Protocol
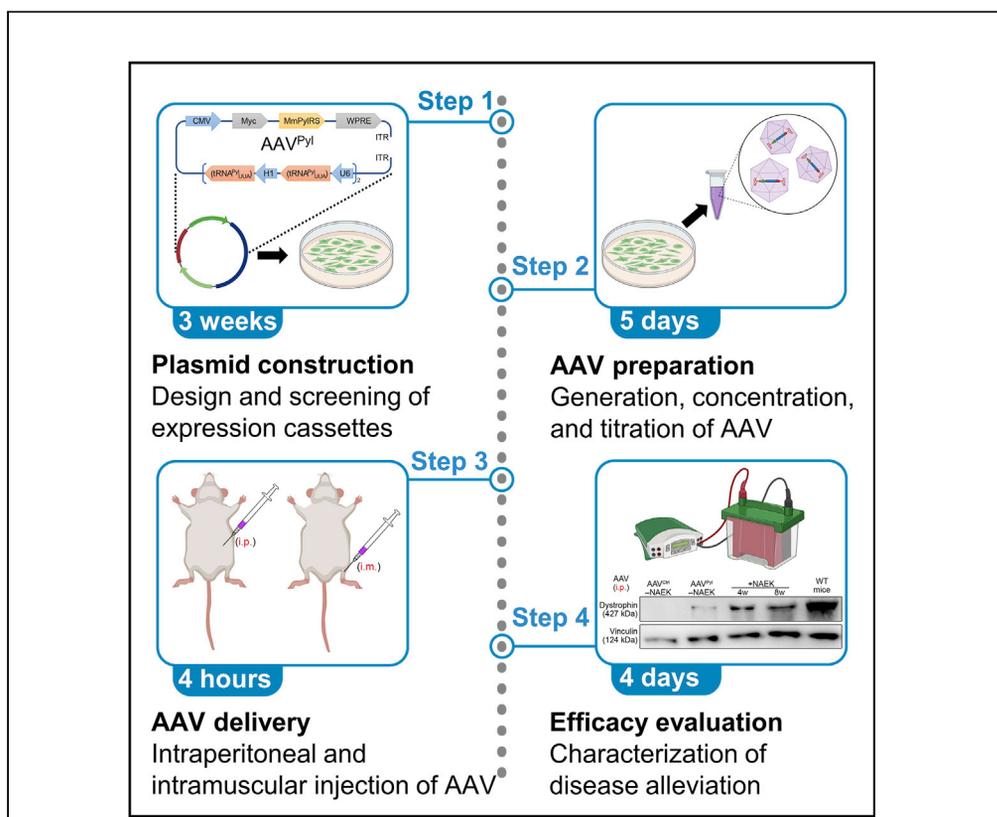
Adeno-associated viral delivery of engineered tRNA-enzyme pairs into nonsense mutation mouse models

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Highlights

Screening optimal tRNA-enzyme pairs for site-specific UAA incorporation

Generate AAV vectors carrying transgenes for expressing engineered tRNA-enzyme pairs *in vivo*

Use UAA incorporation system in *mdx* mice to read through premature termination codons

Evaluate dystrophin restoration and DMD disease alleviation after AAV treatment

In this protocol, we describe how to utilize the unnatural amino acid (UAA) incorporation system to read through endogenous premature termination codons in a Duchenne muscular dystrophy mouse model. We detail how to screen and optimize tRNA-enzyme pairs for efficient UAA incorporation, deliver the system intraperitoneally or intramuscularly in pathogenic mice by an adeno-associated viral (AAV) vector, and evaluate the restoration of endogenous dystrophin and increase in muscle strength after AAV injection.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Protocol

Adeno-associated viral delivery of engineered tRNA-enzyme pairs into nonsense mutation mouse models

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SUMMARY

In this protocol, we describe how to utilize the unnatural amino acid (UAA) incorporation system to read through endogenous premature termination codons in a Duchenne muscular dystrophy mouse model. We detail how to screen and optimize tRNA-enzyme pairs for efficient UAA incorporation, deliver the system intraperitoneally or intramuscularly in pathogenic mice by an adeno-associated viral (AAV) vector, and evaluate the restoration of endogenous dystrophin and increase in muscle strength after AAV injection.
For complete details on the use and execution of this protocol, please refer to Shi et al. (2021).[1]

BEFORE YOU BEGIN

In the past two decades, genetic code expansion has evolved from a fantastic idea to a powerful research tool, enabling not only biosynthesis of proteins with novel or enhanced functions,[2] but also introduction of functionally diverse unnatural amino acids (UAAs) into all types of organisms, from bacteria to mice.[3] To incorporate UAAs into target proteins, several components are needed: a reassigned nonsense codon (UAG, UGA or UAA) in mRNA, an engineered aminoacyl-tRNA-synthase (aaRS)–tRNA pair, and relevant UAAs. Currently, four orthogonal aaRS–tRNA pairs, referred as to the MmPylRS/tRNA^{MmPyl}, MbPylRS/tRNA^{MbPyl}, EcTyrRS/tRNA^{EcTyr} and EcLeuRS/tRNA^{EcLeu} systems,[4] have been developed to specifically recognize a nonsense codon and incorporate a desired UAA in mammalian cells, respectively or simultaneously.[5]

Approximately 11% of monogenic diseases are caused by premature termination codons (PTCs) in human genes,[6] whose transcripts harboring nonsense mutations could be down-regulated by the nonsense-mediated decay mechanism and produce truncated proteins with impaired functions.[7] Duchenne muscular dystrophy (DMD) is a human X-chromosome-linked disease and nonsense mutations in the *DMD* gene account for about 10% of reported cases, presenting the most prevalent and severe phenotypes characterized by progressive muscle weakness and a shortened life span.[8] Therapeutic methods have been developed to restore the expression of dystrophin encoded by pathogenic *DMD*, via exon-skipping,[9] genome editing[10] or PTC read-through.[1,11]

Here we describe specific steps for using engineered UAA incorporation system to read-through the PTC site of *DMD* transcript and alleviate disease symptoms in mice. We anticipate that this potentially curative method could be repurposed for treating more human nonsense mutation diseases.

Institutional permissions (if applicable)

Animal experiments in steps 15–34 were performed according to the Animal Protection Guidelines of Peking University, China. Any use or reproduction of this protocol will require the acquisition of permissions from the relevant institution.
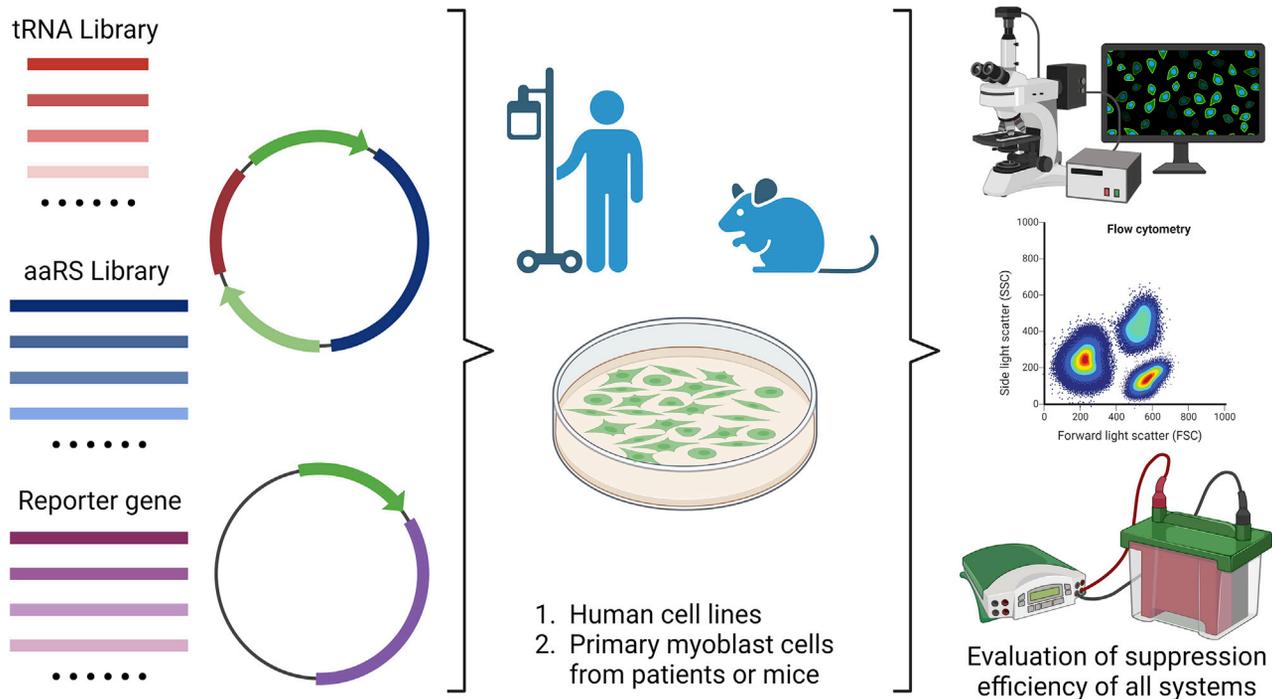
Figure 1. Schematics of cloning and screening strategies

An overview of the procedures described in the protocol that result in the generation of the UAA incorporation system carrying tRNA and orthogonal aaRS that are utilized to suppress nonsense mutations (steps 1–9 in before you begin).

Plasmid construction

⏱ **Timing: 3 weeks (for step 1)**

The preparation method involves the design and construction of UAA incorporation system expression vector and then demonstrate its efficacy for suppressing a specific nonsense mutation *in vitro* primarily. We present an overview of these procedures in Figure 1.

