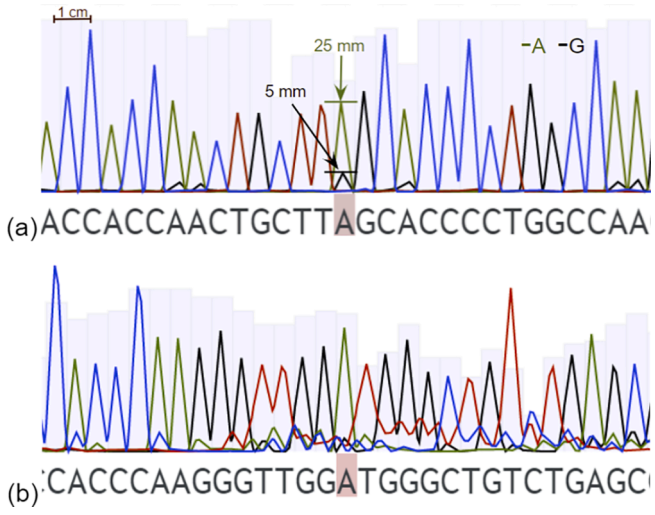

Fig. 5. (a) Example of a good quality trace with visible on-target editing (16.7%) and hyper editing on GAPDH gene, opened with Benchling. Acceptable for quantification. (b) Bad quality trace with background-level on-target editing on ALDOA gene, opened with Benchling. Not suitable for quantification. If high background is only prevalent around the targeted area, we recommend troubleshooting the PCR amplification. Occasionally, alternative PCR clean up protocols or sequencing protocols can improve the trace quality.

4. Notes

4.1. Target selection

1. Pre-existing background editing: Before experimenting on a new target, amplify the targeted region as you would in Subheading 3.6.2 using cDNA prepared from RNA extracted from untransfected cells. This is to ensure the target has no pre-existing background editing and the amplification of your interested region has no pre-existing complications. It is also good practice to look through SNP databases before selecting an editing site. Running negative controls along with the experimental samples for each transfection is always recommended.
2. Low gene expression: Our recommended PCR amplification cycle number is less than 33 cycles. Note that low endogenous gene expression could result in large cycle numbers and may lead to inaccurate quantification results due to amplification bias [18].
3. For efficient transcription from a U6 promoter, ensure that the antisense sequence does not contain a stretch of more than 4 Ts. If such a stretch is present, mutate one of the Ts.

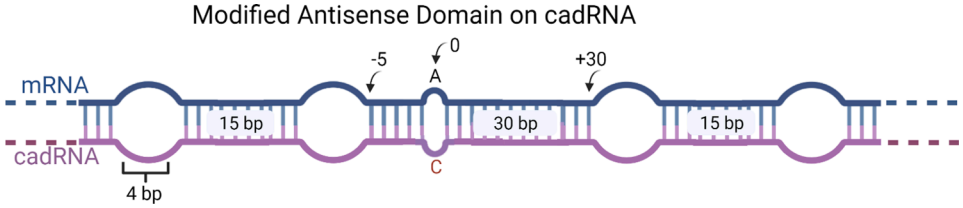

Fig. 6. Schematic of the modified cadRNA binding to mRNA.

4.2. Alternative adRNA construction methods

1. We have presented easy to implement methods to construct cadRNAs and BoxB-MS2 adRNAs. If preferred, one can also design multiple primers and perform primer annealing to construct the DNA inserts for Section 3.2.
2. Simple cadRNAs bearing 200 bp long antisense domains will result in significant bystander A-to-I editing within the dsRNA stretch formed between the antisense RNA and target mRNA. To minimize this, design the antisense domain such that it contains a stretch of 4 bp mismatches every 15 bp starting at locations −5 and + 30 relative to the target [11]. Example antisense sequences targeting the RAB7A transcript are given below:

   Original cadRNA antisense:

```
5′-
GACAGTTGTCCCCCTGGAGAGATGAAAAGCTTGTTGGCTCTTAAGTCTTTGATAAAAGG
CGTACATAATTCTTGTGTCTACTGTACAGAATACTGCCGCCAGCTGGATTTCCCAATTC
TGAGTAACACTCTGCAATCCAAACAGGGTTCAACCCTCCACCTTACAGGCCTGCATTAC
AGGACTTAAACACATAATCCAA-3′
```

   Modified cadRNA antisense:

```
5′-
GACAGTTGTCCCCCTGGAGAGATGAAATCGATGTTGGCTCTTAAGTGAAAGATAAAAGG
CGTACACTTGTCTTGTGTCTACTGTACAGAATACTGCCGCCAGCTGCTAATCCCAATTC
TGAGTATGTGTCTGCAATCCAAACACCCATCAACCCTCCACCTTTGTCGCCTGCATTAC
AGGACTTAAACACATAATCCAA-3′
```

4.3. Circularization validation for cadRNA

1. With the cDNA obtained in subheading 3.6.2, perform qPCRs using a set of primers binding outwards on the antisense. Only successfully circularized guide RNAs will result in non-background level amplification.

4.4. Quantification of target transcripts

1. Using antisense guide RNA binding to specific regions of the mRNA can occasionally lead to knock down of certain gene expression [11]. A qPCR is recommended to check if there is any knockdown of gene expression.

4.5. Improving editing efficiency and specificity

1. RNA editing efficiencies can vary based on the target. While we recommend using a 200 bp antisense RNA sequence with the C-mismatch centrally located at position 100 as a starting point, further optimization by moving the mismatch position around might help improve the editing efficiency.
2. Secondary structure prediction softwares should be used to ensure that the antisense is free to bind its target. Additional elements such as flexible unstructured linkers might allow for this.
3. Inclusion of 8 bp loops at positions −5 and + 30, and periodically along the length of the antisense (as outlined in Fig. 6) can improve on target editing efficiency as well as reduce bystander editing.
4. While the split-ADAR2 system can be utilized to edit adenosines in non-preferred motifs, introduction of additional ADAR mutations might help improve the editing efficiency [16]. For instance, a hyperactive ADAR2 mutant with two mutations (E488Q, N496F) can improve editing efficiencies at 5′-GAN-3′ and 5′NAC-3′ motifs.

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Authors contribution

All authors contributed to writing - original draft.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: P. M. is a scientific co-founder of Shape Therapeutics, Navega Therapeutics, Boundless Biosciences, and Engine Biosciences. The terms of these arrangements have been reviewed and approved by the University of California, San Diego in accordance with its conflict of interest policies. D.K. is an employee of Shape Therapeutics. The remaining authors declare no competing interests.

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