1. Design and screening for aaRS-tRNA pairs and relevant UAA that read-through the target nonsense codon.
    a. *In vitro* synthesis of aminoacyl-tRNA-synthase sequences and tRNA sequences. We recommend that screening at least the four well characterized aaRS–tRNA pairs for selecting the best system to read-through disease-related nonsense codons. DNA sequences of the four aforementioned systems are listed in Table 1.
    b. Cloning aaRS and tRNA into mammalian expression vectors. Troubleshooting 1.
        i. Design primers for PCR of synthetic aaRS and tRNA sequences using SnapGene and note that the length of primers should be 20–35 nt and the melting temperature is between 60°C and 65°C.

Primers used for aaRS and tRNA cloning	
Primer	Sequence(5′-3′)
Primer Forward for aaRS	ggtaccgagctcggatccgccacc
Primer Reverse for aaRS	gtgttaaacgggccctctagactcgag
Primer Forward for tRNA	ctgacgtcgacggatcgggagatct
Primer Reverse for tRNA	cgcctaaccctaagcagattcttcatgcaattg

PCR reaction master mix

Reagent	Amount
DNA template (20–100 ng)/μL	1 μL
Phanta Master Mix	10 μL
Primer Forward (10 μM)	2.5 μL
Primer Reverse (10 μM)	2.5 μL
ddH$_2$O	4 μL

PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	3 min	1
Denaturation	95°C	30 s	25–35 cycles
Annealing	55°C	45 s	
Extension	72°C	10 min (1 min / kb)	
Final extension	72°C	5 min	1
Hold	4°C	Forever	

Alternatives: The primer sets are used for amplifying the synthetic aaRS and tRNA genes and cloning into a single pcDNA3.1 vector by Gibson assembly. If you want to use other vectors, please design other primers for PCR.

Alternatives: You could set up PCR reactions using other DNA polymerase and run the PCR program according to its manufacturer's protocols.

Note: Anticodon of tRNA (the three **BOLD** nucleotides in Table 1) should be mutated for recognizing your target nonsense codon. For example, to read-through the premature UAA codon in mRNA, you should change the anticodon with **TTA** by PCR. We provide a scheme for better understanding the reverse complementary between PTC and anticodon in Figure 2.

    ii. Set up restriction digests for your recipient plasmid (e.g., pcDNA3.1 vector).

Restriction digest reaction for aaRS gene insertion

Reagent	Amount
Recipient plasmid	5 μg
10× FD Buffer (Thermo)	5 μL
FastDigest BamHI (Thermo)	2.5 μL
FastDigest XhoI (Thermo)	2.5 μL
ddH$_2$O	Up to 50 μL

Restriction digest reaction for tRNA gene insertion

Reagent	Amount
Recipient plasmid	5 μg
10× FD Buffer (Thermo)	5 μL
FastDigest BglII (Thermo)	2.5 μL
FastDigest MfeI (Thermo)	2.5 μL
ddH$_2$O	Up to 50 μL

Restriction digest conditions

Steps	Temperature	Time	Cycles
Incubation	37°C	2 h	1
Hold	4°C	forever	

Note: Note that the enzyme sets are used for digesting pcDNA3.1 vectors in this protocol. When using restriction enzymes to cut open the recipient plasmid, we recommend to follow the manufacturer's protocol of the enzyme. And if you want to use more than one enzyme, choosing enzymes with same working conditions is better.

    iii. Gel electrophoresis to determine the successful amplification of aaRS and tRNA genes. Then obtain the right PCR products and digested vectors by gel DNA extraction according to the manufacturer's protocol (QIAGEN, Cat#28705, https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/dna-clean-up/qiaquick-gel-extraction-kit).

    iv. Generate aaRS-tRNA pair expression vectors by Gibson assembly. Make sure that in the expression vector, transcription of aaRS should be driven by a RNA polymerase II gene promoter (e.g., CMV, EF1α), while tRNA should be transcribed by RNA polymerase III (e.g., U6, H1).

Gibson assembly mix	
Reagent	Amount
Gibson Assembly Master Mix	5 μL
PCR product	0.09 pmol (> 200 bp) 0.15 pmol (≤ 200 bp)
Digested vector	0.03 pmol
ddH$_2$O	Up to 10 μL

Gibson assembly conditions			
Steps	Temperature	Time	Cycles
Incubation	50°C	60 min	1
Hold	4°C	forever	

Note: The abundance of tRNA is critical for nonsense read-through. Based on our data, we recommend to use the strong human U6 promoter and construct a multi-copy tRNA expression cassette (Figure 3).

2. Generate reporter plasmids for *in vitro* demonstration of the UAA incorporation system. The reporter could be fluorescent proteins, luciferases or genes-of-Interest harboring nonsense mutations. In this example we construct three pCMV-GFP plasmids, each containing a nonsense codon (TAG, TAA or TGA) located at residue 39 of GFP by a similar cloning method.

3. Transformation of the homologous recombined products with DH5α chemical competent cells. In brief, add the products to the competent cells and incubate on ice for 30 min, then heat at 42°C for 45 s and incubate on ice for 2 min. Add 700 μL of antibiotic-free LB medium to each tube and shake for 60 min at 220 rpm at 37°C.

4. Plate *E.coli* on the pre-warmed plates containing specific antibiotics according to your expression vector. Incubate the plates in a 37°C incubator for 12–16 h.

5. Pick 6 to 10 single colonies, and suspend them into the LB medium of the culture tubes. Send them for Sanger sequencing to select correct DNA clones to continue.

Optional: Though the sequencing company could provide DNA extraction services, we recommend to extract DNA from single colonies using the QIAprep Spin Miniprep Kit before sequencing to obtain the plasmid of high quality. And correct DNA constructs should be stored at −20°C.

⏸ **Pause point:** The correct DNA constructs can be prepared and stored at −20°C, and the *E.coli* pellets should be stored at −80°C after being mixed with glycerol of 10% final concentration).

Table 1. DNA sequences of aaRS–tRNA pairs

Name	Sequence(5′-3′)
tRNA^{MmPyl}	ggaaacctgatcatgtagatcgaacggact**CTA**aatccgttcagccgggttagattcccggggttttccg
MmPylRS	atggagcaaaagctcatttctgaagaggacttggataaaaaaccactaaacactctgatatctgcaaccgggctctggatgtccaggaccggaacaattcataaaataaaacaccacgaagtctctcgaagcaaaatctatattgaaatggcatgcggagaccaccttgttgtaaacaactccaggagcagcaggactgcaagagcgctcaggcaccacaaatacaggaagacctgcaaacgctgcagggtttcggatgaggatctcaataagttcctcacaaaggcaaacgaagaccagacaagcgtaaaagtcaaggtcgtttctgccctaccagaacgaaaaaggcaatgccaaaatccgttgcgagagcccgaaacctcttgagaatacagaagcggcacaggctcaaccttctggatctaaattttcacctgcgataccggtttccaccaagagtcagtttctgtcccggcatctgtttcaacatcaatatcaagcatttctacaggagcaactgcatccgcactggtaaaagggaatacgaacccattacatccatgtctgcccctgttcaggcaagtgcccccgcacttacgaagagccagactgacaggcttgaagtcctgttaaacccaaaagatgagatttccctgaattccggcaagcctttcagggagcttgagtccgaattgctctctcgcagaaaaaaagacctgcagcagatctacgcggaagaaagggagaattatctggggaaactcgagcgtgaaattaccaggttctttgtggacaggggtttctggaaataaaatccccgatcctgatccctcttgagtatatcgaaaggatgggcattgataatgataccgaactttcaaaacagatcttcaggggttgacaagaacttctgcctgagacccatgcttgctccaaacctttacaactacctgcgcaagcttgacagggccctgcctgatccaataaaaaattttttgaaataggcccatgctacagaaaagagtccgacggcaaagaacacctcgaagagtttaccatgctgaacttctgccgagatgggatcgggatgcacacgggaaaatcttgaaagcataattacggacttcctgaaccacctgggaattgatttcaagatcgtaggcgattcctgcatggtctatgggatacccttgatgtaatgcacggagacctggaactttcctctgcagtagtcggacccataccgcttgacccgggaatgggtattgataaaccctggataggggcaggtttcgggctcgaacgccttctaaaggttaaacacgactttaaaaatatcaagagagctgcaaggtccgagtcttactataacgggatttctaccaacctgta
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tRNA^{EcLeu}	gcccggatggtggaatcggtagacacaagggatt**CTA**aatccctcggcgttcgcgctgtgcgggttcaagtcccgctccgggta

Table 1. Continued

Name	Sequence(5'-3')
EcLeuRS	atggaagagcaataccgcccggaagagatagaatccaaagtacagcttcattgggatgagaagcgcacatttgaa gtaaccgaagacgagagcaaagagaagtattactgcttttctggccctccctatccttctggtcgactacacatgggcca cgtacgtaactacaccatcggtgacgtgatcgcccgctaccagcgtatgctgggcaaaaacgtcctgcagccgatcg gctgggacgcgtttggtctgcctgcggaaggcgcggcggtggtgaaaaacaacaccgctccggcaccgtggacgtacg acaacatcgcgtatatgaaaaaccagctcaaaatgctgggctttggttatgactggagccgcgagctggcaacctgtac gccggaatactaccgttgggaacagaaattcttcaccgagctgtataaaaaaggcctggtatataagaagacttctgcg gtcaactggtgtccgaacgaccagaccgtactggcgaacgaacaagttatcgacggctgctgctggcgctgcgat accaaagttgaacgtaaagagatcccgcagtggtttatcaaaatcactgcttacgctgacgagctgctcaacgatctg gataaactggatcactggcctgacaccgttaaaaaccatgcagcgtaactggatcggtcgttccgaaggcgtggag atcaccttcaacgttaacgactatgacaacacgctgaccgtttacactaccgcccggacacctttatggggttgtaccta cctggcggtagctgcgggtcatccgctggcgcagaaagcggcggaaaataatcctgaactggcggcctttattgac gaatgccgtaacaccaaagttgccgaagctgaaatggcgacgatggagaaaaaaggcgtcgatactggctttaaa gcggttcacccattaacgggcgaagaaattcccgtttgggcagcaaacttcgtattgatggagtacggcacgggcgc agttatggcggtaccggggcacgaccagcgcgactacgagtttgcctctaaatacggcctgaacatcaaaccggttat cctggcagctgacggctctgagccagatctttctcagcaagccctgactgaaaaaggcgtgctgttcaactctggcga gttcaacggtcttgaccatgaagcggccttcaacgccatcgccgataaactgactgcgatgggcgttggcgagcgtaa agtgaactaccgcctgcgcgactggggtgtttcccgtcagcgttactggggcgcgccgattccgatggtgactctaga agacggtaccgtaatgccgacccccggacgaccagctgccggtgatcctgccggaggatgtggtaatggacggca ttaccagcccgattaaagcagatccggagtgggcgaaaactaccgttaacggtatgccagcactgcgtgaaaccgac actttcgacacctttatggagtcctcctgggttcttgcgcgctacacttgcccgcagtacaaagaaggtatgctggattccgaa gcggctaactactggctgccggtggatatcgcgattggtggtattgaacacgccattatggagagtctctactgtcgcttctt ccacaaaactgatgcgtgatgcaggcatggtgaactctgacgaaccagttaaacagttgctgtgtcagggtatggtgctggca gatgccttctactatgttggcgaaaacggcgaacgtaactgggtttccccggttgatgctatcgttgaacgtgacgagaaa ggccgtatcgtgaaagcgaaagatgcggcaggccatgaactggtttatatccggcatgagcaaaatgtccaagtcgaag aacaacggtatcgacccgcaggtgatggttgaacgttacggcgcggacaccgttcgtctgtttatgatgtttgcttctccgg ctgatatgactctcgaatggcaggaatccggtgtgtggaaggggctaaccgcttcctgaaacgtgtctggaaactggtttac gagcacacagcaaaaagtgatgttgcggcactgaacgttgatgcgctgactgaaaatcagaaagcgctgcgtcgcg atgtgcataaaacgatcgctaaagtgaccgatgatatcggccgtcgtcagaccttcaacaccgcaattgcggcgattatg gagctgatgaacaaactggcgaaagcaccaaccgatggcgagcaggatcgcgctctgatgcaggaagcactgct ggccgttgtccgtatgcttaacccgttcaccccgcacatctgcttcacgctgtggcaggaactgaaaggcgaaggcga tatcgacaacgcgccgtggccggttgctgacgaaaaagcgatggtgaagactccacgctggtcgtggtgcagg ttaacggtaaagtccgtgccaaaatcaccgttccggtggacgcaacggaagaacaggttcgcgaacgtgctggcc aggaacatctggtagcaaaatatcttgatggcgttactgtacgtaaagtgatttacgtaccaggtaaactcctcaatctggt cgttggcgggcccgtttaa

Characterization of UAA incorporation systems in cell lines

⏱ Timing: 5 days

The protocol below describes the specific steps for manipulating HEK293T cells. However, we have also used the systems in Vero-E6 cells, MDCK cells, and CHO cells, according to this protocol with changes in cell lines culture.

6. Cell lines culture. HEK293T cells were purchased from the ATCC (CRL-11268) and cultured in DMEM (Macgene) containing 10% (v/v) fetal bovine serum (GIBCO) and 1% Penicillin-Streptomycin (Macgene) at 37°C with 5% CO_2.

   Optional: You can pre-warm the media and trypsin before use, while we recommend to incubate trypsin for less than 20 min at 37°C, to avoid the decreased activity of it.

   Optional: We recommend to perform testing and authentication for mycoplasma in cultured cells with a MycAway one-step mycoplasma detection kit (Yeasen) before the next step.
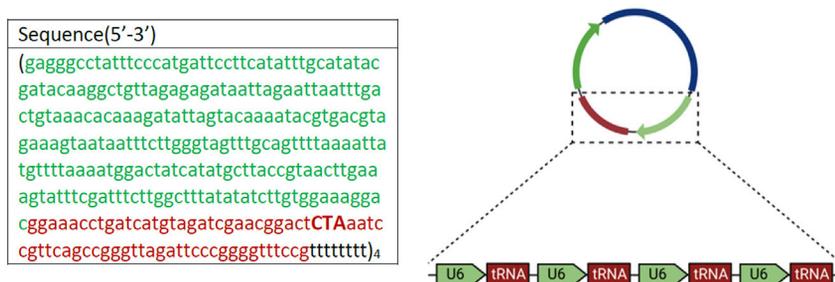
7. Cell seeding for transfection.
   a. Cells that reached 100% confluence were digested into single cells by 1 mL of 0.25% Trypsin-EDTA (Macgene), suspend cells with fresh medium and count cell numbers.
   b. Seed 3×10^5 HEK293T cells per well in six-well plates (Thermo) containing 2 mL growth medium.
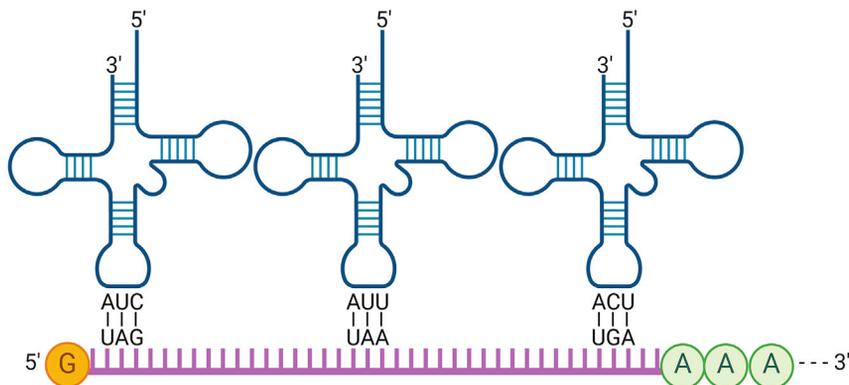
**Figure 2. A scheme for base pairing between anticodons and codons**
Engineered tRNAs could recognize specific nonsense codon by the reverse complementary of anticodon-codon (step 1 in plasmid construction).

   c. Incubate the plates at 37°C with 5% CO_2 until cells are approximately 70% confluent, about 16–20 h.

8. Preparation of UAA solutions. Here we choose three unnatural amino acids shown in Figure 4, N-ε-2-azidoethyloxycarbonyl-L-lysine (NAEK) for MmPylRS/tRNA^{MmPyl}, e-tBoc-lysine (eBK) for MbPylRS/tRNA^{MbPyl}, p-acetylphenylalanine (pAcF) for EcTyrRS/tRNA^{EcTyr}, and 3-(6-acetylnaphthalen-2-ylamino)-2-aminopropanoic acid (Anap) for EcLeuRS/tRNA^{EcLeu}, since they are available for purchase. You can select other UAAs with desired properties and synthesize them according to previous reports.[12] Troubleshooting 2.

   a. NAEK solution: 259 mg of NAEK powder is dissolved in 10 mL of deionized water in a 15 mL centrifuge tube to a final concentration of 100 mM, following sterilization by a 0.2 μm membrane filter and then storing at 4°C.

   b. eBK/PAcF solution: eBK/pAcF is prepared as a 100 mM solution like NAEK. However, we recommend to add 1 M NaOH solution (Sigma) drop by drop to accelerate the dissolution of powders and then adjust the pH to 7.0–8.0. eBK/PAcF solution should be sterilized by a 0.2 μm membrane filter and stored at 4°C away from light.

   c. Anap solution: since the water solubility of this compound is relatively poor, Anap powder should be dissolved in DMSO (Sigma) in a heated ultrasonic bath to prepare a 10 mM solution. Finally, the prepared solution should be sterilized by a 0.2 μm membrane filter and stored at 4°C away from light.

9. Transfection of cell line.

   a. For each constructed UAA incorporation system, mix its plasmids with pCMV-GFP reporter plasmids in a ratio of 1 μg: 1 μg in a centrifuge tube containing 100 μL Opti-MEM (GIBCO).

Sequence(5'-3')
(gagggcctatttcccatgattccttcatatttgcatatac gatacaaggctgttagagagataattagaattaatttga ctgtaaacacaaagatattagtacaaaatacgtgacgta gaaagtaataatttcttgggtagtttgcagttttaaaatta tgttttaaaatggactatcatatgcttaccgtaacttgaa agtatttcgatttcttggctttatatatcttgtggaaagga cggaaacctgatcatgtagatcgaacggact**CTA**aatc cgttcagccgggttagattcccggggtttccgttttttttt)$_4$

Figure 3. Multiple copies of tRNA
A multi-copy tRNA expression cassette is shown in sequences (left) and schematics (right). The sequences of U6 promoter is in green while the tRNA sequence (for example, tRNA^{MmPyl}) is in red, and the "4" means the plasmid should carry four U6 promoter-tRNA-polyT units to improve tRNA abundance (step 1 in plasmid construction).
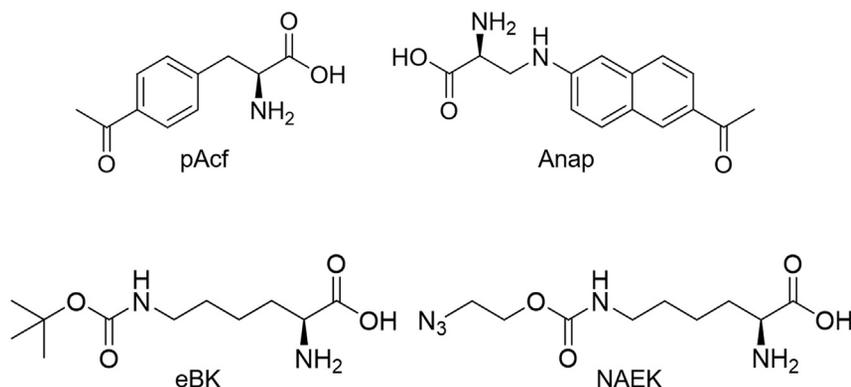
Figure 4. Structural formulas of three unnatural amino acids (step 7 in *in vitro* screening)

  b. Add 6 μL Megatran 2.0 transfection reagent (Origene) to another tube containing 100 μL Opti-MEM (GIBCO). Then vortex the two tubes for 15 s, mix well and incubate the mixture for 15 min at room temperature.

  c. Add the mixture per well dropwise to the six-well plates containing 2 mL of cells in growth medium. Mix gently by rocking the plates for 10–30 s.

  d. Prepare complete medium by adding NAEK, eBK, pAcF, and Anap into growth medium to a final concentration of 1 mM, 1 mM, 1 mM, and 10 μM, respectively. After 6 h, replace with 2 mL of complete medium containing UAAs for each well.

  Note: It is not a must that to refresh cell media before transfection when you are using aforementioned reagents, however, we also recommend you to perform a pre-experiment to validate whether the media would influence cell viability or transfection efficiency or not.

10. Analysis of suppression efficacy by microscopy and flow cytometry.

  a. 48–72 h after transfection, observe and compare the GFP fluorescence of each group (Figure 5A), and then evaluate the fluorescence intensity by flow cytometry.

  b. HEK293T cells were digested by using 0.25% Trypsin-EDTA (Macgene), suspend with fresh medium and centrifuge to collect cells. Wash cell pellets with PBS twice and suspend with 1 mL PBS.

  c. Cells of each group are analyzed on a BD FACSAria (BD Biosciences) with appropriate filter settings (488 nm coherent sapphire laser for GFP excitation). The front scatter and side scatter were used to identify intact cells, and the mean background fluorescence from untransfected cells was subtracted from the measured signal.

  d. Analyze the data by the FlowJo software package (version 10) to determine the number of cells with GFP fluorescence (Figure 5B).

  e. Based on the data of *in vitro* experiments, select the optimal system for further investigation. For example, we observe the relatively high efficiency of MmPylRS/tRNA^{MmPyl} pair for all three nonsense codons read-through as manifested by the GFP positive percentages among transfected cells (Figure 5), and choose it for *in vitro* demonstration.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-dystrophin antibodies (1:3000 dilution)	Proteintech	Cat#12715-1-AP
Anti-vinculin antibodies (1:3000 dilution)	Abcam	Cat#ab91459

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
HRP-conjugated goat anti-rabbit IgG (1:3000 dilution)	Bioss	Cat#bs-0294R-HRP
Anti-dystrophin antibody (1:100 dilution)	Abcam	Cat#ab15277
Goat anti-rabbit IgG Alexa Fluor 594 (1:400 dilution)	LifeTech	Cat#A-11037
Bacterial and virus strains		
Trans1-T1 Phage Resistant Chemically Competent Cell	TransGen	Cat#CD501-02
AAV2/9-CMV-MmPylRS-(U6-tRNA-H1-tRNA)$_2$ (AAV^{Pyl})	This paper	N/A
AAV2/9-CMV-mScarlet-3-Myc flag (AAV^{ctrl})	This paper	N/A
Chemicals, peptides, and recombinant proteins		
1 Kb Plus DNA Ladder	TransGen	Cat#BM211-01
DNA gel loading dye (6×)	Thermo Scientific	Cat#R0611
Agar	Sigma-Aldrich	Cat#A6686
Ampicillin	Sigma-Aldrich	Cat#A9393
N-ε-2-Azidoethyloxycarbonyl-L-lysine (NAEK)	SustGreen	N/A
e-tBoc-lysine (eBK)	Bidepharm	N/A
p-Acetylphenylalanine (pAcF)	Bidepharm	N/A
3-(6-Acetylnaphthalen-2-ylamino)-2-aminopropanoic acid (Anap)	SustGreen	N/A
DMSO	Sigma-Aldrich	Cat#472301
Opti-MEM	Gibco	Cat#31985062
Megatran 2.0 transfection reagent	Origene	Cat#TT210003
DMEM	Macgene	Cat#CM15023
Fetal bovine serum	Gibco	Cat#16000044
Penicillin-streptomycin	Macgene	Cat#CC004
Yeast extract	Sigma-Aldrich	Cat#8013-01-2
Tryptone	Sigma-Aldrich	Cat#91079-40-2
Sodium chloride	Sigma-Aldrich	Cat#S3014-5KG
DNase I, RNase-free	Thermo Scientific	Cat#EN0521
Proteinase K	New England Biolabs	Cat#P8107S
10 × phosphate-buffered saline	Sigma-Aldrich	Cat#P5493-1L
NuPAGE™ 4%–12%, Bis-Tris, 1.0–1.5 mm, Mini Protein Gels	Thermo Scientific	Cat#NP0321BOX
4% paraformaldehyde (PFA)	Biosharp	Cat#BL539A
Sucrose	Sigma-Aldrich	Cat#57-50-1
Tissue-Tek® O.C.T. Compound	SAKURA	Cat#4583
Hoechst 33342	Thermo Scientific	Cat#62249
Click-iT Alexa Fluor 488 sDIBO	Thermo Scientific	Cat#C20020
5% normal donkey serum	Jackson ImmunoResearch	Cat#017-000-121
10 × Tris buffered saline	Sigma-Aldrich	Cat#T5912-1L
Tween-20	Sigma-Aldrich	Cat#9005-64-5
Triton X-100	Sigma-Aldrich	Cat#9036-19-5
SDS-PAGE loading buffer	Solarbio	Cat#P1015
Millipore ECL	Sigma-Aldrich	Cat#WBKLS0500
Critical commercial assays		
2 × Phanta Max Master Mix	Vazyme	Cat#P515-03
Gibson Assembly® Master Mix	New England Biolabs	Cat#E2611L
MycAway one-step mycoplasma detection kit	Yeason	Cat#40612ES25
QIAquick Gel Extraction Kit	QIAGEN	Cat#28705
QIAprep Spin Miniprep Kit	QIAGEN	Cat#27104
QIAGEN plasmid maxi kit	QIAGEN	Cat#12163
AAV Maxi Purification Kit	Biomiga	Cat#V1269-02
2 × SYBR Green mix	Promega	Cat#M7123

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Pierce™ BCA Protein Assay Kit	Thermo Scientific	Cat#23227
Amicon® Ultra-15 Centrifugal Filter Unit	Sigma-Aldrich	Cat#UFC9100
Experimental models: Cell lines		
HEK293T cell line	ATCC	CRL-11268
AAV293 cell line	Agilent	Cat#240073
Experimental models: Organisms/strains		
Mouse: C57BL/6N (B6) strain (wild-type control), 6-week-old	Vital River Laboratories	N/A
Mouse: C57BL/10ScSn-*Dmd* [*mdx*]/JNju strain, 6-week-old	the Model Animal Research Center of Nanjing University	N/A
Oligonucleotides		
Primers for qPCR	This paper	N/A
Recombinant DNA		
Plasmid: pCMV-GFP-Y39TAG	This paper	N/A
Plasmid: pCMV-GFP-Y39TAA	This paper	N/A
Plasmid: pCMV-GFP-Y39TGA	This paper	N/A
Plasmid: pCMV-MmPylRS-(U6-tRNA)$_4$	This paper	N/A
Plasmid: pCMV-MbPylRS-(U6-tRNA)$_4$	This paper	N/A
Plasmid: pCMV-EcTyrRS-(U6-tRNA)$_4$	This paper	N/A
Plasmid: pCMV-EcLeuRS-(U6-tRNA)$_4$	This paper	N/A
pAAV-RC	Hanbio	N/A
pHelper	Hanbio	N/A
pAAV-ctrl	Hanbio	N/A
pAAV-Pyl	This paper	N/A
Software and algorithms		
Image-Pro Plus	Media Cybernetics	https://www.mediacy.com/imageproplus
FlowJo version 10.2	Tree Star	https://www.flowjo.com/
Prism version 8.0	GraphPad software	https://www.graphpad.com/
SnapGene version 6.1	SnapGene software	https://go.snapgene.com/

MATERIALS AND EQUIPMENT

DMEM 10% (v/v) FBS supplemented with antibiotics		
Reagent	Final concentration	Amount
DMEM	89%	44.5 mL
FBS	10%	5 mL
100 × Penicillin-Streptomycin solution	Penicillin: 100 U/mL, Streptomycin: 100 μg/mL	500 μL
Total	N/A	50 mL

Store at 4°C for up to one month.

LB medium		
Reagent	Final concentration	Amount
Yeast Extract	0.5%	2.5 g
Tryptone	1.0%	5.0 g
NaCl	1.0%	5.0 g
RNase-free water	N/A	To 500 mL
Total	N/A	500 mL

Store at 4°C for up to 2 months.

Gently mix the reagents in a 1,000 mL conical flask or a 500 mL glass bottle and autoclave at 121°C (15 psi) for 20 min.

LB agar plates medium

Reagent	Final concentration	Amount
Yeast Extract	0.5%	2.5 g
Tryptone	1.0%	5.0 g
NaCl	1.0%	5.0 g
Agar	1.5%	7.5 g
Ampicillin	100 μg/mL	500 μL
RNase-free water	N/A	To 500 mL
Total	N/A	500 mL

Store at 4°C for up to one months.
Gently mix the Yeast extract, Trypton, NaCl, Bacto Agar, and RNase-free water in a 500 mL glass bottle and autoclave at 121°C (15 psi) for 20 min. After the autoclaved media is cooled down to around 60°C, add 500 μL of ampicillin (100 mg/mL), and pour 15 mL of the LB/Agar solution into the petri-dishes. Allow the LB/Agar solution to solidify in room temperature and keep LB Agar plates in 4°C for storage.

TBST buffer

Reagent	Final concentration	Amount
10 × Tris Buffered Saline	N/A	100 mL
Tween-20	0.02%	1 mL
RNase-free water	N/A	To 1,000 mL
Total	N/A	1,000 mL

Store at room temperature for 2–3 months.

PBST buffer

Reagent	Final concentration	Amount
10 × Phosphate-Buffered Saline	N/A	100 mL
Triton X-100	0.1%	1 mL
RNase-free water	N/A	To 1,000 mL
Total	N/A	1,000 mL

Store at room temperature for 2–3 months.

STEP-BY-STEP METHOD DETAILS

After accomplishing the screening and evaluation of UAA incorporation system *in vitro*, we attempted to deliver this orthogonal tRNA-enzyme pair into *mdx* mice using an AAV viral vector, to further advance this method for clinical purposes. The step-by-step method details can be divided into two main parts. The first is to prepare an AAV, and the second is to systematically analyze and evaluate the *in vivo* efficacy and safety after AAV injection in mice. If a fresh virus is to be used for delivery, the administration of AAV should be performed 72 h after transfection of the packaging cells.

Part 1 preparation of AAV
Generation of AAV^{Pyl}

⊙ Timing: 5 days

In this protocol, we constructed AAV2/9 carrying the MmPylRS/tRNA^{MmPyl} (AAV^{Pyl}) system by transfecting AAV-293 cells with three packaging plasmids, and purified the collected virus to obtain the concentrated AAV virus with high titers.

1. Day 1: AAV-293 cells are grown and plated for transfection of packaging plasmids.
   a. Seed 2.0×10^6 AAV-293 cells in 100 mm cell culture plates containing 10 mL of growth medium (DMEM (Macgene) containing 10% (v/v) fetal bovine serum (GIBCO) and 1% Penicillin-Streptomycin (Macgene)).
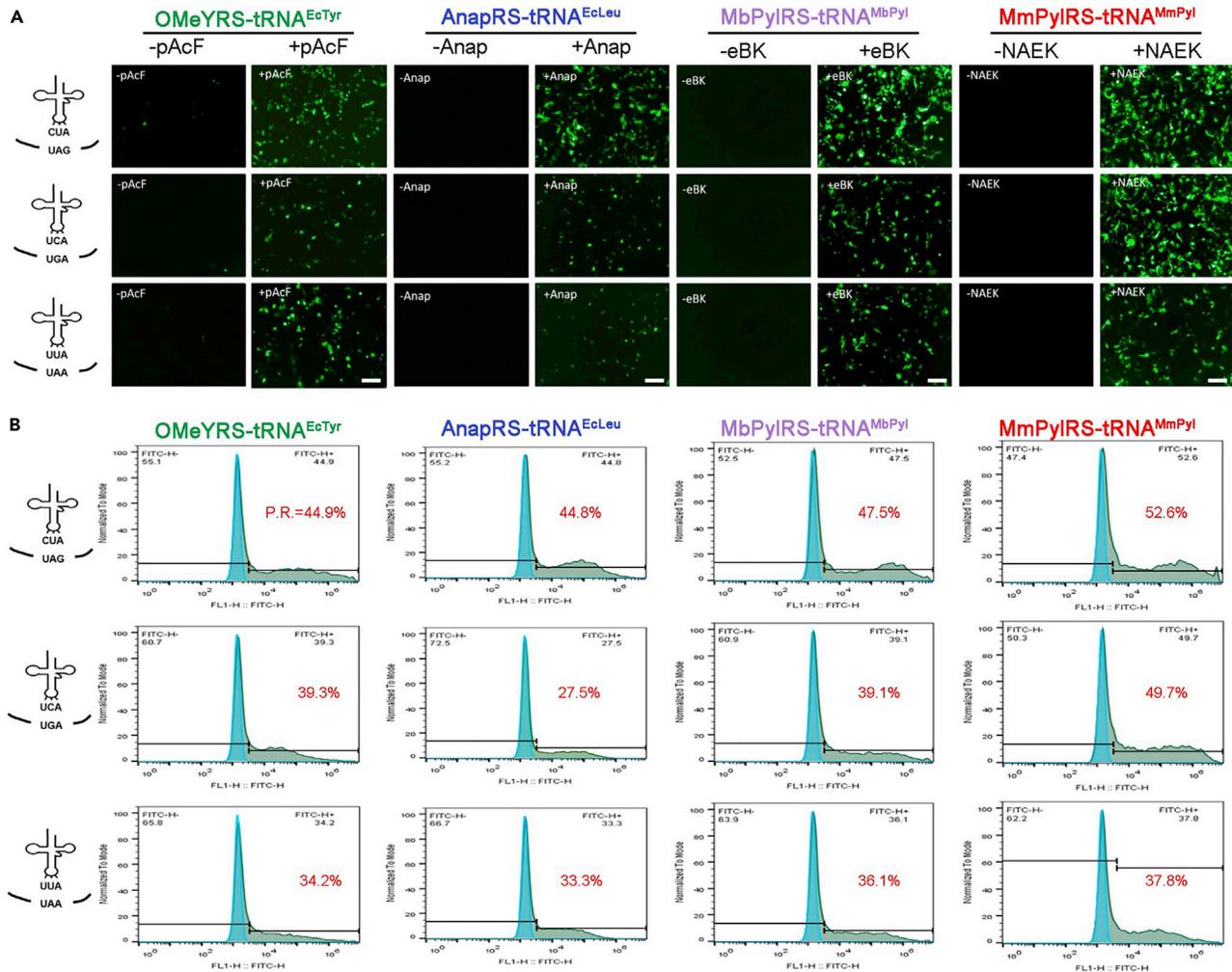
Figure 5. Evaluation of suppression efficacy by microscopy and flow cytometry *in vitro*

(A and B) Utilization of the four aaRS/tRNA pairs to identify the three stop codons of mutant GFP transcripts in HEK293T cells respectively. And the GFP recovery is observed and measured by fluorescence microscopy (A) and flow cytometry (B) (Shi et al.[6]) (step 9 in *in vitro* screening).

   b. Incubate the cells in a 5% CO2 incubator at 37°C until the confluent is approximately 80%, about 12 h.

2. Day 2: Transfection of the plasmids into the packaging cell line. Troubleshooting 3.

   a. Add plasmids and transfection reagents into two clean tubes containing Opti-MEM medium, respectively.

   b. Please mix them thoroughly.

   c. Incubate at room temperature for at least 15 min.

Note: Amounts of plasmids and reagents needed per plate are listed below.

AAV packaging system		
Plasmid	Amount	Function
pAAV-RC	10 μg	Containing the gene sequence for AAV serotype 2 replication (rep) protein paired with either serotype 9 capsid (cap) protein.

(Continued on next page)

Continued

Plasmid	Amount	Function
pAAV-Pyl/pAAV-ctrl	10 μg	Containing the gene sequence for MmPylRS/tRNA^{MmPyl} system or an control vector that only contains a reporter gene, e.g., mScarlet, constructed in a similar method of **Plasmid construction**.
pHelper	20 μg	Providing adenoviral helper genes, E2A, E4, and VA for AAV packaging.
Transfection Reagent	Amount	
Megatran 2.0	120 μL	

    d. Replace with 10 mL of fresh growth medium 6 h after transfection. Transfected AAV-293 cells would be in a 5% CO2 incubator at 37°C for another 72 h.

Alternatives: You could use HEK293 cells for AAV generation as well according to this protocol.

⚠ CRITICAL: Since the life cycle of AAV is less than 6 h, the discarded medium might contain viruses released from packaging cells, be careful when you replace the medium and remember to sterilize the items by ultraviolet germicidal irradiation and autoclaving used in virus manipulation.

3. Day 5: Collection of AAV virus.
    a. 72 h after media refresh, cells containing AAV particles were digested by 0.25% Trypsin or gently scraped off into a 15 mL centrifuge tube, centrifuge at 800 rpm for 3 min. Discard the supernatant and wash cell pellets once with PBS. Finally, suspend the pellet with 300 μL PBS and transfer cells to a clean 1.5 mL tube.
    b. Put the tube containing cells in liquid nitrogen for quick freezing (about 40 min) and transfer to a 37°C water bath for thawing (about 8 min). Repeat the freezing-thawing step three times for thorough lysis of cells and release of AAV virus. Centrifuge the tube at 4°C, 10,000 rpm for 20 min and collect the supernatant containing AAV particles to continue.

Note: We recommend to package a AAV virus containing the gene of MmPylRS/tRNA^{MmPyl} system and GFP, which could help you measure the transfection efficiency using the GFP signal and refine your experimental process.

Concentration of AAV^{Pyl}

⏱ Timing: 3 h

4. Add 0.1 μL Benzonase Nuclease (Sigma) per 1 mL of the supernatant from last step. Incubate the mixture at 37°C for 1 h to remove the cell genomes and plasmid DNA. Centrifuge at 4°C, 600 rpm for 10 min and collect the supernatant.
5. The AAV-containing supernatant is concentrated using a commercial kit (Biomiga, Catalog #: V1269-02, https://biomiga.com/wp-content/uploads/2021/09/V1269.AAV_.Maxi_.pdf) according to the manufacturer's protocol. Briefly, the supernatant is loaded to the column and gradually run through the column. Then wash the column with binding buffer to exclude the nonspecific bindings and elute the AAV by elution buffer at last.
6. The purified virus sample (about 4 mL) was added to an ultrafiltration tube and centrifuged at 4°C, 1,400 rpm for 30 min to obtain approximately 1 mL of concentrated AAV.
7. Aliquot and store the final purified virus in PBS or DMEM at −80°C.

⏸ **Pause point:** The purified AAV^{Pyl} could be stored at −80°C for about 6 months. But if the fresh virus is needed in your experiment, we recommend continue to the next step without freezing the virus.

Titration of AAV^{Pyl}

⏱ Timing: 4 h

This AAV Titration protocol is to determine the number of AAV genome-containing particles of your AAV prep using SYBR green technology by quantitative PCR (qPCR).

8. Design and synthesis of the required primer sets for amplifying the conserved components, WPRE and ITR, in AAV genome.

Primers	Sequence(5′-3′)
Forward primer-WPRE	ACGCTATGTGGATACGCTGC
Reverse primer-WPRE	CGGGCCACAACTCCTCATAA
Forward primer-ITR	CGGCCTCAGTGAGCGA
Reverse primer-ITR	AGGAACCCCTAGTGATG

9. Treat the sample of your AAV virus using DNase I (Thermo) to exclude the AAV DNA that is not packaged in a viral particle.

DNase I treatment mix	
Reagent	Amount
DNase I, RNase-free	10 μL
10 × reaction buffer with MgCl$_2$	6 μL
AAV sample	3 μL
ddH$_2$O	41 μL

DNase I treatment conditions			
Steps	Temperature	Time	Cycles
Incubation	37°C	60 min	1
DNase I Inactivation	100°C	10 min	1
Hold	4°C	forever	

10. Lyse the sample of DNase I-treated AAV virus using Proteinase K (Thermo).

Proteinase K lysis mix	
Reagent	Amount
Proteinase K Solution (20 mg/mL)	2 μL
DNase I-treated AAV sample	60 μL

Proteinase K lysis conditions			
Steps	Temperature	Time	Cycles
Incubation	55°C	60 min	1
Proteinase K Inactivation	100°C	10 min	1
Hold	4°C	forever	

11. Dilute the lysed AAV sample by ten-fold.

   △ CRITICAL: DNase I and Proteinase K treatment are both important for tittering AAV.

   Alternatives: We recommend to make a serial dilution of your lysed AAV sample, but no less than 1: 10^6 dilution. The original lysed AAV sample and dilutions can all be used for qPCR.

12. Dilute the plasmids containing AAV genome sequences to copies of 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10}, respectively as standards in qPCR.
13. Set up qPCR reactions for the diluted AAV sample and standards. Two technical replicates were carried out for each sample.

qPCR reaction mix	
Reagent	Amount
2 × SYBR Green mix	10 μL
AAV samples/standards	1 μL
Primer Forward (10 μM)	0.5 μL
Primer Reverse (10 μM)	0.5 μL
ddH$_2$O	8 μL

qPCR cycling conditions			
Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	3 min	1
Denaturation	95°C	30 s	40 cycles
Annealing	62°C	30 s	
Extension and data collection	72°C	1 min	

14. Create the standard curve by plotting Ct values against the corresponding plasmid copies, and calculate the genome titer of your AAV virus based on the curve (Figure 6A). Troubleshooting 4.

   Optional: Before starting animal experiments, you can measure the activity of AAV^{Pyl} by infecting cell lines or primary myoblast cells derived from pathogenic mice (Figure 6B).

Part 2 *In vivo* restoration of dystrophin by AAV^{Pyl} delivery
In this part, we will investigate the *in vivo* efficacy of MmPylRS/tRNA^{MmPyl} pair delivered by AAV in two routes. Remember to make sure that your pathogenic animals are ready for following experiments. For *mdx* mice used in this protocol, all mice are reared in house (temperature: 20°C–25°C, humidity: 40%–70%) under specific-pathogen-free conditions with lighting controlled (light: 06:00–18:00). All mice had free access to food and water. Before experiments, male and female *mdx* mice at 6 weeks old are randomly allocated to each experimental group (Table 2).

   △ CRITICAL: Here we provide a scheme for arranging four experimental groups, which is a relatively low configuration for this research. We recommend to add groups for evaluation of efficacy in different time points if you need, for example, we recommend to evaluate the efficacy and safety in 4, 6, and 8 weeks after injection.

   Note: Though the AAVs used in this protocol are replication-incompetent in conventional cells, the leakage of AAVs would result in undesired expression of the translation system in control or mock mice, and even the experimenters. Please wear the medical mask and gloves to do experiments and do not to handle mice from different groups at the same time.
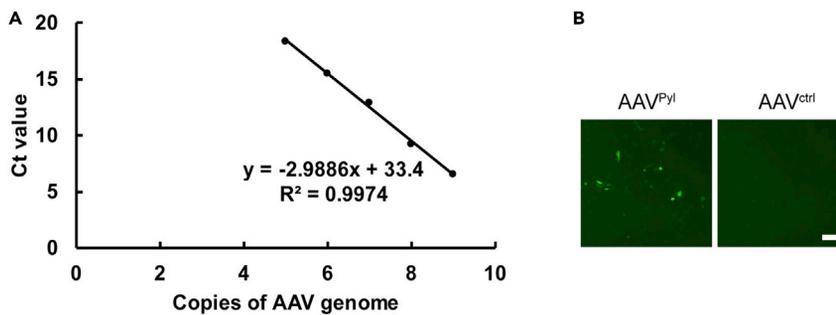
Figure 6. Preparation of AAV
(A) The standard curve by plotting Ct values (Y axis) against the AAV genome copies (X axis). The titer of your AAV could be calculated using the equation.
(B) AAV infect primary cells and the activity is measured by the GFP positive cells percentages (mentioned in step 14 in AAV titration).

Intraperitoneal injection of AAV^{Pyl} in mdx mice

⏱ Timing: 1 h

15. Gently remove the mouse from the cage and restrain appropriately in the head-down position (Figure 7A).

   Optional: You can comfort the mice by gently scratching its back and head.

16. Insert needle with bevel facing "up" into the lower right quadrant of the abdomen towards the head at a 30–40° angle to horizontal. Insert needle to the depth in which the entire bevel is within the abdominal cavity (no more than 2 cm).

   Note: Be careful to feel that the needle passed through the mouse skin and avoid damage to the urinary bladder.

17. Pull back on the plunger to ensure negative pressure prior to injecting.

   ⚠ CRITICAL: The presence of blood in the syringe indicates an abdominal blood vessel has been punctured. We recommend to discard the syringe and syringe contents and carry out experiments in other mice.

18. Depress the plunger until the AAV solution (about 4.0×10^{12} AAV^{Pyl} or AAV^{ctrl} in 100 μL) has been fully administered. The injection can better be completed in 2 s. Troubleshooting 5.

   Note: The titer of AAV here is suitable for DMD therapy in *mdx* mice. If you want to investigate other nonsense diseases, or in other animal models, or via other injection routes, please adjust the titer based on your needs.

19. Pull the needle straight out and place the syringe/needle directly into a sharps container without recapping. Use a new needle and syringe and repeat steps 15–18 for each mouse.

   Note: Please make sure there is no reflux of the injected solution.

20. Place the mouse back into its cage and monitor closely for any signs of pain, distress or illness induced by injection.

Table 2. Experimental mice groups

Group	Mice	AAV injection	Delivery route	NAEK administration
Treatment (+)	*mdx* mice, *n* = 3–5	AAV^{Pyl}	Intraperitoneal or intramuscular	50 mg per two days
Treatment (-)	*mdx* mice, *n* = 3–5	AAV^{Pyl}		0 (0.9% NaCl only)
Control	*mdx* mice, *n* = 3–5	AAV^{ctrl}		0 (0.9% NaCl only)
Mock	C57BL/6 mice, *n* = 3–5	/		/
	mdx mice, *n* = 3–5	/		/

Intramuscular injection of AAV^{Pyl} in mdx *mice*

⏱ Timing: 1–2 h

21. The mouse is weighed and injected intraperitoneally with 4 mL / Kg of 1% pentobarbital sodium and placed in a rearing cage for about 10 min.

   Note: For most 6-week old *mdx* mice, their weight is about 25 g and 100 μL of 1% pentobarbital sodium is enough for anesthesia.

22. Restrain the anesthetized mouse in a prone position and secure the rear foot nearest you.
23. Isolate the muscle mass to be used with your fingers or cotton balls. Troubleshooting 6.

   ⚠ CRITICAL: Please use the muscle cranial to (in front of) the femur (that is, quadriceps), do not use the thigh muscles caudal to (behind) the femur (Figure 7B).

24. Direct the needle cranially (toward head) and insert into the quadriceps muscle. Be careful to avoid the sciatic nerve (Figure 7B).

   Note: Be careful not to let the needle penetrate the muscles, and if necessary, hold the needle with your finger so that it does not move around.

25. Aspirate to ensure negative pressure prior to injecting.

   ⚠ CRITICAL: The presence of blood in the syringe indicates the needle has entered a blood vessel. We recommend to discard the syringe and syringe contents and carry out experiments in other mice.

26. If no blood is aspirated, depress the plunger slowly until the AAV solution (about 2.0×10^{12} AAV^{Pyl} or AAV^{ctrl} in 50 μL) has been fully administered. The injection can better be completed in 10 s.
27. Withdraw the needle and discard the syringe/needle directly into a sharps container without recapping. Use a new needle and syringe and repeat steps 21–26 for each mouse.

   Note: Please make sure there is no reflux of the injected solution and no bleeding in the injection site.

28. Return the mouse to its cage and monitor closely for any signs of pain, distress or illness induced by injection.

Daily monitoring of experimental mice

⏱ Timing: 4–8 weeks
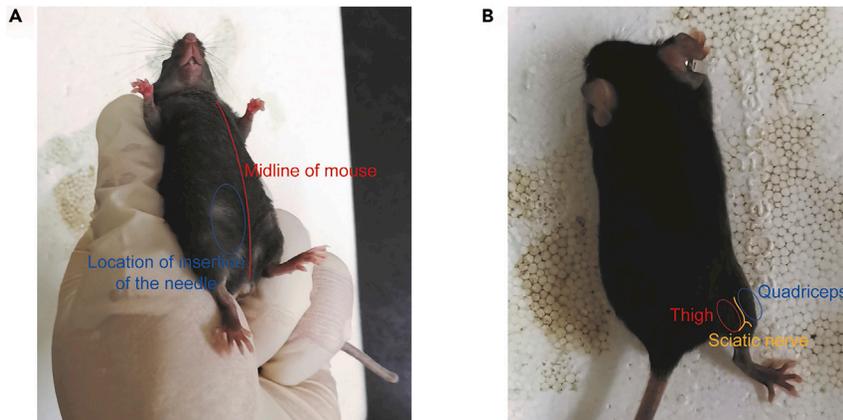
Figure 7. Handling of mouse
(A and B) Restrain the mouse in a head-down (A) or prone (B) position before AAV injection intraperitoneally (i.p.) and intramuscularly (i.m.) (steps 15–16 and 22–24 in intraperitoneal or intramuscular AAV injection).

29. Injection of the mouse intraperitoneally with 100 μL of 500 mg/mL NAEK solution or 0.9% NaCl solution every two days. Remember to vary the side injected between right and left for multiple administrations. Troubleshooting 7.

    Note: The concentration of NAEK solution can be adjusted between 250 and 1,000 mg/mL if you need. Ensure that the pH of solution is about 7.0 and the injection volume is between 50 and 200 μL (50 mg NAEK).

30. Daily record the body weight and survival rate of the experimental group (injected with NAEK or 0.9% NaCl solution) mice, the control group (injected with 0.9% NaCl solution) mice, and the mock group mice (healthy wild-type C57BL/6 mice).

Grip strength tests for experimental mice

⏱ Timing: 3–4 h (for step 31)

Recovery of grip strength is a sign of disease alleviation in *mdx* mice. Here we design a double-blind study of grip strength for mice from each group, to measure the outcomes after treatment with no bias.

31. The four groups, AAV^Pyl^-treated *mdx* mice with NAEK or 0.9% NaCl solution, AAV^ctrl^-treated *mdx* mice, and wild-type C57BL/6 mice of different time points (4, 6 and 8 weeks) are randomly picked.
32. Take the mouse out of the cage and lift it by the tail, causing the four limbs or a single limb to grasp the pull-plate assembly connected to the grip strength meter (Methods videos S1 and S2).
33. The mouse is drawn along a straight line leading away from the sensor until the grip was broken and the peak amount of force in grams was recorded.
34. Test 4–6 times for each mouse and collect the average grams of force.

    Note: Please test all experimental mice in a group once in turn, and then retest them in the same order for at least three times.

Measurement of restored dystrophin after treatment
To further evaluate the dystrophin recovery in experimental mice, we should quantify and compare the protein levels in every group. We recommend to detect dystrophin expression in multiple ways,

and we will provide two general approaches, western blot analysis and immunofluorescence staining in details here. For other diseases treatment, you could choose other approaches to demonstrate the restoration, according to the protein's characteristics.

Western blot analysis for dystrophin expression

⏱ Timing: 12 h

35. Isolate the tibialis anterior muscles from experimental mice.

   Note: If you inject AAV intramuscularly, be careful to tell the two anterior muscles from different legs, injected with AAV or not.

36. Tissue is broken with a homogenizer at 3,000 rpm for 1 min and tissue debris was removed by centrifugation at 4°C, 16,000 rpm for 15 min.
37. Add RIPA buffer and protease inhibitor to extract total proteins from supernatant and quantify them by BCA assay, according to the manufacturer's protocol (Thermo, catalog #: 23227, https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets. thermofisher.com/TFS-Assets%2FLSG%2Fmanuals%2FMAN0011430_Pierce_BCA_Protein_Asy_ UG.pdf).
38. 100 μg of protein from each sample is boiled with loading buffer respectively.

   Alternatives: The high amount of loaded proteins is because of dystrophin characteristics. You could load 20–100 μg of sample proteins for electrophoresis, but we recommend to determine an optimal usage for your target proteins at first.

39. Electrophoresis: Separate samples on 4%–12% NuPAGE (Invitrogen) with a voltage of 120 V.
40. Activate a polyvinylidene difluoride membrane with methanol for 30 s before use, and remove air bubbles between the layers with a glass rod.
41. Electroblotting in a 4°C refrigerator with a voltage of 100 V for 1 h. Troubleshooting 8.
42. Block the membrane with 5% (v/v) non-fat milk in TBST (50 mM Tris-HCl, 150 mM NaCl and 0.02% Tween-20, pH 7.5) at room temperature for 1 h.
43. Incubate the membrane with anti-dystrophin antibodies (1:3,000, 12715-1-AP, Proteintech) and anti-vinculin antibodies (1: 3,000, ab91459, Abcam) diluted in TBST containing 5% (v/v) of de-fatted milk, overnight at 4°C.
44. Rinse the incubated membrane with TBST at a orbital shaker, 130 rpm for 10 min, repeat for three times.
45. Incubate the membrane with horseradish peroxidase-conjugated goat anti-rabbit IgG (1: 3,000, bs-0294R-HRP, Bioss) at room temperature for 1 h.
46. Rinse the incubated membrane with TBST for three times.
47. Mix two components of Millipore ECL luminescent solution at a ratio of 1:1 (v/v), and apply the luminescent solution evenly to the membrane.
48. The protein bands are visualized in a Fuji Las-3000 dark box (FujiFilm), and the integrated optical density (IOD) of western blot bands is semi-quantified using the ImagePro Plus software package (version 6).

Immunofluorescence staining for in situ *dystrophin detection*

⏱ Timing: 24 h

49. Isolate the tibialis anterior muscles from experimental mice.
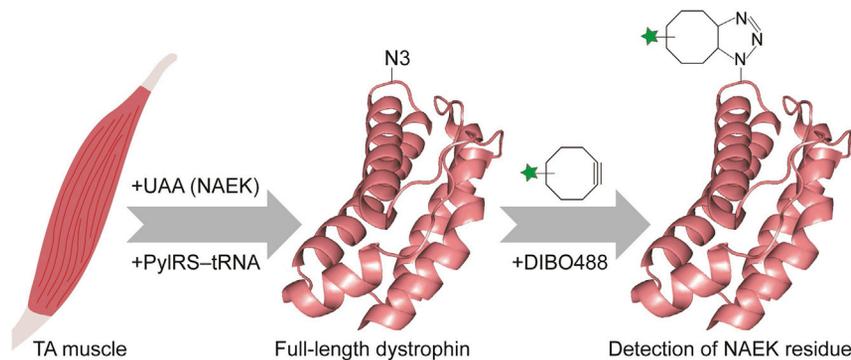
Figure 8. Bioorthogonal labeling of dystrophin by click reaction
Schematic illustration of the bioorthogonal click-chemistry of incorporated NAEK in dystrophin with the fluorescent dye DIBO488 (Shi et al.[1]) (steps 56 and 57 in immunostaining).

> *Note:* We recommend to cut tissues into small pieces (no larger than 1 cm^3) for efficient fixation and dehydration.

50. Fix tissues pieces from different mice in 4% paraformaldehyde (PFA) at room temperature for 1 h.
51. Wash fixed tissues with PBS three times.
52. Immerse tissues in 30% (w/v) sucrose overnight.

> *Note:* Tibialis anterior muscles ought to be immersed over 8 h, depend on the size of cut pieces. The submersion of tissues is a sign for entire dehydration.

53. Embed tissues in Optimal Cutting Temperature (OCT) compound (Tissue-Tek) and freeze them at −80°C.
54. Cryo-sectioning of embedded tibialis anterior muscle tissues at −20°C using a cryostat (Leica) to obtain serial 12-μm sections.

> *Alternatives:* The sections should be 8- to 14-um.

55. Block Cryosections with 5% normal donkey serum (017-000-121, Jackson ImmunoResearch) in PBST (PBS with 0.1% Triton X-100) for 30 min.
56. Incubate the sections with anti-dystrophin antibody (1:500, ab15277, Abcam) diluted in blocking buffer overnight at 4°C.
57. Incubate the sections with secondary goat anti-rabbit IgG Alexa Fluor 594 (1:400, A-11037, LifeTech) at room temperature for 1 h.

> *Alternatives:* Since we have incorporated an unnatural amino acid, NAEK, into dystrophin, the functional side chain could be conjugated with fluorescent dye by click reaction (Figure 8). We could incubate the slides with Click-iT Alexa Fluor 488 sDIBO (1: 1,000, C20020, Invitrogen) at room temperature for 1 h, instead of the primary and secondary antibodies. This labelling method is simpler in manipulation and efficient in imaging, while after incubation you need to carefully wash the sections by PBS to exclude the unconjugated dye.

58. Stain the sections with 0.5 μg/mL Hoechst and mount them in mounting medium (e.g., PBS).
59. Photograph stained sections under a Nikon Ti-S microscope and analyze the expression and distribution of dystrophin measured by fluorescence (Figure 9).
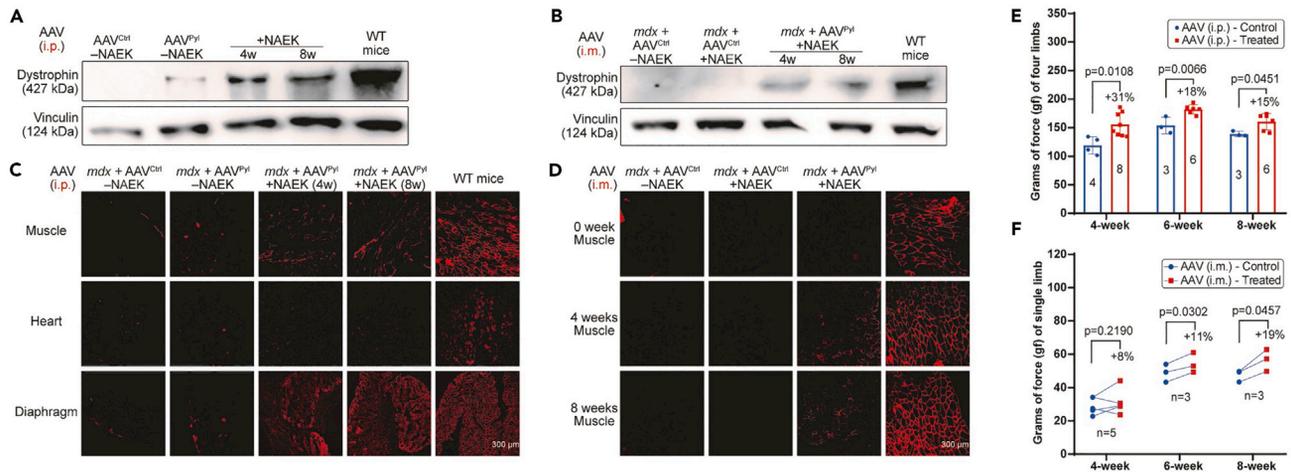
Figure 9. *In vivo* efficacy of the UAA incorporation system in dystrophin restoration

(A and B) Western blot analysis of the dystrophin restoration in *mdx* mice after intraperitoneal (A) and intramuscular (B) AAV injection. NAEK (50 mg) was injected intraperitoneally every two days. w, weeks.

(C) Immunostaining of dystrophin in tibialis anterior muscle (top), heart (middle) and diaphragm (bottom) of *mdx* mice at 4 and 8 weeks after AAV[Pyl] and NAEK administration, compared with *mdx* mice injected with AAV[ctrl], and wild-type C57BL/6 mice. Scale bar, 300 μm.

(D) Immunostaining of dystrophin in tibialis anterior muscle of mdx mice at 0 (top), 4 (middle) and 8 (bottom) weeks after AAV treatment (intramuscularly) and NAEK uptake, compared with *mdx* mice injected with AAV[ctrl] and wild-type C57BL/6 mice. Scale bar, 300 μm.

(E and F) Grip strength for the intraperitoneal (E) and intramuscular (F) delivery routes was measured at 4, 6 and 8 weeks to reflect the muscle functions. Data are mean ± s.d. (Shi et al.[1]) (expected outcomes).

EXPECTED OUTCOMES

For *DMD* therapy in mice, we anticipate that the dystrophin would be restored in tibialis anterior muscles by both the intraperitoneal (Figure 9A) and intramuscular (Figure 9B) AAV routes as shown by western blot analysis, After the administration of 50 mg NAEK intraperitoneally every two days for 4 and 8 weeks. Moreover, we expect to observe significant fluorescence by immunostaining or click reaction of *in vivo* dystrophin at 4 weeks, after AAV[Pyl] injection and following NAEK uptake. And we would observe the accumulation of restored dystrophin in muscle, diaphragm, and heart as continuously NAEK administration, from 4 to 8 weeks, inferring that the long-term efficacy of AAV-delivered aaRS-tRNA pairs (Figures 9C and 9D). To validate the efficacy, we analyze the grip strength changes in experimental mice from each group and find a significant increase in muscle strength of AAV[Pyl]- and NAEK-treated mdx mice at 6 and 8 weeks compared with AAV[ctrl]-treated mice, and intramuscular topical therapy is also feasible for strength recovery of the injected muscles (Figures 9E and 9F). We predict that disease symptoms in heart and respiratory system of treated mice could also be reduced. In conclusion, these results including protein levels recovery and disease symptoms alleviation, could demonstrate the robustness of the AAV-delivered UAA incorporation system in *DMD* therapy in mice and highlight the potential and possibility of this approach applied to clinical applications in the future.

LIMITATIONS

This presented protocol provides a novel approach for nonsense mutation rescue using the UAA incorporation system. Unlike genome editing or transcripts engineering, this system could generate a bioorthogonal translation machinery to read-through nonsense codons and restore functional proteins expression, without undesired changes in DNA or RNA. However, the approach has two limits. Firstly, the UAA incorporation system is not suitable for suppressing those out-of-frame nonsense mutations, which cannot produce healthy proteins just relying on the read-through of nonsense codons. Secondly, the bioavailability of unnatural amino acids *in vivo* is also a constraint for treatment. For DMD therapy in this protocol, systemic delivery of AAV and UAAs could alleviate symptoms efficiently in disease-related organs, especially the muscles, while the dosage is twice-fold than the

intramuscular administration. Though DMD patients usually exhibit progressive muscle weakness, severe heart disease and respiratory failure are responsible for the shortened life span as well. Therefore, the systemic delivery via intravenous or intraperitoneal injection needs to be further investigated for clinical use, instead of local injection. If you want to further improve the efficacy or treat other diseases, we recommend to investigate the robustness of delivery in targeted tissues at first.

TROUBLESHOOTING

Problem 1
Inefficiency of your constructed system in *in vitro* read-through (related to step 1 in before you begin).

Potential solution
Please check your design of expression vectors at first. The Inefficiency is mainly due to inappropriate promoters for aaRS and tRNA, too few copies of tRNA (we recommend to utilize at least four copies of tRNA), or inefficient cassettes construction (for instance, we recommend four copies of tRNA to be driven by four Pol III gene promoters respectively, instead of four copies of tRNA to be driven by one Pol III gene promoters, please also see Figure 3).

Problem 2
Observe poor efficiency in read-through nonsense codon and cytopathic effect of UAA-incubated cells (related to steps 7–9 in before you begin).

Potential solution
Though the pH of UAA solutions would influence the cell viability significantly, especially the low pH. Please adjust the pH of solutions to about 7.0 before use.

Problem 3
Observe significant changes in AAV-293 morphology after transfection, following by obtain of AAV viruses with low titers (related to steps 1–3).

Potential solution
The quality of packaging plasmids is pivotal for efficient AAV generation. We recommend to remove the endotoxin of plasmids, and measure the absorbance ratio of A260/A280 (1.8–1.9) and A260/A230 (2.2–2.5) before use.

Problem 4
R^3 of the standard curve is low (related to steps 8–14).

Potential solution
The Ct values of standards or your samples could vary in each experimental set, carry out more technical repeats could eliminate the randomness to some extent. But if you find the high copies standards resulted in high Ct values while low copied standards are attributed to low Ct values, you need to check whether there are some mistakes in the standard dilutions or PCR procedures. And titration of AAV sample by infecting cells and analyze delivered-gene expression could be an alternate if qPCR cannot work well.

Problem 5
Find bleeding or death in experimental mice after injection (related to steps 15–18).

Potential solution
Generally, bleeding indicates an abdominal blood vessel has been punctured. And if you also draw back green or yellow materials into the needle, this means the bowel or bladder of mouse has been

punctured. Be careful to Insert needle to the abdominal cavity no more than 2 cm and depress the plunger quickly, to avoid undesired injuries.

Problem 6
Find extravasation of injected AAV solution or lameness in mice (related to steps 21–26).

Potential solution
Please ensure that you have conducted injection in quadriceps muscles and the volumes of injection is no more than 50 μL at first. Unlike intraperitoneal injection, intramuscular injection needs to be more slowly when inject the solution, to avoid damages in mice muscles or even the sciatic nerve.

Problem 7
Observe inflammation in the abdomen of some mice with weight loss (related to step 29).

Potential solution
Inflammation might be due to irregular intraperitoneal injection with NAEK. And the abnormal pH or too high concentration of NAEK solution would resulted in weight loss.

Problem 8
Observe non-specific bands or not the expected size of dystrophin band in your western blot analysis (related to steps 37–41).

Potential solution
Since the molecular weight of dystrophin is relatively high (427 kDa), the electroblotting for dystrophin is more difficult and inappropriate conditions would result in loss and even disappearance of the specific band. Lower voltage with longer electroblotting time could obtain a clearer protein band. And we recommend to conduct a pre-experiment to find an optimal voltage in your laboratory.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Qing Xia (xqing@hsc.pku.edu.cn).

Materials availability
Plasmids and reagents used in this study are available upon request.

Data and code availability
This study did not generate new datasets or codes.

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101950.

AUTHOR CONTRIBUTIONS
Q.X. supervised the project. Z.Z. and N.S. conducted the experiments and data analysis. Z.Z. wrote the original draft of manuscript. Q.X. reviewed and edited the manuscript.

DECLARATION OF INTERESTS

Q.X. has filed patents regarding the engineered tRNA-enzyme pairs for dystrophin read-through and DMD therapy (PCT/CN2017/075577 and CN2016/10134657.6). The other authors declare no competing interests.

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